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Table of contents

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Beneficial impacts of goat milk on the nutritional status and general well-being of human beings: Anecdotal evidence

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ABSTRACT

Goats provide an essential food supply in the form of milk and meat. Goat milk has distinct qualities, but it shares many similarities with human and bovine milk regarding its nutritional and therapeutic benefits. Because of their different compositions, goat and cow milk products could have different tastes, nutrients, and medicinal effects. Modification in composition aid of goat milk determining the viability of goat milk processing methods. Comparatively, goat's milk has higher calcium, magnesium, and phosphorus levels than cow's or human milk but lower vitamin D, B12, and folate levels. Goat milk is safe and healthy for infants, the old, and healing ailments. Capric, caprylic, and capric acid are three fatty acids that have shown promise as potential treatments for various medical issues. Considering the benefits and drawbacks of goat milk over cow milk is essential; goat milk is more digestible, has unique alkalinity,

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Casein

Human health

has a better buffering capacity, and has certain medicinal benefits. Acidifying goat milk shrinks fat globules and makes protein friable (with less αs1-casein and more αs2-casein). Goat milk treats malabsorption illnesses because it has more short- and medium-chain triglycerides that give developing children energy. In wealthy countries, goat milk and its products—yoghurt, cheeses, and powdered goods—are popular with connoisseurs and persons with allergies and gastrointestinal issues who need alternative dairy products. A food product category containing fermented goat milk with live probiotic microbes appears promising nutritionally and medicinally. This article presents anecdotal evidence of the therapeutic effects of consuming goat milk for human health and its nutritional value.

1 Introduction

Milk has always played an important role in the human diet, which has not changed over history. It has been used for decades by individuals of all ages. In addition, milk is regarded as a great food due to its balanced composition of proteins, vitamins, lipids, and minerals. Most of the milk consumed presently ought to be cow milk (Chandran et al. 2021a; Prakash et al. 2021a;). Cow's milk provides the full spectrum of nutrients, including proteins, calcium, vitamin B12, and iodine, essential for healthy human growth and development. The good news comes with a catch, though. Some people have sensitivities to the proteins found in cow's milk (Chandran et al. 2019; Chandran 2021b). Cow milk consumption is also related to lactose intolerance and other health problems. The answer to this predicament lies with the goat, the rich man's substitute for the cow. India has many high-return, low-risk industries, including goat husbandry (Khan et al. 2019; Gallier et al. 2020; Chandran 2021a). Farmers on tight budgets and with restricted land can still make a living by raising goats due to this endeavor's minimal risk and cheap cost (Prakash et al. 2021b). Investment returns from goats are higher than those from cows. Hence the adage that goats are the poor man's cow is correct. Goat farming appeals to many subsistence farmers because of its low input costs and relatively low grazing space requirements (Martemucci and D'Alessandro 2013; Li et al. 2020). Two indicators may show how the worldwide popularity of goat farming is developing. A growing number of people are keeping goats for two reasons: either as a means of subsistence or as a hobby (Chandran and Athulya 2021; Manuvanthra et al. 2022). The goat industry is crucial to the economic well-being of rural communities because of the income generated from the sale of goat meat. Anyone can raise goats without access to land by simply giving them leftovers and herbs from the kitchen. Their mild demeanor and diminutive stature make them a breeze to house and handle (Lejaniya et al. 2021a).

The market for dairy goats around the world is expanding quickly. Keeping dairy goats provides smallholders with a sustainable source of income and assets in the form of healthy milk products and helps them maintain a more self-sufficient lifestyle. Estimates put the global goat population at 1 billion, with 94 percent residing in Africa and Asia's poorest nations (Chandran and Arabi 2019; FAOSTAT 2019; Prakash et al. 2021b). The 20th Livestock Census of India found 535.78 million cattle in the country in 2019, with 148.88 million goats making up 10.14 percent of the total (GOI 2019). Goats are valuable and necessary livestock for many reasons. Their adaptability to many environments, dietary requirements, and climate extremes is a bonus to their already helpful versatility and moderate output. Dairy, meat, fleece, and manure are just a few products that may be derived from goats. Milk production from goats begins at around 16 to 17 months of age, and the animals reproduce quickly. Goats are helpful to rural areas since they are inexpensive to raise, have a rapid reproduction rate, require little food, and consistently yield only a tiny amount of milk that is perfectly adequate for the needs of a single family (Clark and Mora García 2017; Chandran et al. 2021c).

Unlike cows and sheep, goats spend most of their time browsing rather than grazing on the grass to cover more ground on their way to a water source. Goat milk, in comparison to the other two kinds of milk from the same species, can have a substantial effect on human nutrition due to the presence or lack of specific proteins, vitamins, carnitine, lipids, minerals, glycerol ethers, enzymes, fat globule size, orotic acid, and casein polymorphisms. This is because goat milk has a distinct flavor compared to sheep and cow milk (Deepak et al. 2020a; Chandran et al. 2021a). Due to species-specific metabolic, nutritional, physiological, and anatomical distinctions, goat milk and its derivatives may command a premium over cow's milk. Goat milk has been recognized as a functional beverage. Its smooth consistency and high mineral and vitamin content make it an appealing substitute for various common dietary supplements (Roy et al. 2021). Goat milk's improved digestion makes it a better option than cow's milk; its medicinal advantages, buffering ability, and alkalinity are also significantly higher. Goat milk fat has more incredible physical qualities than cow milk fat, including increased surface tension, viscosity, and specific gravity (Turck 2013; Patange et al. 2022a; Patange et al. 2022b). The global goat milk production profile and top-producing countries are depicted in Figure 1.

The principal carbohydrate in goat milk, lactose, is beneficial because it increases the body's absorption of calcium, magnesium,

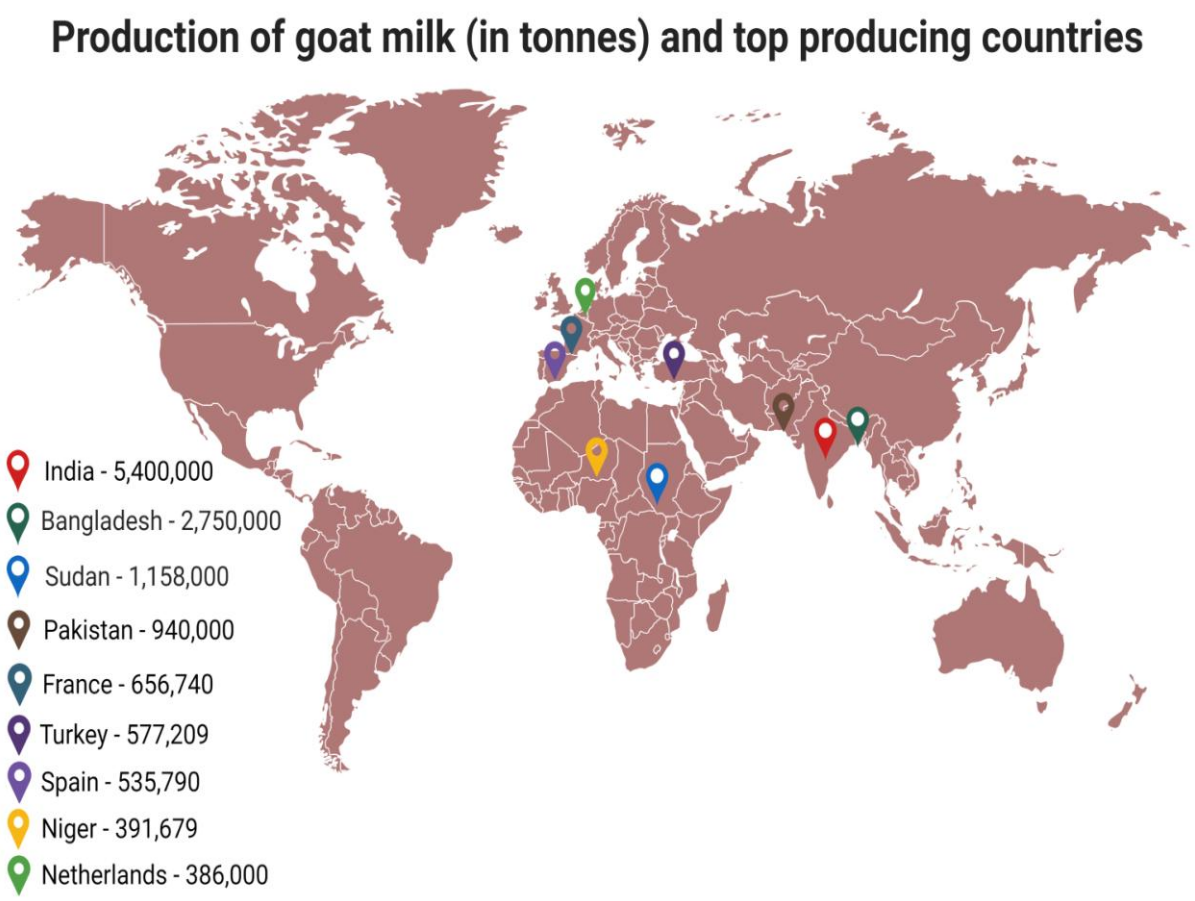

Figure 1 Analyzing the state of goat milk production throughout the world
(Designed with Biorender premium software; https://app.biorender.com/)

phosphorus, and vitamin D. Because goats convert all of the beta-carotene in their food into vitamin A, goat milk naturally contains higher vitamin A than cow milk. Goat milk outshines cow milk in many minerals, including potassium, selenium, zinc, calcium, chloride, phosphorus, and copper. Goat milk is a better alternative for persons who cannot tolerate lactose. The popularity of goat milk may be linked to the fact that it is digested more easily than cow's milk (Stergiadis et al. 2019). Cheese and yoghurt made from goat's milk can soothe stomachs that have problems digesting cow's milk. Given its composition, human breast milk represents a biologically typical diet for babies. Its chemical makeup helps prevent inflammation and infection while providing nutrients, easing digestion, promoting healthy organ development, and protecting against food poisoning. Yet, issues of time, health, and living in an urban environment can all contribute to the premature end of breastfeeding. During the COVID-19 pandemic, even India's government guidelines warned that nursing was unsafe under the current circumstances and should be avoided if possible (Chawla et al. 2020; Chauhan et al. 2021; Kaur and Pareek 2022). Hence, it is vital to find a way to provide an alternative to breast milk for babies who cannot get enough of it.

Dairy products, especially milk, have undeniable health and practical benefits. Several people undoubtedly consume goat milk or goat cheese. Goat's milk is processed into various dairy products, including ice cream, paneer, channa, srikhand, condensed milk, dried whole milk, butter oil, and flavored milk (Martemucci and D'Alessandro 2013). The nutritional and therapeutic benefits of goat milk for illnesses like lactose intolerance, inflammatory bowel disease, antibiotic resistance, cardiovascular disease, and lactose malabsorption should not be overlooked (Collard et al. 2021). Goat milk is known for its health benefits. Goat milk helps cow milk-allergic or digestively challenged infants (Jirillo et al. 2010). Figure 2 shows how goat milk benefits health. Individuals' awareness of the problems with conventional medical treatments for certain ailments also contributes to this rising demand. Goat milk has gained popularity as a functional food in recent years since it is easily absorbed and causes fewer allergic reactions than bovine milk. Goat milk is a promising alternative to traditional dairy products that can help those with lactose intolerance and other digestive issues (Lejaniya et al. 2021a). Milk has very high nutritional benefits and has recently been a popular part of the diet in industrialized countries. Milk is drunk either on its own or as

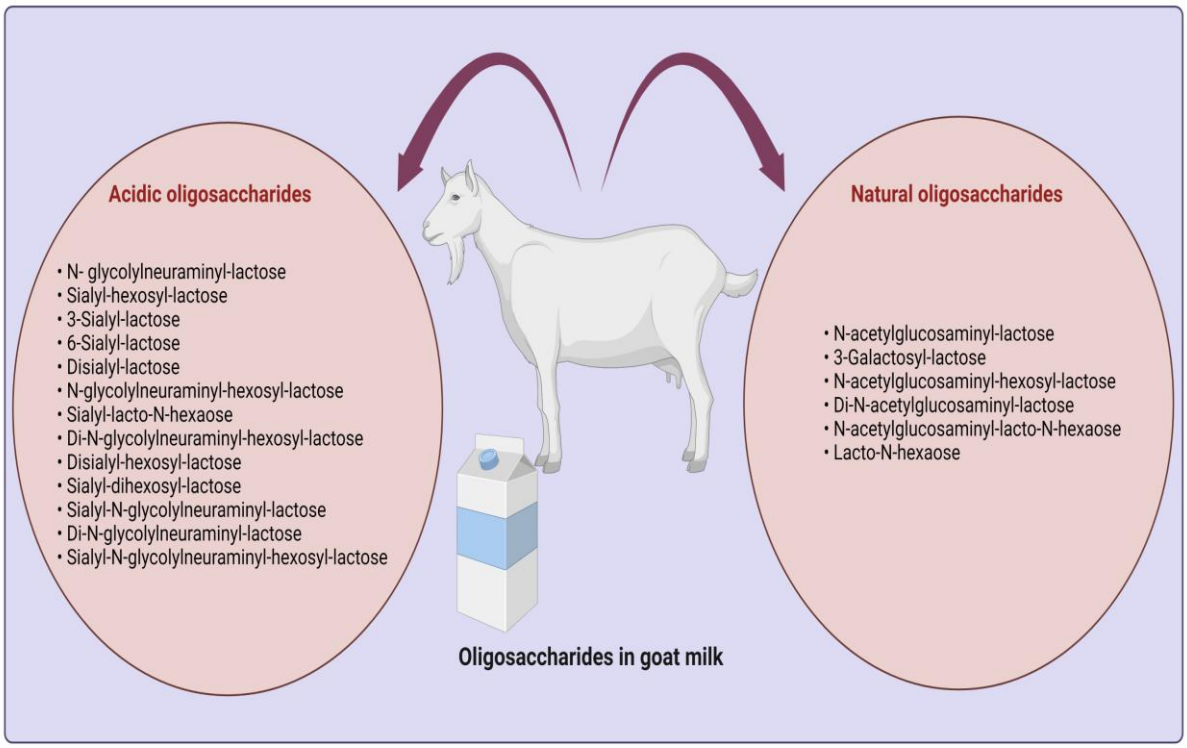

Figure 2 Oligosaccharides in goat milk (Designed with Biorender premium software; https://app.biorender.com/)

part of a new category of functional dairy goods in industrialized countries. Physiologically active metabolites and probiotic bacterial strains are common ingredients in these products (Kumar et al. 2016; Rai et al. 2022). The article below looks at anecdotal evidence supporting the nutritional and physiological advantages of consuming goat milk by humans.

2 Nutritional composition of goat milk

Goat milk has more therapeutic characteristics than cow and human milk because it is more easily digested, has a higher buffer capacity, is more alkaline, and has a higher pH. The fat in goat's milk has a higher specific gravity, greater density, and more viscosity than cow's milk. Steroids and glycerides can make up as much as 99 percent of fat. The fat in milk emulates oil and water (Siefarth and Buettner 2014). While the fat globules in goat milk are chemically and physically comparable to cow milk, agglutinin is lacking in goat milk. Globules in goat milk range in size from 1.5 to 2 mm, whereas those in cow milk range from 2.5 to 3.5 mm, and there are 28 percent more of them in goat milk than cow milk (10 percent). Fat globules in goat milk are typically much smaller than in cow milk, with about 65 percent of fat globules being less than 3m. Goat's milk is sometimes referred to as "self-homogenized" because of its quality (Cebo et al. 2010; Toral et al. 2015), offers better values than cow milk in terms of free lipids, and has twice the C8, C10, and C12 fatty acids as cow milk (Patange et al. 2022a). Medium-chain triglycerides are a healthy fat that may be burned for fuel instead of stored as fat, and they also help lower cholesterol. Chyluria and chylothorax (lung conditions) are also treated with these. Medium-chain triglycerides are used to treat a variety of digestive disorders, including those that result from abnormal food absorption, such as diarrhea, steatorrhea (fat indigestion), celiac disease, liver illness, short bowel syndrome, and digestive problems caused by the surgical removal of a portion of the stomach (gastrectomy) or intestine (Collard et al. 2021).

Milk proteins can be separated into a stable micellar phase, made up of casein, and an insoluble whey protein phase. Compared to cow's milk, goat's milk has a little lower αs-casein concentration, a much greater β-casein concentration, and a comparable κ-casein concentration (Stergiadis et al. 2019; Chandran et al. 2021b). Goat milk has more αs1-casein than cow milk, which has more β-casein. Micelles derived from goat milk are more soluble in β-casein, have higher calcium and phosphorus concentrations, and are less heat stable than those derived from cow milk. Compared to cow's milk, less allergen αs1-casein is found in goat's milk (Ballabio et al. 2011; Dhasmana et al. 2022).

The mammary gland needs beta-lactalbumin to make milk from glucose and galactose. Lactose aids protein digestion, intestinal mineral absorption (especially calcium, magnesium, and phosphorus), and vitamin D usage. The udder's duct system secretes milk from it (He et al. 2022). Goat milk contains

monosaccharides, oligosaccharides, glycopeptides, glycoproteins, and nucleotides. Lactose-derived oligosaccharides are much more significant in goat milk than in cow's milk. Milk oligosaccharides have prebiotic and antibacterial properties, which are thought to contribute to human nutrition. Goat milk oligosaccharides inhibit colitis-induced inflammation in mice (Kiskini and Difilippo 2013). These findings may treat inflammatory bowel disease. Goat milk has fewer oligosaccharides than human milk, but more than bovine and ovine milk, and its structures are like human milk. Human milk oligosaccharides are prebiotic and anti-infective, making them advantageous for newborn feeding (Khan et al. 2019; Li et al. 2020). Goat milk oligosaccharides are illustrated in Figure 2.

Goats convert all β carotene in their food into vitamin A, so their milk has more vitamin A and is whiter than cow milk. Goat and cow milk lack the essential vitamins and minerals that infants require, including vitamins B6 and D (He et al. 2022). Vitamin A levels in the milk of goats and humans are comparable. Vitamin A is necessary for cell-mediated immunity and antibody responses, two immune system aspects crucial for fighting infection and maintaining health (Kumari et al. 2022). Goat milk has higher concentrations of the well-known water-soluble antioxidant vitamin C than cow milk (Stergiadis et al. 2019). The immune system is just one of the many things this vitamin has been shown to influence. It has antiviral and antioxidant effects, which help regulate the immune system. Goat milk contains all of the B vitamins (particularly thiamine, riboflavin, and niacin) and vitamins D and E. Folate levels are low in goat's milk (Gallier et al. 2020).

Each species' milk has a particular mineral pattern, which may indicate the element's nutritional value. Goat milk has more minerals than cow's milk, making it more mineral-rich (Stergiadis et al. 2019). Cow milk is different because it has lower sodium, phosphorus, zinc, copper, and manganese concentrations than goat and human milk. Goat milk is enriched with a higher concentration of nutrients, making it a healthier alternative to cow's milk. Goat milk, like cow milk, is not a suitable replacement for human milk, although it can be used as a supplement for newborns and toddlers (Patange et al. 2022a; Patange et al. 2022b). Goat milk's mineral richness exceeds cow and human milk, reflecting that it may be used as a supplement if more people know about it. Goats' mineral metabolism is distinctively different from that of cows and sheep, especially in molybdenum, copper, iodine, selenium, magnesium, and iron (Siefarth and Buettner 2014). Goats, as opposed to cattle or sheep, spend most of their time browsing rather than grazing in a grassland setting, meaning they may cover more ground on foot and require less frequent watering. Proteins, vitamins, carnitine, lipids, minerals, glycerol ethers, enzymes, fat globule size, orotic acid, and casein polymorphisms distinguish goat milk from cow, sheep, and human milk (Martemucci and D'Alessandro 2013; Khan et al. 2019). Differences in anatomy, physiology, metabolism, and nutrition contribute to goat milk and its derivatives having their own properties. Goat milk and other dairy products are more popular because they contain a higher concentration of nutrients (Toral et al. 2015). While goats' milk has identical amounts of protein, lipids, and lactose as cows' milk, the protein and fat structures are less digestible and nutritious. The chemical composition, secondary protein structures, and amino acid profile of goat milk differ from cow milk, making it hypoallergenic. Due to its high nutrient content and functional components, such as prebiotic chemicals and probiotic microorganisms, goat milk can be used in various products. Goat's milk can be a good alternative for lactose intolerant or suffering from gastrointestinal conditions like ulcers or colitis (Sousa et al. 2019; Rai et al. 2022).

3 Dietary and therapeutic significance of goat milk

The dietary and medicinal benefits of goat milk on human health and nutrition are summarized in Figure 3.

3.1 Alleviation of lactose intolerance

Sugar lactose is present in goat milk, just as in human and cow milk. Due to its lower lactose content, goat's milk is more easily absorbed by the human digestive system than cow's milk. Lack of lactase, the enzyme responsible for digesting lactose, is the root cause of lactose intolerance (milk sugar). Lactose is the primary carbohydrate in milk (Turck 2013). Lactose is formed when two molecules of D-glucose and D-galactose are joined together. Goat milk is a better choice for lactose intolerant people since it has lesser (22 to 27 %) lactose content as compared to cow milk (33 to 40 %) (Martemucci and D'Alessandro 2013; Lund and Ahmad 2021). High lactose content in milk gives it a pleasant flavor and facilitates calcium absorption in the small intestine. It has a critical role in establishing healthy bone structure in infants.

However, many lactose-intolerant persons find that goat's milk suits their tastes. It is hypothesized that this is due to the better digestion of goat's milk (Gallier et al. 2020). Because goat's milk has a more remarkable lactose absorption ability than cow's milk, fewer people experience the discomfort of lactose intolerance when drinking it. Some people may be allergic to cow's milk despite not being lactose intolerant because of the protein as1-casein, which is uncommon in goat's milk or non-existent in some cases. Similar symptoms accompany lactose intolerance and milk protein allergy (Arasi et al. 2022; Liu and Zhang 2022). Patients with lactose intolerance have difficulty digesting lactose because the sugar is absorbed by the large intestine undigested. Microbes fermenting this unhydrolyzed lactose in the large intestine produce gas and free fatty acids, which in turn cause bloating, cramping, and other gastrointestinal symptoms (Lund and Ahmad 2021). Due to its softer curd, goat milk is often advised as an alternative to cow's

milk. Higher casein content in goat's milk facilitates lactose digestion and reduces the chance of lactose intolerance by hastening the process by which the sugar is absorbed by the large intestine (Quigley et al. 2013; Rai et al. 2022).

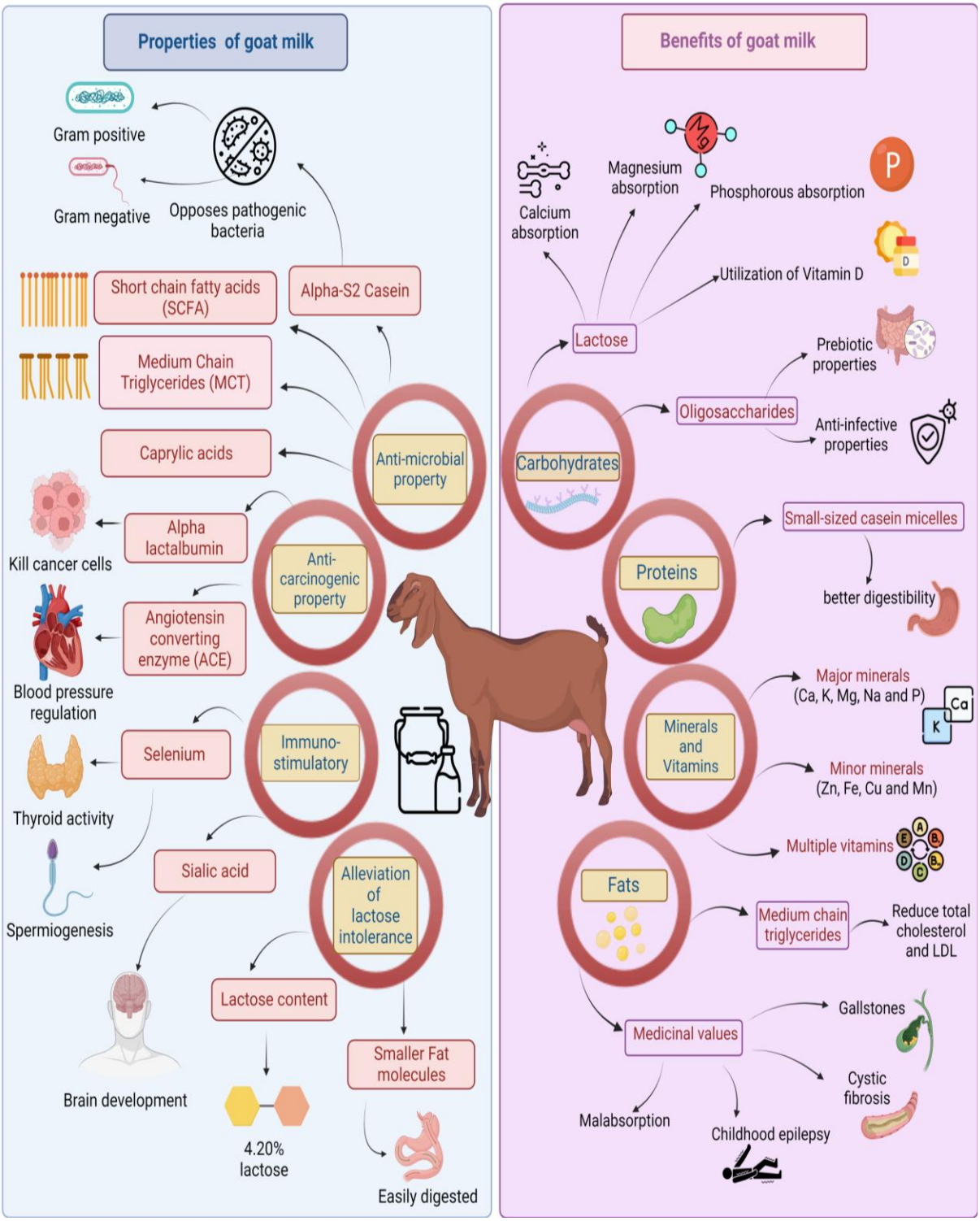

Figure 3 Dietary and therapeutic implications of goat milk on the human health and nutrition (Designed with Biorender premium software; https://app.biorender.com/)

3.2 Digestibility, gastrointestinal function, and prevention of malabsorptive disorders

Goat milk fat contains more short- and medium-chain (C4:0-C12:0) fatty acids than cow milk. Goat's milk absorbs more easily since it has fewer fat globules. Goat milk's unique protein profile allows for forming of a milder curd, which is easier on the digestive system and more comfortable to eat (Roy et al. 2021). Compared to bovine casein micelles, goat casein micelles are less heat stable, less soluble, and lose β-casein faster due to their higher inorganic phosphorus and calcium content (Chandran et al. 2021b). Less αs-casein can be found in goat's milk, and sometimes αs2-casein is more prevalent than αs1-casein. Proteins in goat milk are absorbed more effectively because they are easier to digest. The decreased αs1-casein level in goat milk results in a more tender and crumbly curd when acidified (Clark and Mora García 2017; Dhasmana et al. 2022).

Several studies have linked goat milk to fewer cases of diarrhea, constipation, and other gastrointestinal issues. The weaker goat milk acid-induced coagulation and faster stomach emptying may affect abdominal pain perception; however, this has not been proven (Carneiro et al. 2018). Recent animal research suggests that goat milk may help prevent some of the harm that heat stress and intestinal inflammation may cause to the digestive tract. In animal models of colitis, goat milk reduces inflammation and alters short-chain fatty acid fermentation markers in mice. Goat milk had a higher impact on mice's metabolism and intestinal flora than cow milk. Goat milk inhibited even the most stubborn bacteria from attaching to Caco-2 cells, including *Escherichia coli* and a *Salmonella typhimurium* strain. Goat milk oligosaccharides increased *Bifidobacteria longum* subsp. *infantis* adherence to HT-29 intestinal cells eight-fold (Kiskini and Difilippo 2013; Khan et al. 2019; Liu and Zhang 2022). Further study, including clinical trials involving human participants, is needed to determine whether or not goat milk influences the gastrointestinal environment and metabolism in a way distinct from cow milk (Li et al. 2020).

Diseases such as malabsorption disorders stem from problems with nutrient and food digestion and absorption. Many forms of cancer can produce this, often as a side effect or symptom. Inadequate absorption of nutrients such as vitamin B12, folic acid, iron, and other minerals, vitamins, and macronutrients can lead to anemia (Liu and Zhang 2022). The rat model of malabsorption syndrome is widely used because it can be induced by a reaction in roughly half of the rodent's small intestine (Carneiro et al. 2018). Protein and fat are digested and absorbed better from goat milk diets, while calcium, phosphorus, magnesium, iron, copper, zinc, and selenium are also better absorbed. Goat milk may require more minerals for metabolism because it contains more protein, cysteine, and vitamins C and D than cow milk (Khan et al. 2019; Chauhan et al. 2021; Saikia et al. 2022).

3.3 Hypoallergenic property

Many infants have a cow milk allergy (CMA); however, its reasons remain unknown. CMA has been related to beta-lactoglobulin, the most prevalent whey protein in cow milk but missing in human breast milk. Many proteins in cow's milk, including caseins, beta-lactoglobulin, and beta-lactalbumin, have been identified as potential allergens (Ballabio et al. 2011). Although nearly all newborns younger than three years old have circulating milk antibodies, roughly 7 percent of children in the United States and possibly all western countries have signs of CMA. CMA symptoms usually appear between 2 and 4 weeks and never after six months. The immune system reacts to milk in the gastrointestinal tract, the respiratory system, the skin, and occasionally the rest of the body (He et al. 2022). CMA symptoms include vomiting, epigastric discomfort, malabsorption, bronchitis, erythraemia, hyperactivity, migraines diarrhea, colitis, eczema, urticaria, rhinitis, asthma, anaphylaxis, and many others (Carneiro et al. 2018). Rhinitis, abdominal discomfort, diarrhoea, anaphylaxis, and urticaria were the most common CMA symptoms that resurfaced. Mothers who cannot breastfeed their babies need to find a suitable substitute (Novac and Andrei 2020; Rai et al. 2022). Infants and food allergy sufferers can switch to goat milk. Goat's milk is safe for cow-milk allergy sufferers since its proteins are different. Goat milk is vital for CMA patients, milk consumers and producers, and human nutrition in general because of its therapeutic and hypoallergenic effects on newborns and CMA patients (Chauhan et al. 2021; He et al. 2022). Despite this, most research has demonstrated that goat milk therapy can help children with cow milk allergies or chronic enteropathy. However, some caprine milk proteins are immunologically cross-reactive with cow milk proteins (Hirsiger et al. 2022).

Further clinical feeding experiments are needed, but governments, corporations, and universities focusing on goat milk have not received funding from cow-milk-centric communities (Manuvanthra et al. 2022). Genetic variations in caseins and whey proteins can complicate CMA cases, making identifying the protein most likely to produce an allergic reaction more challenging. Nevertheless, milk protein genetic polymorphisms could be used in clinical studies to detect allergens (Pastuszka et al. 2016; Lund and Ahmad 2021). Cross-immune reactivity between cow and goat milk is common. Goat milk containing αs1- casein, a protein polymorph found solely in cow milk, produced allergic reactions in guinea pigs. However, guinea pigs given αs2-casein, which lacks the αs1-casein polymorph, had just a 40 percent allergic reaction, concluding that αs2-casein-lacking goat milk is less allergenic than regular goat milk (Ballabio et al. 2011). Due to differences in cheese manufacturing and renneting, several countries are selecting goats for or against αs1-casein, which could benefit or hurt goat breeding programs. Breeding techniques that aim to increase protein and casein content in milk for use in cheese

production could take advantage of the isoelectrofocussing gene (Sousa et al. 2019; Chauhan et al. 2021). Goat milk with low αs1-casein and high αs2-casein has lower curd yield, longer rennet coagulation time, higher heat lability, and lower curd stiffness. Because of significant mutation rates at each locus encoding the four casein genes, goat casein polymorphism research is arduous (Novac and Andrei 2020). Protein and DNA analyses have examined the polymorphisms. Protein synthesis rates are related to 16 alleles. The CSN1S1, CSN2, and CSN2S2 genes encode three calcium-sensitive caseins: αs1-casein, β-casein, and αs2-casein. The casein micelle requires κ-CN (Dhasmana et al. 2022). Ulcers can be treated with goat milk because it has a higher buffering capacity than cow milk. The casein and phosphate systems in milk are two protein components that contribute to the beverage's buffering ability (Chilliard et al. 2003; Khan et al. 2019). Buffering capacity is higher in goat milk than in cow milk because goat milk, regardless of breed, contains more nitrogen phosphate nitrogen (NPN) and more nitrogen moieties and phosphate. Infant formulas made from soy contain lower levels of total nitrogen and NPN than goat and cow milk, suggesting that the enhanced buffering capacity of goat milk may have therapeutic benefits for people (Hirsiger et al. 2022). The higher concentration of short- and medium-chain fatty acids in goat milk makes it helpful in treating various malabsorption disorders. Diseases and conditions that are treated by removing the gallbladder include hyperlipoproteinemia, coronary bypass, pediatric epilepsy, steatorrhea, intestinal resection, chyluria, cystic fibrosis, gallstones, and feeding a premature baby (Pastuszka et al. 2016). The therapeutic effects of these medium-chain triglycerides on cholesterol metabolism include hypocholesterolaemia action, cholesterol deposition inhibition, and gallstone dissolving. Goat's milk products, like regular milk products, can help humans in need of healing as well (Kumar et al. 2016; Pastuszka et al. 2016; Saikia et al. 2022). People in many different countries worldwide enjoy several indigenous cultured goat milk products. The nutritional value of these foods is enhanced because lactic starter cultures pre-hydrolyze the primary milk constituents, allowing for a better synthesis or availability of certain minerals. Several examples of yoghurt and other cultured foods are used in medicine. Typical uses include treating diarrhea, infantile gastroenteritis, and constipation (Li et al. 2020; Saleena et al. 2022a; Tiwari et al. 2022). Studies have indicated that yoghurt has a more significant effect on lowering cholesterol than milk, perhaps due to the presence of hydroxymethyl glutarate in yoghurt, which blocks the formation of cholesterol from acetate (Chilliard et al. 2003). Orotic acid, lactose, calcium, and casein are only a few of the many dietary components suggested as possible contributors to hypocholesterolaemia. Goat milk producers and consumers, especially in industrialized nations, have shown a consistent interest in this hypothesis because of the hypoallergenic and medicinal benefits of goat milk and products (Saleena et al. 2022a; Saleena et al. 2022b).

3.4 Prevention against inflammatory bowel disease (IBD)

While Crohn's disease and ulcerative colitis are each unique, they are classified as inflammatory bowel diseases (IBDs). Although IBD and Crohn's disease are defined by intestinal inflammation that persists and flares, they are treated differently. In contrast, Transmural inflammation is the hallmark of Crohn's disease, which can manifest in any part of the gastrointestinal system but most commonly affects the ileocolonic region (Carneiro et al. 2018). Because of its increasing frequency and negative impact on patient well-being, IBD has become a major public health issue in recent years. Although researchers have looked into what causes IBD, they still do not know much about it. Irritable bowel syndrome (IBS) is usually treated pharmaceutically and occasionally additionally with prebiotics and/or probiotics (Chilliard et al. 2003; Selvaggi et al. 2014). Contrarily, there should unquestionably be more therapeutic options available. In Spain, two studies were conducted on induced colitis in rats using goat milk oligosaccharides. Studies have shown that oligosaccharides in goat milk have anti-inflammatory properties. Weight loss predicted colon enlargement, and necrotic lesion progression are all halted by the oligosaccharides. The clinical symptoms (diarrhea, and bloody stools) were also less severe, and the immune response was less severe (less neutrophil infiltration). The untreated rats were provided a standard diet devoid of oligosaccharides (Basnet et al. 2010; Lund and Ahmad 2021).

3.5 Antimicrobial, immunostimulatory, anti-inflammatory, and anti-carcinogenic properties

Goat milk's lactoperoxidase protein is efficient against a wide range of bacteria, including those that cause pneumonia (*Klebsiella pneumoniae*), cholera (*Vibrio cholerae*), dysentery (*Shigella dysenteriae*), typhoid (*Salmonella typhi*), and food poisoning (*Staphylococcus aureus*). Bovine lactoperoxidase has been used in trials showing that goat milk has antimicrobial effects (Quigley et al. 2013; Clark and Mora García 2017). Goat milk oligosaccharides have anti-inflammatory properties due to their ability to bind to and remove a wide variety of pathogens, as well as to inhibit the heat-stable enterotoxin produced by *Escherichia coli* and to prevent the contact between leukocytes and endothelial cells (Novac and Andrei 2020). It has been shown that medium-chain fatty acids have antimicrobial activity, particularly against gram-negative bacteria (Kumar et al. 2016). When goat milk is digested by pepsin, antimicrobial peptides are generated that are active against gram-negative bacteria. There is evidence that fermented goat milk, like fermented cow milk, can inhibit the growth of *Serratia marcesens* (Chauhan et al. 2021).

Selenium is an important mineral for maintaining a healthy immune system. Selenium levels in cow milk are low, while goat milk levels are much higher, suggesting that it and its derivatives

can help people stay healthy by boosting their immune systems. Natural killer (NK) cells, T lymphocytes (T-cells), and B lymphocytes are crucial to the immune system's innate and adaptive responses (B-cells). Despite their structural similarities, IgG and IgA comprise most serum immunoglobulins and are linked to many biological properties (Faccia et al. 2020; Poppitt 2020). Similar immunological classes include IgM, IgD, IgA, IgG, and IgE. The body's immune response can be partly predicted by nutritional status, among other variables (Ra et al. 2022). Recent *in-vitro* and human research has shown that goat milk has immunomodulatory effects; thus, it may be a good option for those with a cow milk allergy and looking for an alternative. In recent research, goat milk has been demonstrated to have a number of impacts on human blood cells, including inducing NO release and driving cytokine production (IL-10, TNF-α, and IL-6). In addition to revealing antibacterial activity that may assist milk drinkers in avoiding becoming sick, the release of nitric oxide (NO) may also safeguard the heart of milk consumers (van Leeuwen et al. 2020; Kazimierska and Kalinowska-Lis 2021).

Goat milk protein does not contain allergens. Cow milk's higher lipid content than goat milk may also cause mucus buildup. Goat milk does not promote digestive system inflammation since its fat globules are a tenth the size of cow milk (Novac and Andrei 2020; Hirsiger et al. 2022). Antioxidant and anti-inflammatory goat milk is necessary for all biological functioning. Oxidation causes numerous diseases, including cancer, and inflammation is the body's principal response to infection (Kumar et al. 2016). Goat milk protects against pathogenic infection allergies, but keeping a healthy gut microbiota with probiotics and prebiotics is also important (both of which are found in goat milk) (Faccia et al. 2020; Hirsiger et al. 2022).

Conjugated linoleic acid (CLA) concentrations are exceptionally high in goat milk. *In vitro* research on human melanoma, colorectal and breast cancer, and animal studies on mammary and colon cancer have demonstrated that CLA slows tumor growth. Fermented goat milk has been hypothesized to have antioxidative properties, interfere with the receptor-mediated actions of estrogen, and disrupt the eicosanoid-dependent cell signalling systems, all of which may be involved in CLA's tumor-inhibitory effects (Lund and Ahmad 2021; Mirzaei et al. 2022).

3.6 Functional food, food intake, and mineral absorption

The technological community is concerned about the novel structures that develop when milk's calcium and proteins react (Deepak et al. 2020a). Milk and colostrum include several beneficial bioactive components that help control weight and hypertension. Digestion and health are also affected. Goat milk can be considered a functional and neutraceutical beverage because of its high concentration of these substances. Goat milk is readily absorbed because its chemical makeup is similar to human milk. Hence, it increases the bioavailability of the nutrients inside it (Hirsiger et al. 2022). Researchers are increasingly interested in antioxidant peptides due to their potential to reduce or postpone the oxidative spoilage of foods. Hydrolysis of goat milk proteins in vitro with enzymes or fermentation with lactic acid bacteria can yield powerful antioxidant peptides. Antioxidant peptides have many beneficial properties, including the ability to scavenge free radicals, chelate iron, and stop the autooxidation of polyunsaturated fatty acids (Chen et al. 2020).

Humans get more iron and copper from goat milk. Unlike cow milk, goat milk has a similar concentration of oligosaccharides as human milk. It is widely established that they function as prebiotics in the gut and boost digestive health. Bifidobacteria, the good bacteria in the gut, are their doing. Bifidobacteria improve lactose maldigestion and have been linked to several other health benefits, such as increased immunity, protection from pathogenic infections, reduced risk of cancer, and lower cholesterol levels (Clark and Mora García 2017; van Leeuwen et al. 2020).

The goat is a bioorganic sodium animal, while the cow is a calcium animal in naturopathic medicine. Bioorganic sodium is essential to keep joints flexible and dynamic. Goat milk delivers 35% of the calcium humans require daily in one cup (Novac and Andrei 2020; Saikia et al. 2022). In addition, only one cup of goat milk supplies as much as 20 percent of the recommended daily value for riboflavin. Furthermore to phosphorus, goat milk is a superb supplier of the nutrients such as potassium and vitamin B12. Zinc (a mineral with antioxidant potential) bioavailability is enhanced by goat milk. Goat milk's lower TBARS levels may be attributable to the fact that its fat is more efficiently used for nutrition, which in turn reduces its availability as a substrate for lipid peroxidation and, in turn, its formation of free radicals (Marius et al. 2020; Kazimierska and Kalinowska-Lis 2021). Goat milk's greater bioavailability of magnesium and zinc and its improved fat quality may explain why it has a good effect on genomic integrity even when consumed regularly by animals subjected to an iron-overloading feeding regime. Genomic stability is improved through magnesium metabolism because DNA is constantly being destroyed by exogenous mutagens and the body's mechanisms (Lund and Ahmad 2021). Evolutionarily, cells have adapted various DNA repair mechanisms to limit mutation frequency. Magnesium is a cofactor essential for almost all of the stages of nucleotide excision repair, which is the primary repair mechanism for DNA damage induced by environmental mutagens. The principal tool for mending endogenous DNA damage is base excision repair (Kumar et al. 2016; Faccia et al. 2020).

After consuming goat milk for breakfast, people reported feeling less hungry and less compelled to eat, indicating that the milk had a somewhat more satiating effect than cow milk. After an

overnight fast, fortified goat or cow milk did not influence appetite, fullness, or preferences for sweet, salty, savory, or fatty meals. When given the option, mice, and rats of all ages favored goat milk over cow milk (Lund and Ahmad 2021). By studying gene expression in the brain circuit involved in feeding, scientists confirmed that this hedonic food preference for goat milk is regulated centrally. There has to be an analysis of the implications for either adult or infant feeding habits (Marius et al. 2020).

Goat milk enhanced mineral uptake more than cow milk in mouse models of poor intestinal absorption. Bone health and iron absorption were significantly improved by goat milk in iron-deficient rats (Deepak et al. 2020a; Deepak et al. 2020b; Lund and Ahmad 2021). Calcium and vitamin D absorption were similar in vitamin D-deficient rats, whether given goat or cow milk enriched with vitamin D (Costa et al. 2016; Deepak et al. 2020b). In a 3-week-old piglet digestive model, goat and cow milk had similar mineral absorption. Hence, goat milk or cow milk fortification may absorb minerals better, depending on the person. This research implies that goat milk would be as helpful as cow milk in the newborn formula, including minerals and vitamins (Kok and Hutkins 2018; Gallier et al. 2020).

3.7 Heart health and cardiovascular diseases

Heart disease and stroke are the two leading killers among the wealthy world's population. Coronary heart disease, irregular heartbeats, high blood pressure, and atherosclerosis are just a few of the many disorders included under this umbrella term. Most cardiovascular occurrences may be traced back to CVD, primarily caused by the buildup of atherosclerotic plaque in the artery walls. Atherosclerosis is exacerbated by several risk factors, including a sedentary lifestyle (smoking, poor nutrition, and lack of exercise), high blood pressure, abnormal lipid profiles, diabetes, and obesity. Although its origin is a mystery, one of its fundamental mechanisms appears to be the accumulation of atherogenic lipoproteins within the artery walls (Costa et al. 2016; Lund and Ahmad 2021). Low-density lipoprotein (LDL), an atherogenic lipoprotein that transports cholesterol from the liver to the arteries, is often called "bad cholesterol". High-density lipoprotein (HDL) transfers cholesterol away from blood vessels, transforming it into oxidized low-density lipoprotein (ox-LDL), accelerating atherosclerosis. So, it comes to reason that antioxidants, which can prevent LDL oxidation, can aid in the decrease of atherosclerosis (Clark and Mora García 2017; Lund and Ahmad 2021).

Goat milk contains the enzyme angiotensin-converting enzyme (ACE), the peptide inhibitory peptide (IP), and the peptide antihypertensive (AHP). They are effective in halting the spread of disease and stopping the spread of bacteria. Immunoglobulins, proteose peptone, lactoferrin, transferrin, calmodulin (calcium-binding protein), ferritin, prolactin, and folate-binding protein are minor milk proteins (Chandran et al. 2021b). NPN levels are higher in goat and human milk than in cow's milk. Taurine, a sulphur-containing amino acid, and carnitine, a vitamin for neonates, are found in goat milk and help the body's metabolic functions. Goat milk contains higher minerals and vitamins than cow milk (Prosser 2021).

Medium-chain triglycerides, polyunsaturated fatty acids, and omega-3 fatty acids are all present in higher concentrations in goat's milk than in cow's milk. As a bonus, these are good for the heart. Yet, unlike cow's milk, goat's milk has a lower percentage of cholesterol. Goat milk's fatty acid composition offers cardiovascular disease protection (Poppitt 2020). Due to its high potassium content, goat milk effectively lowers blood pressure and cholesterol levels. Plasma triglyceride levels were lower in goat milk drinkers, indicating a beneficial influence on lipid metabolism. Goat milk has lower and more steady levels of total cholesterol and hepatic intoxication indicators (transaminases; glutamate pyruvate transaminase and glutamate oxaloacetate transaminase) than cow's milk. Goat milk can also combat and prevent heart disease (Carr et al. 2021).

3.8 Consumption in infancy

In many areas, goat milk is still widely used as an infant formula substitute. Regarding baby nutrition, there is a conflicting scientific opinion regarding goat milk. Unpasteurized goat milk is safe for adults and older children, but experts agree that it should not be given to newborns and toddlers (Gallier et al. 2020). Infectious diseases such as brucellosis, tuberculosis, and brucellosis can be spread through drinking unpasteurized milk. Pasteurized goat milk or formula made from goat milk is another option as a cow milk substitute (Roy et al. 2021). Due to insufficient essential nutrients, including folic acid, vitamin B12, and iron, in ordinary goat milk, commercially made formulas are strongly advised. Megaloblastic anemia, brought on by a lack of folic acid or vitamin B12, has been documented in infants fed homemade goat milk formulae. Goat milk alone induced hypernatremia, brain hemorrhages, and azotemia in a newborn because goat milk has more salt than human milk (Lund and Ahmad 2021; Prosser 2021). Due to their immature kidneys, infants should avoid consuming high sodium levels. Fortified goat milk formulae may be a viable alternative to cow milk, which lacks certain nutrients (He et al. 2022). Researchers evaluated goat and cow milk on development and fat absorption in healthy newborns and underfed children (Carr et al. 2021). Malnourished youngsters (aged 1–5) who were given either goat or cow milk showed similar weight and fat absorption increases. The study milk was supplemented with the same vitamins and minerals the subjects usually took to make it nutritionally equal. Healthy infants have been studied again regarding the differences between goat and cow milk formula (with comparable nutrient contents). Neither group outgrew the

other significantly faster than the other (Lund and Ahmad 2021; Prosser 2021).

3.9 Goat milk products and their importance in human nutrition

Typically, milk from goats would be produced on smaller farms. People have been processing goat's milk and eating the resulting goods since ancient times. Allergens are absent from the fresh milk of well-cared-for, well-fed goats (Ballabio et al. 2011; Kumar et al. 2016). Roquefort cheese and Leben are both favorite goat milk products. But goat milk is incompatible with ghee-making because its fat globules are too tiny, creating issues with the separation process and the resulting aroma and flavor. Baby formulas made from goat milk are of high quality. Yoghurt, cheese, evaporated milk, ultra-high-temperature milk, ice cream, milk powder, pasteurized beverages, and traditional milk products are made from goat milk (Faccia et al. 2020; Saleena et al. 2022b). This trend toward using goat milk in product manufacturing is likely attributable to the milk's well-documented functional properties and health benefits. Yet, goat milk produces an unpleasant "goaty" or "muttony" taste. Furthermore, because goat milk is low in folic acid, it is necessary to augment replacement diets with folic acid when goat milk products are used (Sousa et al. 2019; Lund and Ahmad 2021).

Lactic acid bacteria as a probiotic starter culture increase intestinal microflora, lactose intolerance, immune system activation, antibacterial activity, anti-tumor, anti-cholesterolemic, and antioxidative capabilities (Kok and Hutkins 2018). The rising demand for nutritious foods has prompted the creation of cutting-edge scientific items in the food business. Many studies have been done on fermented milk (Quigley et al. 2013; Lejaniya et al. 2021b; Saleena et al. 2022a). Many anecdotal reports of positive health effects from consuming goat milk suggest this may become the next big thing in probiotic fermented milk. Fermented goat milk (*Lactobacillus fermentus* ME-3) has antioxidative and antiatherogenic effects in healthy people (Chauhan et al. 2021). Fermented goat milk containing a mixed starter culture (*Lactobacillus helveticas* PR4, *Streptococcus thermophilus* CR12, and *Lactobacillus plantarum* 1288) reduced hypertension by producing GABA (gamma amino-butyric acid) as an inhibitory neurotransmitter in the central nervous system (Kok and Hutkins 2018; Mirzaei et al. 2022). Recently, it has been demonstrated that consuming fermented goat milk (*Lactobacillus rhamnosus* CRL1505) can improve mucosal immunity and resistance to intestinal and respiratory infections in an immunosuppression mouse model. As previously established, goat milk has a nearly non-existent folic acid level. This issue could be addressed in fermented food by including microorganisms that produce folate during fermentation. Goat milk fermented with a combination of *Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus*, making a yoghurt having high folate content and favorable sensory qualities (Quigley et al. 2013; Kumar et al. 2016; Saleena et al. 2022b).

Researchers conclude that goat milk can potentially serve as a neutraceutical health beverage. Those who are intolerant to or allergic to the proteins in cow's milk can get what they need from goat's milk (Lejaniya et al. 2021a). Anyone with anemia, osteoporosis or malabsorption issues might also benefit from consuming goat milk. Due to its purported health benefits, goat milk's popularity and demand have soared in recent years. Children and infants benefit more from goat milk than cow's milk; however, parents should be aware that goat milk is low in essential nutrients like folic acid (Lund and Ahmad 2021; He et al. 2022).

4 Conclusion and future perspectives

This review report suggests that goat milk's nutritional value and flavor make it a good substitute for cow and human milk. Due to its high fat, protein, mineral, and vitamin content, goat milk is healthy for all ages. Goat milk's medicinal potential, ease of digestion, and buffering capacity make it a popular ingredient in many products. To a chemical degree, goat milk is equivalent to, if not superior to, human and cow milk. Goat milk's functional and nutritive properties can help with various human health and wellness aspects, including expansion, maturation, and upkeep. Goat's milk, unlike cow's milk, is better for infants, the elderly, and anyone recovering from illness or injury because of its higher nutrient content and nutraceutical characteristics. Goat milk is highly relevant to the food sector because of its number of bioactive compounds and its multiple physiological roles. Dengue, cardiovascular disease, immunological problems, etc., are only some chronic disorders that can benefit from this therapy method. As we have seen, goat milk has many advantages over other milks, including cow and human milk. The above benefits explain why goat milk has become more popular than cow and human milk. Due to its exceptional nutritional, therapeutic, nutraceutical, and physiological benefits, goat milk should be promoted in areas with high rates of poverty and a poorly functioning health sector, where malnutrition is of the most concern.

The unique characteristics of goat milk have led to extensive study of its nutritional value and the effects it may have on health. Goat milk, on average, does not differ significantly in composition from cow milk. Research is needed to find ways to mask the "goaty flavor" of goat milk, which turns off many potential consumers. Goat milk's medicinal components, fatty acid profile, and ease of digestion all point to it being a potential treatment or preventative measure for various health problems. Researchers have found that goat milk appears to help with their animal models of malabsorption disorders and inflammatory bowel diseases. There is some evidence that drinking fermented goat milk can lower the

danger of cardiovascular disease. Goat milk is highly cross-reactive with cow milk; hence cow milk allergy sufferers should avoid it. Most studies conduct their analyses on animals to extrapolate their findings to human subjects. Although there is some evidence that consuming goat milk products is beneficial, more research is needed.

Goat milk appears to have features that make it helpful in treating or avoiding certain medical diseases due to its high digestibility, balanced fatty acid profile, and abundance of bioactive chemicals. Animal studies suggest that goat milk may help with malabsorption and inflammatory bowel diseases. Fermented goat milk's antioxidant and antiatherogenic qualities may reduce cardiovascular disease risk. Due to severe cross-reactivity, goat milk is not recommended for cow's milk allergy sufferers. Most animal research shows prospective results. Goat milk products' health advantages need human research.

Even if there is still a lot to understand about goat milk, it is evident that it provides an alternative to those looking for dairy-free options. According to compositional analysis, infant formulae made from whole goat milk that maintains milk fat and is enriched with essential fatty acids, lactose, and vitamins can meet compositional parameters without whey. Clinical trials have shown that a formula produced with whole goat milk proteins and lipids is safe and effective. Goat milk is distinct from cow milk in many important respects, including composition and function, and these differences may be significant when evaluating the biological role goat milk plays for humans. Growing evidence from animal studies, for example, suggests that goat milk may alter the gut microbiome and immunological pathways important in allergy treatment in a way distinct from that of cow's milk. These results are significant for infants because of the immaturity of their digestive and immunological systems at birth.

Further clinical trials and translational studies are needed to verify the beneficial effects of this treatment on babies' gastrointestinal health and eczema. Current knowledge originates from either in vitro or animal trials. Further human studies are needed to compare the gut microbiota profile of infants fed goat or cow milk formula and assess whether adding natural milk oligosaccharides to goat milk formula improves microbiome diversity.

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Sheep Associated-Malignant Catarrhal Fever: Past, present, and future

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ABSTRACT

Members of *Artiodactyla* can contract the infectious disease Malignant Catarrhal Fever (MCF), which has a wide range of symptoms. Ten known viruses contribute to the disease, the two most significant ones being *Ovine gamma herpes virus 2* (OvHV-2) and *Alcelaphine gamma herpes virus 1* (AIHV-1). In the African subcontinent, AIHV-1 is seen in most MCF cases. In the Indian scenario, *Ovine gamma herpes virus-2* is the main culprit. MCF is reported in certain pockets of India. Its threat to wildlife is not yet completely understood. In AIHV-1, wildebeests serve as the primary MCF reservoir, whereas with OvHV-2, the primary MCF reservoir is sheep. In India, OvHV-2 causes MCF in deer species, bison, and water buffaloe. The life cycle and properties of this virus are not yet wholly deciphered. To understand the impact of the disease and the threat it may pose in the future, we need to have diagnostic techniques in place. Currently, PCR is the most commonly used diagnostic technique. Work should be done on field-oriented tests like ELISA and LFA, which are helpful in areas without sophisticated lab facilities. Treatment protocols must be in place, as culling bovines is not an accepted policy in India. Probable plans for overcoming all these problems are discussed in this article.

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1 Introduction

Malignant Catarrhal Fever (MCF) is an intriguing lethal condition that affects *Artiodactyla* members like cattle, bison, deer, water buffalo, and pigs (Russel et al. 2009; Cordduzza et al. 2022). Lymphoid hyperplasia in lymphoid organs, lymphoid cell accumulation in non-lymphoid organs, and various organ failures brought on by immunological dysregulation are the disease's hallmarks (Nelson et al. 2010). Currently, 10 viruses of the genus *Macavirus* (Subfamily: *Gammaherpesvirinae*, Family: *Herpesviridae*) causes MCF disease (O'Toole and Li 2014). Wildebeests commonly harbor *Alcelaphine gamma herpes virus-1*(AIHV-1), and sheep harbor *Ovine gamma herpes virus 2* (OvHV-2) without showing any clinical signs and act as a source of infection to susceptible animals. The AIHV-1 is mainly found in Africa and some zoological enclosures in different parts of the world. OvHV-2 cases are reported worldwide and affect deer species, bison, and water buffaloe. OvHV-2 infections are also reported in some zoological collections.

OvHV-2 has never been isolated in cell culture, although lymphoblastoid cell lines from clinically sick animals contain viral DNA despite AIHV-1's lengthy history of cell culture isolation (OIE 2018). MCF illness can manifest as an acute form in which mortality may occur within hours or as a chronic type consisting of symptoms like high temperature, excessive nasal and ocular discharge, bilateral corneal opacity, and muzzle necrosis (Headley et al. 2020a; Iván et al. 2022). Diagnosis of the disease is possible by histopathology, DNA detection by PCR, and serological assays (OIE 2018).

2 History

In Africa, Malignant Catarrhal Fever linked with wildebeest (WA-MCF) was discovered in the early 1900s. In 1929 transmission of sheep-associated MCF (SA-MCF) from blood was observed. Plowright et al.(1960) isolated the *Alcelaphine gamma herpes* virus from wildebeest for the first time and successfully propagated it in cattle, rabbits, and monolayer tissue cultures. Based on a histological investigation, sheep-associated MCF (SA-MCF) was first described in India in 1975 (Parihar et al. 1975). OvHV-2 identification in sheep provides proof of the presence of SA-MCF illness (Wani et al. 2006; Banumathi et al. 2008; Sood et al. 2014; Kumar et al. 2021). The secrets this pandora's box holds are not yet wholly unveiled.

3 Virion properties

Order *Herpesvirales* is divided into 3 families (*Herpesviridae, Alloherpesviridae,* and *Malacoherpesviridae).* Our interest here is in *Herpesviridae*, which affects mammals and birds. The *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae* subfamilies make up the *Herpesviridae* family. Double-stranded DNA viruses from the *Macacvirus* genus of the *Gammaherpesvirinae* subfamily are the agents of MCF illness (Davison et al. 2009). *Macavirus* consists of the 10 MCF-causing viruses, including *Alcelaphine gamma herpes virus 1, Alcelaphine gamma herpes virus 2, Hippotragine herpes virus, Oryx MCF, Ovine gamma herpes virus 2, Caprine herpes virus 2, Caprine herpes virus 3, Ibex MCF virus, Muskox MCFV, and Aoudad MCFV*. Common reservoirs and susceptible hosts of some MCF-causing viruses are given in Table 1.

Among these, *Alcelaphine gamma herpes virus 1* and *ovine gamma herpes virus 2* are the most important ones. In Indian conditions, *Ovine gamma herpes virus 2* is the primary pathogen causing the disease in water buffaloe, cows, deer species, and gaur. Sheep act as reservoirs for OvHv-2 and result in disease transmission to susceptible hosts.

Table 1 Common reservoirs and susceptible hosts of some MCF-causing viruses

Virus	Reservoir	Clinically susceptible
Alcelaphine gamma herpes virus 1	Wildebeest	Cattle, Deer
Alcelaphine gamma herpes virus 2	Hartebeest	Cattle, Deer
Hippotraginegamma herpes virus 1	Roan antelope	No reported cases of MCF
Oryx MCF *virus*	Oryx	No reported cases of MCF
Ovine gamma herpes virus 2	Sheep	Cattle, Gaur, Deer, Pig, Giraffe
Caprine gamma herpes virus 2	Goat	White-tailed deer
Caprine gamma herpes virus 3	Goat	Reindeer
Ibex-MCFV	Ibex	Bongo, Anoa, Pronghorn
Muskox-MCFV	Muskox	No reported cases of MCF
Aoudad-MCFV	Aoudad	No reported cases of MCF

Source: Li et al. 2014

Alcelaphinegamma herpes virus 1 has an estimated genomic size of approximately 110 kbps (Seal et al. 1989). The essential structural components of the *Herpesvirus* are nucleocapsid, tegument, and envelope. DNA is covered by a capsid that protects, transports, and delivers nucleic acid inside the host cell. Tegument has multiple functions, such as capsid transport, regulation of transcription, translation, apoptosis, replication, and viral assembly (Kelly et al. 2009). Amines, lipids, and glycoproteins are present in the envelope interacting with the host immunity.

Hart et al. (2007) used a clinically MCF-affected bovine lymphoblastoid cell line for complete sequencing of the OvHV-2 genome and reported the genome size was 130 kbps. Sequencing disclosed the presence of 73 ORFs (Open Reading Frame), of which 62 showed homology with other *Gamma herpes viral* genes. This research also revealed a few unique genes that could be utilized in planning control strategies, such as Ov7 and Ov8 genes that code for viral glycoprotein. It's a fact that the virus's surface glycoproteins are crucial in viral attachment to the host cell, which is the first step of viral replication. Products of these genes can be effectively used in designing a vaccine (Russel et al. 2009). The details of the same will be discussed in control strategies.

4 Geographical distribution

AIHV-1-associated MCF is predominantly seen in the African subcontinent, where a significant wildebeest population intermixes with cattle during grazing periods. AIHV-1 is a severe concern in Africa. In susceptible domestic species and ruminants in international wildlife parks, OvHV-2 is the primary cause of MCF.

5 Disease transmission

5.1 *Alcelaphine gamma herpes virus*-1 (AIHV-1)

The virus is transmitted through direct contact, contaminated pastures, and aerosols from reservoir animals. Newborn wildebeests are exposed to the AIHV-1 virus within three months of their age, and it's calves secrete the virus from their nasal and ocular secretions. Interestingly, wildebeests never formally manifest the disease, but they shed viruses that are the primary source of infection in vulnerable animals (Mushi et al. 1981).

5.2 *Ovine gamma herpes virus 2* (OvHV-2)

Transmission is predominantly through aerosols and direct contact (Kim et al. 2003). Lambs are exposed to viruses and become reservoirs as early as 3 months of age. Sheep transmit the disease to susceptible animals. The disease is reported when reservoirs and cattle are separated by 70 meters and in bison up to 5 km (WOAH 2022).

6 Replication of virus

Not much research has been done to understand the complete replication of different MCF-causing viruses. However, as they are herpes viruses, let's consider that MCF-causing viruses share similar replication steps (Figure 1). The entry of the virus occurs after membrane fusion or endocytosis of an associated virion, both of which are facilitated by glycoprotein complexes that contain glycoprotein B, gH, and gL (Myster et al. 2020). Immediate early genes carry out the regulation of succeeding gene expressions. The DNA replication complex, as well as many enzymes and other proteins involved in altering host cell metabolism, are encoded by early (E) genes, while late (L) genes largely encode virion proteins (Gatherer et al. 2021). After primary infection, on activation of immediate early genes, lytic replication will be seen in the case of herpes viral infection. If immediate early genes are not activated, there will be an establishment of latency in neurons, lymphocytes, etc. (Riaz et al. 2017). This unique phenomenon of herpes viruses has led to the introduction of a postulation called 'Herpes harmony'. It explains that in herpes viral infection if the host responds with a robust immune response, it leads to the establishment of latency. If the host's immune response is weak, it leads to lytic replication and active infection. However, suppose there is an

Table 2 Geographical distribution of the MCF

S. N.	MCF causing virus	Geographical distribution
1	*Alcelaphine gamma herpes virus 1*	Africa
2	*Alcelaphine gamma herpes virus 2*	Africa
3	*Ovine gamma herpes virus 2*	Africa, Asia, Europe, North America, South America
4	*Caprine gamma herpes virus 2*	Europe, North America, Asia
5	*Caprine gamma herpes virus 3*	North America
6	*Hippotragine gamma herpes virus 1*	North America
7	*Ibex*-MCFV	North America

Source: Headley et al. 2020b

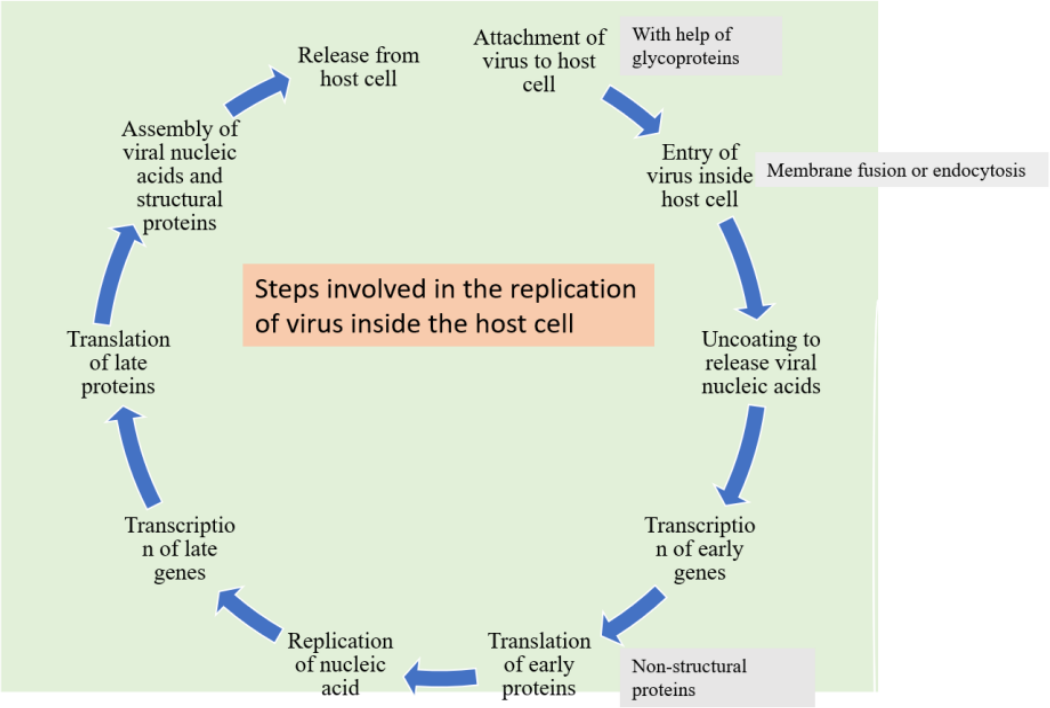

Figure 1 Replication of virus inside the host cell

immune-compromised condition in latency-established animals due to host-associated (very young or very old, immunodeficiency due to chemotherapy, other concurrent infections, organ failures)/ pathogen-associated (altered tissue tropism, condition of the non-native host)/latency may be converted to lytic replication, and active disease may be seen (Sehrawat et al. 2018).

6.1 Intranuclear events in herpes viral infection

Herpes viral naked DNA that enters the host cell's nucleus resembles double-stranded breaks (DSB). Naked viral DNA help in the triggering of DNA damage response proteins (DDRPs). DDRPs are ubiquitously present in the host cell, and they result in the initiation of a cascade of events which in turn activates p53, a tumor suppressor protein, and the result is the cell cycle arrest. This mechanism is in place to avoid replicating cells with damaged DNA. Since viral DNA resembles damaged DNA inside the nucleus, its presence inhibits the cell cycle (Full and Ensser 2019).

6.2 Importance of *ORF 73* in *MCF* infection

AIHV-1 genome is maintained as latent episomes and is a classic example of herpes viral latency. A unique genome maintenance protein coded by ORF 73 is responsible for latency induction and maintenance. It was once believed that ORF 73 was solely necessary for installing latency. However, it is now known that ORF 73 is also crucial for the induction of infection. So ORF 73 deleted recombinant virus can be effectively tried as a vaccinal candidate (Palmeira et al. 2013).

6.3 Immune evasion strategies by herpes viral infection

The Herpes virus utilizes multiple strategies (Figure 2) to evade the host immune system (Griffin et al. 2010). The methods included in viral evasion are (a) Downregulation of peptide transport to MHC (Major Histocompatibility Complex) class I molecules, (b) MHC class I molecule expression is downregulated, (c) MICA (MHC class I polypeptide-related sequence A) and MICB shedding inhibits NKG2D (Natural killer group 2D) receptors of NK cells, (d) The HLA-G is expressed and secreted (Human leucocyte antigen-G) that binds to KIR2DL4, ILT2 (Immunoglobulin-like transcript 2), ILT4 (Immunoglobulin-like transcript 4) and inhibit NK (Natural killer) and cytotoxic cells, (e) Increased expression of PDL1 (Programmed death Ligand-1) which results in avoiding attack by immune cells, and (f) Viral micro RNAs inhibit the production of pro-inflammatory cytokines.

6.4 Interference of virus in host *micro-RNA* generation

Host micro-RNAs are essential in cell development, immunity, maintenance, and death. Mature mi-RNAs generated by host cells are inhibited by *Alcelaphine gamma herpes virus*1 and *Ovine gamma herpes virus* 2, which results in the availability of ribosomes for viral transcription and translation (Bruscella et al. 2017).

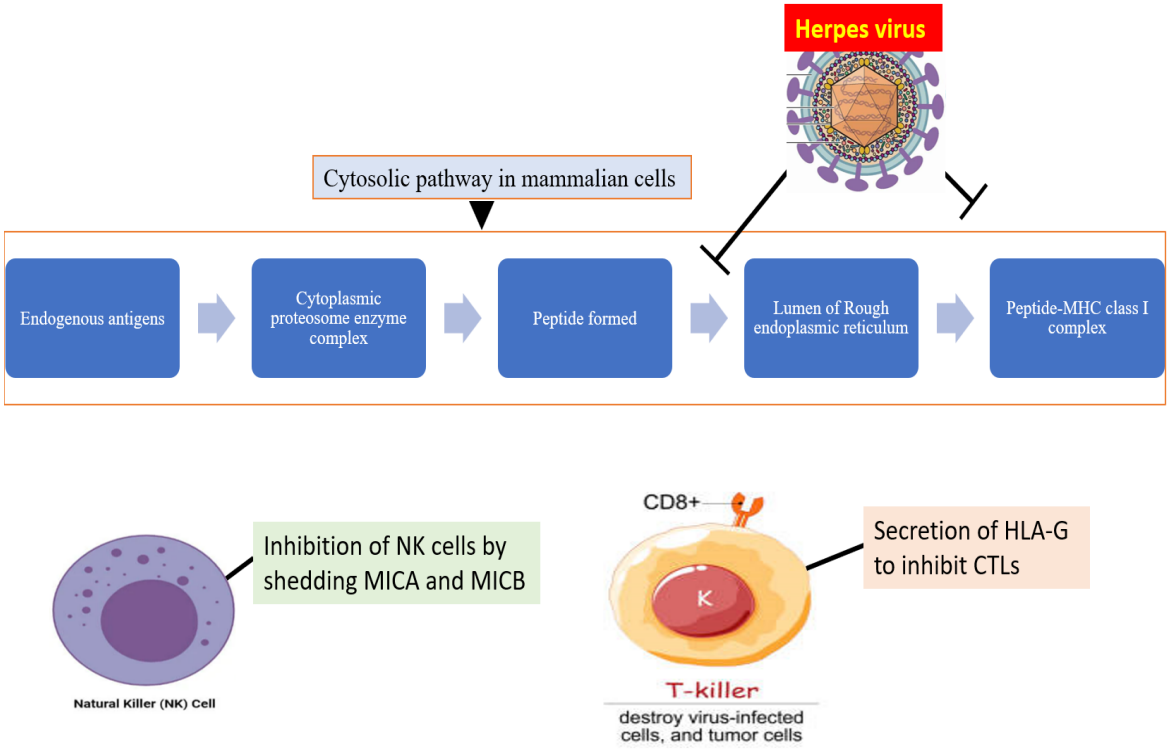

Figure 2 Herpes virus strategies to evade the host immune system (Source: Jasinski et al. 2020)

7 Clinical signs

Affected animals developed fever, corneal opacity, ocular discharges, nasal discharges, and rarely abortion. Suffering animals will have dried muzzles, lack of appetite, and dehydration. Less frequently, interdigital ulcerations, glossitis, and ulcerative gingivitis are also seen (Bildfell et al. 2017; Headley et al. 2020a).

8 Diagnostic techniques

8.1 Histopathology

Histological changes such as epithelial deterioration, vasculitis, hyperplasia, and necrosis of lymphoid organs, as well as significant interstitial accumulations of lymphoid cells in non-lymphoid organs, have been used to demonstrate the presence of MCF (OIE 2018). In the infected animals, Cytotoxic T lymphocyte has been seen in higher number than Helper T cells (Dewals and Vanderplasschen 2011). Epithelial degeneration and necrosis are seen in multiple organs (Sharma et al. 2019).

Recently conventional histopathology has been replaced by Immunohistochemistry, Immunofluorescence, and in-situ PCR (Headley et al. 2020b). Among them, in-situ PCR is helping to understand the complicated life cycle of multiple viruses causing MCF (Simon et al. 2003).

8.2 PCR

To specifically detect Ovine gamma herpes virus 2 DNA in naturally occurring and experimentally induced cases of sheep-associated Malignant Catarrhal Fever, Baxter et al. (1993) developed a hemi-nested PCR. This method helped identify the tegument protein coded by the ORF75 region of the *Ovine gamma herpes virus 2*. This standardized PCR is utilized to identify MCF in suspect cases. The sheep population in Karnataka was subjected to a cross-sectional study (Premkrishnan et al. 2015). A Hemi-nested PCR test was used to identify the OvHV-2 genome in blood samples, revealing that 24.4% of sheep were carriers.

One more unique gene in the case of SA-MCF is glycoprotein B, coded by the Ov-8 gene. PCR is standardized to identify the Ov-8 gene present in different variants. Dunowska et al. (2001) noticed the similarity in glycoprotein (gB) sequences from bovine and healthy ovines indicating transmission between them.

8.3 Viral isolation

As discussed earlier, Dr. Plowright isolated the *Alcelaphine gamma herpes virus 1* for the first time in 1960. In 1970 he isolated the C500 strain in bovine thyroid cells from an ox suffering from the clinical disease. To this day C500 strain is considered a wild-type strain (maintained with as minimum passages as possible) and used in challenge studies.

AIHV-1 can be isolated in bovine embryonic bovine tracheal cells, VERO cells, and Madin-darby bovine kidney cell line (Hristov and Peshev 2016). It has been challenging to gather enough virus DNA to decode the genome of OvHV-2 because the virus has not yet been propagated in vitro. However, a lymphoblastoid cell line BJ1035 is maintained, which consists of a virus and is used for sequencing (Hart et al. 2007).

8.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Li et al. (1994) developed a competitive inhibition ELISA using mAb-15A for an antigen conserved in all types of MCF viruses to understand the antibody response in different animals for different MCF viruses. Powers et al. (2005) also observed that cattle could become infected with OvHV-2 without developing clinical signs of MCF and that PCR assay and CI-ELISA can be readily used to detect OvHV-2 and MCFVs infected cattle, respectively. Russel et al. (2022) developed an indirect ELISA using recombinant ORF65 (capsid protein) expression to identify antibodies against OvHv-2 in sheep and cattle. An effective commercial kit is not yet available based on the above-described method.

8.5 Electron microscopy

To comprehend the structure of the WC 11 strain of the Alcelaphine gamma herpes virus, Castro and Daley (1982) used electron microscopy.

9 Prevention and control

The best way of prevention is to keep infected and carrier animals apart from susceptible species. Strict quarantine and testing of new animals entering the herd in the zoo and domestic enclosures is necessary. Infection can also be prevented by providing separate grazing and water-drinking areas for sheep and bovines. Extension activities regarding disease symptoms in endemic regions are essential to facilitate better reporting and control of the disease. In zoological enclosures, only seronegative animals should be introduced.

9.1 Vaccination

Plowright et al.(1975) injected formalized preparations of *Alcelaphine gamma herpes virus 1* developed in cells, mixed with Freund's incomplete adjuvant, and reported the presence of neutralizing antibodies. Despite this, there was no discernible defense against parenteral challenges involving pathogenic viruses. The reason may be due to MCF viruses' multiple immune evasion strategies.

The significance of ORF 73 in the induction of infection and latency was established by Palmeira et al. (2013), and the disruption of ORF 73 may be a potential candidate for a vaccine. Further, Myster et al. (2020) identified that the spreading of AIHV-1 between cells was held with the help of the A7 gene and propagated through the A8 gene. Strains with altered or deleted A7 and A8 genes can be effectively tried as a vaccine candidate. Similarly, Shringi et al. (2021) used recombinant BoH-4 to deliver OvHV-2 glycoprotein. It conferred partial immunity against MCF in the Rabbit model.

9.2 Antiviral drugs

Anti-herpes viral drugs could be used, but their efficacy in treating animals is poorly documented. Thymidine analogs such as iododeoxyuridine and guanosine analog antiviral drugs such as acyclovir could also be tried.

9.3 New strategies

ATF3, which is known as a stress-induced transcription factor, functions in a way relatable to ORF A2 and ORF Ov2. ATF3 results in the induction of latency-associated transcript (LAT). It might be possible to inhibit the induction of LAT and the development of latent infection if the mechanism of ATF3 induction were to be identified. Increased LAT leads to increased neuronal survival, host micro-RNA silencing, and decreased apoptosis of infected cells. Thus, ATF3 inhibition may help in putting a well-waited pin in the coffin of herpes viruses (Knipe et al. 2015).

Conclusion

To this date, PCR-based identification is most commonly used. As of right now, sheep-specific commercial ELISA kits are not accessible. Field-oriented antigen detection tests are not researched until now, which could be a good tool for early disease identification. The culling policy is not a well-accepted option for disease-affected bovines in India. So, some treatment protocols could be standardized to care for infected animals. Much research regarding epidemiology, life cycle, and novel diagnostic techniques has to be carried out.

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The use of medicinal plants for combating breast cancer: A comprehensive review

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ABSTRACT

Breast carcinoma is a common illness among females. Various therapies, including hormone therapy, surgery, radiotherapy, chemotherapy, and targeted treatment, have been available to treat existing breast cancer. These therapies can potentially halt the development and spread of cancer, especially if the disease is at an early stage, but all these treatments have various adverse effects on human health. Cancer cells proliferate more rapidly than most normal cells, so chemotherapy is the most suitable treatment. Certain medications can cease dividing cells by destroying the cell's control center region. Other drugs can inhibit the chemical processes essential for cell division. On the contrary, because cancer is frequently identified at a late phase, treating the disease is extraordinarily challenging. Therefore, it is advisable to avoid this fatal condition from occurring. Multiple studies have revealed a continuous inverse connection between cancer and natural materials, such as plant extracts, their fractions, and active principles. These bioactive phytochemicals' have synergistic or cumulative effects in the treatment of cancer disease. This review article examined the effect of various extracts/fractions/active principles obtained from diverse plant origins against breast cancer disease. Information regarding the most commonly used plants, including *Alpina galaga*, *Urtica dioica*, *Annona muricata*, *Rosmarinus officinalis*, *Ficus carica*, *Nigella sativa*, *Murraya koenigii*, and *Urtica dioica* have been presented in this study. Owing to the information in this study, these plants exhibited anticancer activities in preclinical MCF-7 carcinoma models by decreasing cell proliferation, inducing programmed cell death, and triggering cell-cycle arrest. The information generated from this review will significantly contribute to developing knowledge of the scientific and medical communities in developing innovative breast cancer treatments.

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1 Introduction

Random proliferation and differentiation of breast cells are the cause of breast cancer. It is common among females and reported as an important medical problem (Saha et al. 2021). Several factors including family history, the existence of BRCA1 or BRCA2 genes, pregnancy after age 30 or infertility, lack of physical exercise, being overweight or obese, and having dense breast tissue, are responsible for developing breast cancer. Along with these, increased usages of alcohol, oral contraceptives, and hormone replacement treatment (HRT) containing just estrogen may also be responsible for the development of breast cancer (Dhama et al. 2018; Abd El-Hack et al. 2022; Rafeeq et al. 2023). Other factors include cigarette smoking, exposure to chemicals like digoxin, ethylene oxide, polychlorinated biphenyls, a prior history of breast cancer (Figure 1), and previous treatment with radiation, particularly before the age of 30, are also associated with the development of cancer.

Over many years, numerous illnesses have been treated with naturally occurring medications. Historically, these treatments depend on herbs, plant extracts, and other plant-based compounds. The advantages of these treatments are the synergistic effect of phytochemicals and other plant-based products without any side effects. Previous studies have already well established the role of various phytochemicals in cancer treatment; these plant products may affect cancer development by altering and detoxifying carcinogens (Abdel-Basset et al. 2020). The four most common anticancer drugs with plant derivatives are vinca alkaloids, epipodophyllotoxins, taxanes, and camptothecin (Upreti et al. 2022). In the 1960s, researchers discovered the role of *Taxus brevifolia* bark extract in cancer treatment (Wani et al. 1971). Taxol and vinca alkaloids were also found effective in stopping the cell cycle by preventing the depolarization of microtubules (Zajączkowska et al. 2019). Recent review article have comprehensive information on some selected plant sources, including *Annona muricata*, *Nigella sativa*, *Ficus carica*, *Alpina galaga*, *Murraya koenigii*, *Urtica dioica*, and *Rosmarinus officinalis*, and their role in the treatment of breast cancer (Dhama et al. 2018; Abd El-Hack et al. 2022; Rafeeq et al. 2023). This study emphasizes the protective and therapeutic impacts of various herbal extracts, fractions, and bioactive constituents on treating breast cancer.

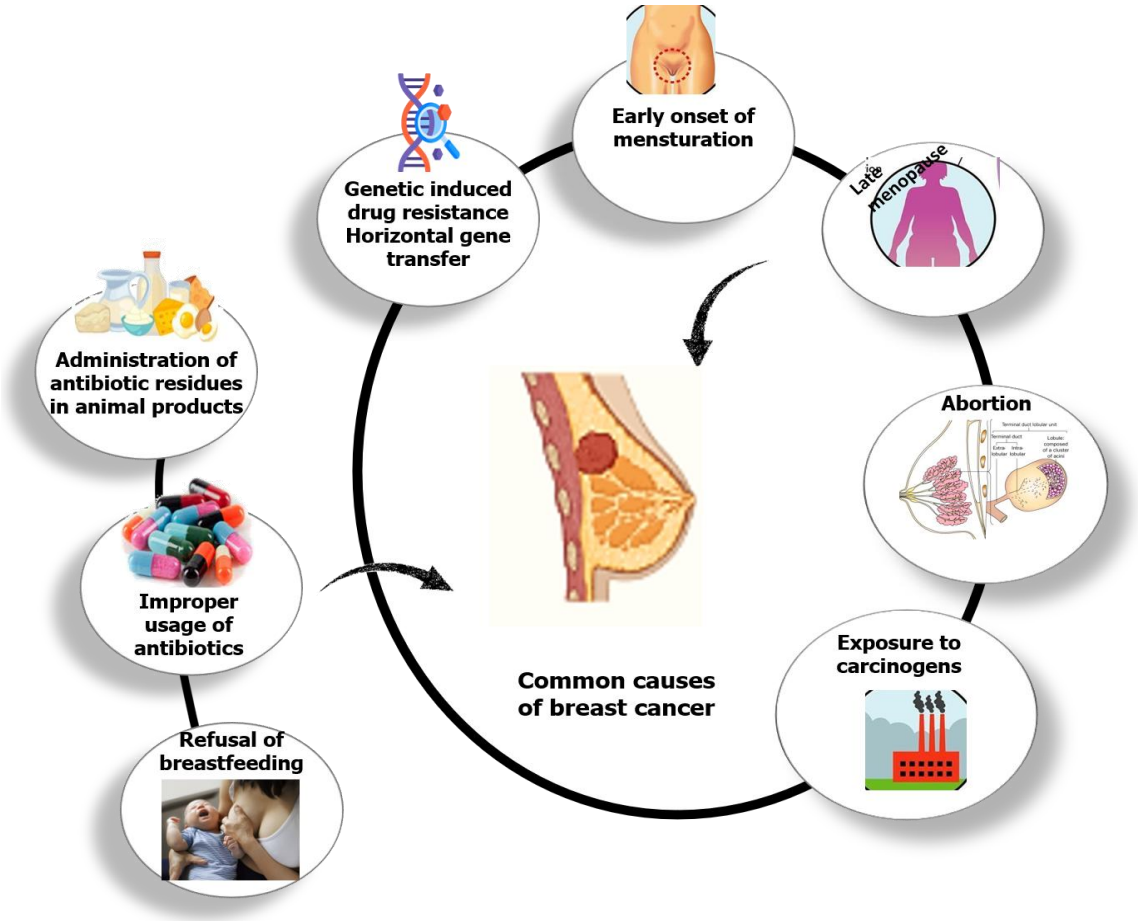

Figure 1 Common causes of breast cancer

2 Classification of various cancers

Cancer is a severe form of the disease that is characterized by uncontrolled cell development. The growth, differentiation, and death of body cells are generally well-programmed. In contrast, some cells, especially those bearing somatic and epigenetic mutations, may escape destiny and grow out of control (Alshaeri et al. 2018; Alshaeri et al. 2020). This kind of abnormal growth can form the so-called neoplasm. A localized neoplasm restricted to the tissue of origin is a benign tumor, while neoplasms can invade other tissues and form secondary tumors, malignant tumors, namely cancer (Alshaeri et al. 2018; Alshaeri et al. 2020). Cancer is also caused by a succession of gene changes that alter the cell's functions (Hassanpour and Dehghani 2017). There are around 100 different forms of cancer that have been identified. The origin and kind of cell are the most critical factors in classifying malignancies (Hiatt et al. 1977). Cancer invades the body's immune system by controlling the body cells in a way that leads to lumps and masses of tissue, otherwise called abscesses. Owing to annual studies, cancer is responsible for over 2% of fatalities worldwide.

Furthermore, according to the American Cancer Society (ACS), 14.1 million people were newly diagnosed with cancer cases in 2012, of which 8.2 million died. It is expected that around 21.7 million people will be diagnosed with tumors by 2030, among these 13 million being in their terminal stage of cancer (Edge et al. 2010). Many lifestyle difficulties, including smoking, physical inactivity, poor diet, and low pregnancies, contribute to cancer risks, particularly in the American cancer community's developing countries (Srivastava and Tiwari. 2022).

Somatic mutations in oncogenic and tumor suppressor genes may ultimately lead to cancer development. In the case of human beings, cancer can begin almost anywhere in the human body. In the case of man, lung, prostate, bronchus, colon, rectum, and urinary bladder cancer is the most common type. While in the case of females, breast, lung, bronchus, colon, rectum, uterine corpus, and thyroid cancer are common. Owing to this data, prostate and breast cancer account for a significant part of the tumor in males and females, respectively. Blood cancer and malignancies of the brain and lymph nodes are responsible for the highest percentage of cancer patients among youngsters. The top six cancer types for both sexes account for more than 50% of newly detected cancer patients and deaths worldwide. Breast cancer in females is the most estimated tumor type (11.7% of total cases) in 2020, subsequent by lung (11.4%), prostate (7.3%), colorectal (10.0%), gastric (5.6%), and liver (4.7%) cancers (Prasad et al. 2020). The most estimated cancer mortality came from lung cancer (18%), subsequent colorectal (9.4%), stomach (7.7%), liver (8.3), and breast (6.9%) cancers. For males, prostate cancer is responsible for 32.5% of estimated new cancer patients in 2020, followed by colorectum (10.5%), bladder (6.6%), lung (6.4%) cancers, and melanoma of the skin (6.4%) (Naik and Sellappan 2021). For females, breast cancer currently accounts for 26.2% of estimated new cancer cases in 2020, followed by colorectum cancer (11.2%), lung cancer (8.4%), melanoma of the skin (7.4%), and corpus uteri cancer (5.1%). Cancer-related mortality is variable in different cancer types. The most significant cancer mortalities in Sweden are expected from lung cancer (15.7%), colorectum cancer (12.7%), prostate cancer (9.8%), and pancreatic cancer (8.3%) (Prasad et al. 2020; Kariyil et al. 2021). The major types of cancer can be classified into five border groups as follows

1 Carcinoma is cancer that affects cells within and outside the body, including the mammary gland, lungs, and intestine colon.
2 Sarcoma can be identified by the location of cells in the body, such as bones, adipose tissue, muscles, etc.
3 Lymphoma is a malignancy affecting lymph nodes and other immune system organs.
4 Leukemia is a bone marrow malignancy that frequently manifests in the blood.
5 Adenomas originate in secretary glands, such as the thyroid, adrenal, pituitary, and other thyroid tissues (Dhama et al. 2018; Abd El-Hack et al. 2022; Rafeeq et al. 2023).

3 Medicinal plants as anticancer agents

Herbs have a long history of being used in cancer therapy. In the analysis of cancer-fighting plants, Hartwell included over 3000 plants that can be used in cancer treatment (Arain et al. 2022; Abd El-Hack et al. 2022). The Greek physician Hippocrates (460-370 BC), known as the "Father of Medicine," created the term "cancer." Abscesses and ulcers were referred to as "carcinoma" by Hippocrates. Another Roman physician, Galen (130-200 AD), used the term once (a Greek word meaning swelling) to denote tumors (Zishan et al. 2017). Cancer is the world's 2nd largest etiology of death. Cancer was becoming more common in 2014, and around 1,665,540 people in the United States had cancer, with 585,720 dying (Zishan et al. 2017).

Surgery, chemotherapy, and radiotherapy are most commonly used in cancer treatment but are effective only in the condition of early diagnosis (Agarwal et al. 2013). Instead of these therapies, medicinal plants can provide raw materials and high-quality food for livelihoods which can fight against illnesses. Based on traditional applications and scientific data, much study has been done on these herbs for tumor treatment, and several plant products have been commercialized as anticancer medications (Chanchal et al. 2018). Medicinal plants are estimated to have over 8,000 species, and some of these plants have been effectively used for medicinal purpose; their significance is also scientifically established, while traditional healer still utilizes others, but their scientific medicinal value has yet to be discovered. Western uses of these data are now being scrutinized more closely, and most

academic and industrial researchers now respect national and indigenous rights to these resources (Chanchal et al. 2018). Approximately three-quarters of the world's population use plants and other traditional medicine to treat ailments. There are at least 250,000 different plant species, with over a thousand having anti-cancer properties (Chanchal et al. 2018).

Medicinal herbs continue to have a significant part in the healthcare system of the world (Abd El-Hack et al. 2019, 2022). Plants' medical and economic advantages are being more widely recognized and developed in emerging nations and enterprises. Herbal supplements, botanicals, and phytomedicines are the products of botanicals used to cure or improve an individual's health. In herbal treatments, raw herbal medications were used to cure illness problems, typically incurable, or to reach or maintain a better state of health were used in medical treatment for an extended period (Chanchal et al. 2018). Because of natural antioxidants, plant-based herbal products serve as decreasing agents and natural remedies that can combat cancer. Bioactive compounds, like isoflavones, flavones, flavonoids, coumarins, anthocyanins, catechins, lignans, and iso-catechins, account for a significant portion of their antioxidant activity, and these natural products can mitigate or reduce the toxic side effects of radiation and chemotherapy by strengthening their anti-cancer action (Nema et al. 2013).

4 Cytotoxicity of some medicinal plants against breast cancer

The study of cancer treatment has expanded significantly. Both traditional and highly contemporary methods are used for the treatment of cancer. Chemotherapy, radiation therapy, and surgery are some standard methods widely used to treat cancer, but each has certain drawbacks. Alternative therapeutic options are required due to the increased cancer incidence worldwide, and herbal therapy offers a convenient alternative to conventional cancer treatment (Yeap et al. 2015).

After going through the previously published literature, some plants were shortlisted which have significant effects against many cancers cell lines, including those found in the breast, gastric, colon, oral, lung, liver, cervical, and blood systems. This review article chose plants based on their reported solid anticancer properties. The presence of secondary metabolites in the plant extracts has shown anti-cancerous properties by inhibiting cancer cell lines via DNA destruction or activating enzymes inducing apoptosis. Herbal medicines are a safe, non-toxic, and widely accessible source of cancer-fighting rather than chemical therapeutics. Because of their various features, medicinal plants are thought to neutralize the impacts of diseases on the body and improve body performance (Aisyah et al. 2020).

Phaleria macrocarpa and *Fagonia indica* have historically been used as anticancer drugs. Active compounds such as gallic acid derived from the abovementioned plant cause apoptosis in cancer cells. Gallic acid was isolated as an active ingredient from *P. macrocarpa's* fruit extract and has been shown to cause apoptosis in lung cancer, leukemia, and colon adenocarcinoma cell lines (Aisyah et al. 2021). Figure 2 has shown the antitumor potential of some selected medicinal plants against breast cancer cell lines. A brief description of these plants have been given in the next section of this review article.

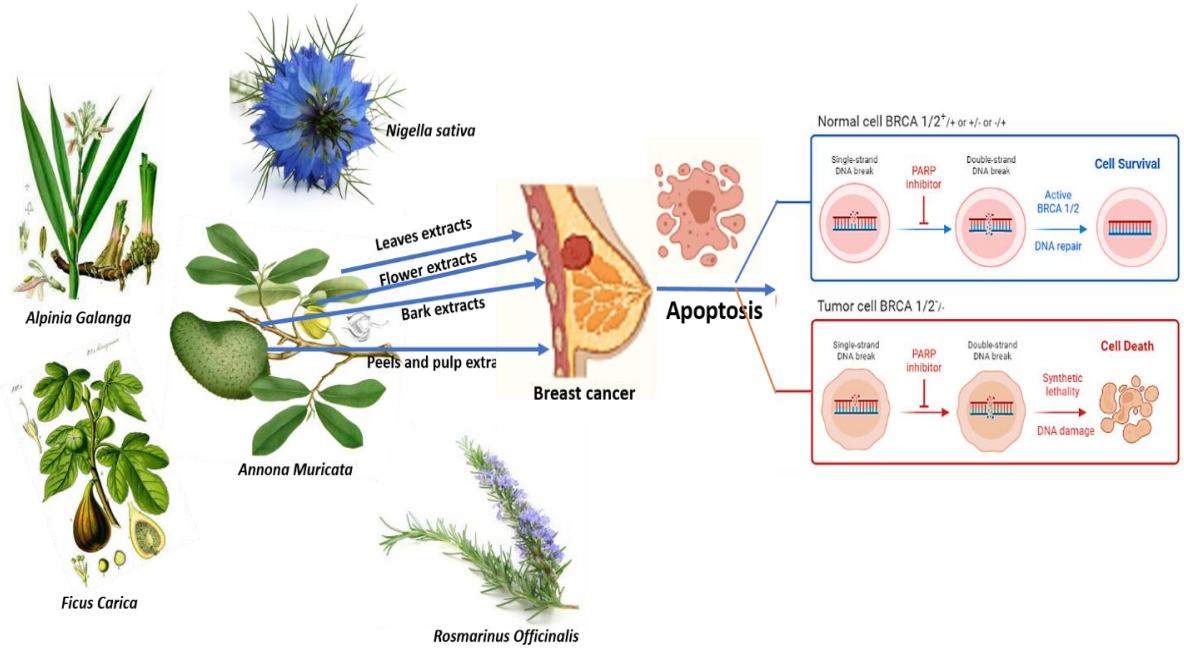

Figure 2 The antitumor potential of some medicinal plants against breast cancer, following examples of some medicinal plants.

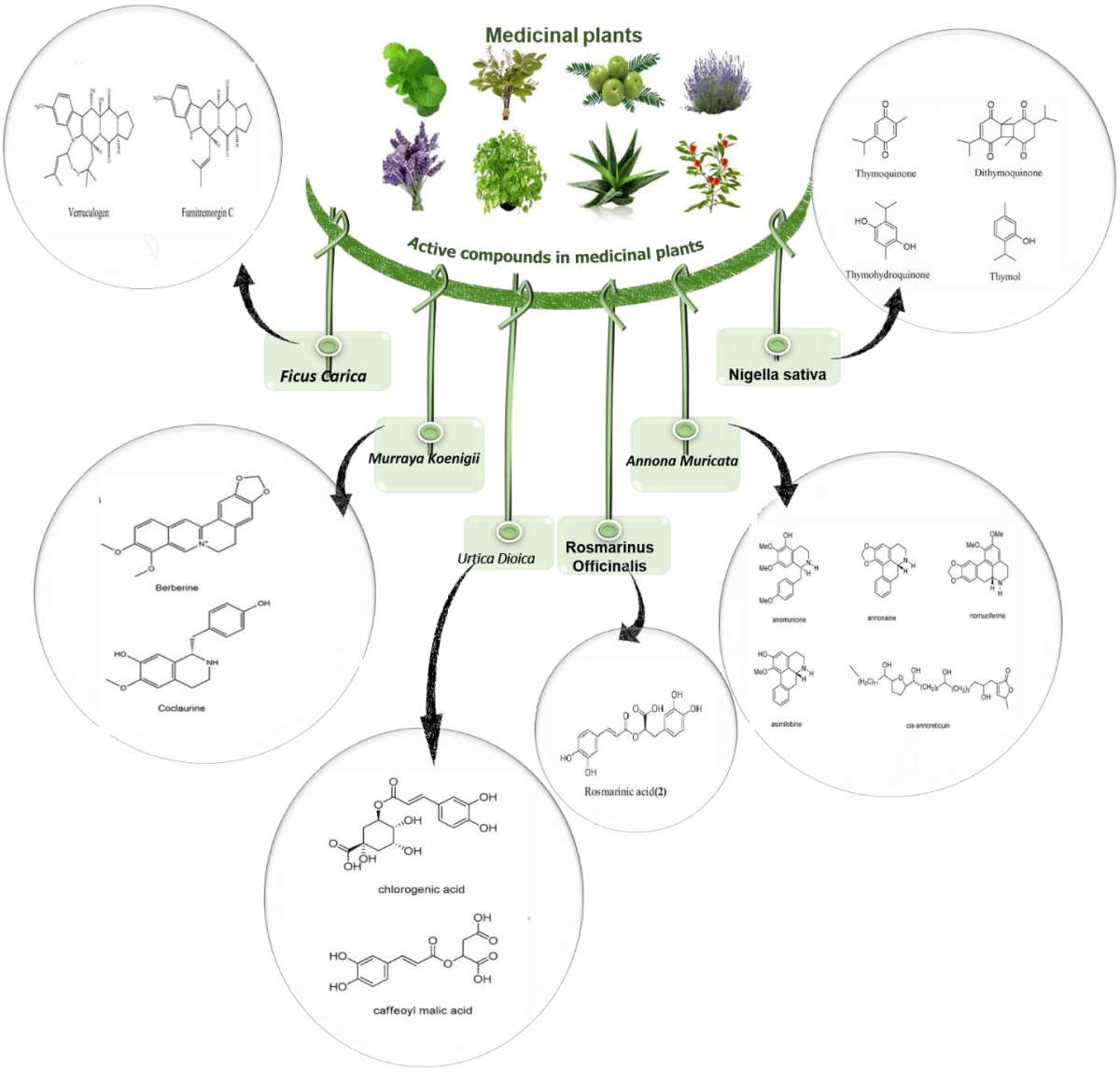

Figure 3 Active compounds in medicinal plants.

4.1 *Annona muricata*

Annona muricata (AM) is a tropical evergreen tree belonging to the family Annonaceae. Its pharmacological characteristics of this plants are associated with various activities like anti-inflammatory (Oliveira et al. 2017), anti-cancerous (Paul et al. 2013), antidiabetic (Spector et al. 2006), antioxidant (Spector et al. 2006), and antimicrobial activities (Pai et al. 2016). The plant contains mostly annonaceous acetogenins, acetogenins, and Cyclohexapeptides active ingredients (Figure 3) (Gajalakshmi et al. 2012). In a mouse model of breast cancer, the group treated with *A. muricata* raw extract had a smaller tumor size than the group that received no treatment (271.714.24 mm vs. 37525.98 mm). Histological analysis of tumor tissue revealed that treatment with *A. muricata* crude extract diminished the number of mitotic cells per tumor segment contrasted to the control group. In addition, *A. muricata* crude extract promoted programmed cell death in 4 T1 breast tumor tissues, reduced metastasis in most studies, regulated immunity, and lowered cancer-related inflammation (Syed et al. 2016). Ethanol extract of *A. muricata* leaves at a dose of 200 mg/kg bw enhanced MDA, SOD, and the histological portion of breast cells demonstrated a reduction in mammary epithelial hyperplasia (Muchtaromah et al. 2015). The highest efficacious dose of *A. muricata* leaf extract against DMBA-induced breast cancer development was 300 mg/kg (Sulistyoningrum et al. 2017). Further, Alshaeri et al. (2018, 2020) also reported that *A. muricata* extract suppresses proliferation by inhibiting EGFR-mediated signaling pathways, including

AKT/MAPK/NF-B and cyclin D1. Similarly, genotoxic annonacin isolated from *A. muricata* inhibited the growth of MCF-7 cells. ER actions as a ligand-dependent gene transcription enhance cancer progression in breast cancer. Due to its high piperine concentration, the MTT assay indicated that *A. muricata* extracts significantly affected MCF7 cancer cells. Similarly, Gudykunst and Nishida (1984) reported that solid lipid nanoparticles (SLNs) produced from *A. muricata* extract have a significant apoptotic impact against the MCF7 cancer cell line. Zeweil et al. (2019) suggested that treatment with *A. muricata* down-regulates the ER gene increases antioxidant characteristics and reduces lipid peroxidation. Also, Daddiouaissa et al. (2019) found that *A. muricata* fruit ionic liquid extract displayed an anti-proliferative effect on MCF-7 breast tumor cell lines by inducing apoptosis, arresting the cell cycle and lowering the number of new cells generated. Ethanolic extract of *A. muricata* inhibits T47D cell proliferation (Fertilita et al. 2020), stimulates mitochondrial cell death, inhibits cell growth, & reduces cellular motility in MDA cells (Kim et al. 2018). The IC50 value of the methanolic extract of *A. muricata* leaves was 85.55 g/mL against the MCF-7 cell line (Naik and Sellappan 2021). In another Prasad et al. (2020) research, *A. muricata* seed extract also induced apoptosis-mediated G0/G1 cell cycle arrest. In MCF-7 cell lines, *A. muricata* treatment significantly weakened the integrity of mitochondrial membranes, resulting in the death of breast cancer cells. The anti-proliferative effect of the ethyl acetate extract of AM leaf was due to a larger quantity of cytotoxicity on breast tumor tissue, which was caused by the *A. muricata* extract (Hadisaputri et al. 2021). Incubation of MCF-7 and MDA-MB-231 cells with scaffolds, including *A. muricata* leaf extract, inhibited their growth (Akpan et al. 2021). Kariyil et al. (2021) also demonstrated the lethal effect of *A. muricata* seeds extract by cellular membranes lysis and S-stage arrest. Similarly, Salsabila et al. (2021) suggested that 13 and 25 g/mL of *A. muricata* extract induced apoptosis in the G1 phase and G2/M arrest in 4T1 cells when paired with DOX by decreasing intracellular reactive oxygen species (ROS) levels. The essential oil of *A. muricata* leaves four sesquiterpenes, i.e., Z-caryophyllene, selinene, pinene, and elements, which are responsible for a decrease in MDA, VEGF levels & a rise in GSH degrees in a mouse model (Rojas-Armas et al. 2022; Figure 2).

4.2 *Alpinia galanga*

Alpinia galangal (AG) is a member of the Zingiberaceae family and is commonly found on Asian continents. It has been widely used as a culinary spice and herbal medicine to cure different illnesses for many years. Many pharmacological properties including antioxidant (Malik et al. 2016; Tang et al. 2018), anti-inflammatory (Baldo et al. 2016; Subash et al. 1970), antimicrobial (Saptiani et al. 2016), antibacterial (Hamad et al. 2016), and antiosteoarthritic activities (Chouni and Paul 2018) have been associated with this plant. This plant contains 1, 7-bis (4-hydroxyphenyl)-1, 4, 6-heparin-3-one (BH), 1,′S-1′-acetoxyeugenol acetate, and (E)-8, 17-epoxylab-12-ene-15 (BDMC) (Figure 3). In a mouse model of breast cancer, *A. galangal* extract exhibited apoptotic & anti-angiogenic effects by stimulating the caspase-3 pathway & preventing the NF-kB, NO, & COX-2 ways (Asri and Winarko 2016). The ethanolic extract of *A. galangal* was cytotoxic to MCF7 breast cell lines (Suhendi et al. 2017). Owing to Song et al. (2017), in human breast tumor cells, the active compound of *A. galangal*, i.e., galangin, inhibits the signaling cycle of TRAIL/Caspase-3/AMPK. *A. galangal* substantially reduced the development of 4T1 cells at IC50 concentrations of 135 µg/mL, whereas the cytotoxic impact increased at 50 and 100 µg/mL. In addition, *A. galangal* inhibited 4T1 cell migration & Dox-induced MMP-9 production. Leaves extract, and cisplatin demonstrated a synergistic impact on various cell lines involving MCF7, HepG4, CaCo2, & PANC1(Ahlina et al. 2020; Awad et al. 2020). This effect is achieved by stimulating apoptosis and lowering drug resistance genes (MAPK1 and MDR1). Moreover, *A. galangal* enhances p53 expression and apoptosis (Ahlina et al. 2020) and acts as an antiproliferative agent by triggering S phase arrest in the cell cycle (Raveesha et al. 2021). *A. galangal* boosted the lethal impacts of cytotoxic T-cells by reducing the abundance of human triple-negative cancerous cells (Alif et al. 2021). Further, *A. galangal* extract may also cause cell senescence, and its anti-proliferative effect against HER2-overexpressing breast cancer has been connected to internal ROS levels, delaying cell cycle progression (Jenie et al. 2021). In humans, the 1′-acetoxychavicol acetate constituent of AG suppressing the pERK1/2, pAKT, epidermal growth factor receptor 2, cyclin D1, estrogen receptor coactivator, and MYC proto-oncogene in a time and concentration-dependent manner (Jenie et al. 2021).

4.3 *Achillea wilhelmsii*

Plant *Achillea wilhelmsii* is a member of the Asteraceae family of the genus Compositaea. Leaf methanolic extract of this plant has cytotoxic effects on colon cancer cells line (HT-29). Various research has also demonstrated the benefits of plant leaf methanol extracts on colon, stomach, and breast cancer cell lineage. The methanolic extract of the plant has high concentrations of phenolic compounds mainly flavonoids inhibit cancer cell multiplication by triggering apoptosis. Kooti et al. (2017) reported that 1,8-cineole and a-piene isolated from the leaf essence of *A. wilhelmsii* are the two most significant monoterpene chemicals that trigger apoptosis in human melanoma cells.

4.4 *Camellia sinensis*

The buds and petals of this plant have been used for tea production. Tea leaves contain caffeine, thianin, and theophylline, like active ingredients, which are responsible for the antioxidant

properties of the tea leaves. Leaves of green tea also inhibited the production of 5-alfardoctase enzymes in mice. This enzyme transforms testosterone into dihydrotestosterone, a prostate cancer-causing substance. As a result, it has been found that green tea can help prevent prostate cancer (Wang et al. 2015). Green tea leaves are an essential source of epicatechin, epigallocatechin, and epigallocatechin-3 polyphenols which have anti-cancerous properties. Green tea's cytotoxic effect has also been reported against breast cancer cell lines. Drinking green tea regularly reduces the hazardous effect of stomach tumors (Srivastava et al. 2010).

4.5 *Ficus carica*

Ficus carica (FC) belongs to the family Moraceae, and this plant was initially endemic to West Asia & the Middle East. However, now it is prevalent in different parts of the word (Idrus et al. 2018). Numerous plant constituents are exploited for their medicinal properties in treating various ailments, including respiratory, inflammatory, cardiovascular, and gastrointestinal disorders (Bouyahya et al. 2016; Idrus et al. 2018). This plant also has antibacterial, antioxidant (Harzallah et al. 2016; Mahmoudi et al. 2016), anticancer (Hashemi and Abediankenari 2013; Tian et al. 2014), anti-acne (Vaghasiya et al. 2015), and antipyretic (Bouyahya et al., 2016) pharmacological properties. This plant species has several bioactive ingredients, like arabinose, amyrins, carotenes, glycosides, sitosterol, and xanthotoxol, which have various medicinal advantages (Figure 3). Zubair et al. (2015) reported significant cytotoxicity of ethyl acetate extract of *F. carica* against breast cancer (MCF-7) cell lines. By boosting the expression of proapoptotic (BAX) and tumor suppressor genes, the extract of FC leaves suppressed the growth of MDA-MB-231 cells (TP53 and TP21). In addition, Zhang et al. (2018) reported that FC extract administration decreased the breast cancer marker gene (GATA3) and also had an impact on the expression of exproto-oncogene (ELF5). Ghandehari and Fatemi (2018) reported that breast tumor volume and size decreased in rats treated with a latex extract of *F. carica*. Further, the histological study of fig latex-treated rat breast cells showed a reduction in angiogenesis, mitotic features, & necrosis. In another Lightbourn et al. (2008) study, *F. carica* leaves extract shortening the S and G2/M stages of the cell cycle and causing apoptosis by a p53-independent mechanism, also inhibited the spread of MDA-MB-231 mammary tumor cells (Sánchez-Valdeolívar et al. 2020). A combination of olive oil and fig extract demonstrated a cytotoxic effect against T-47D and MCF-7 cells (Widyaningrum et al. 2020; AlGhalban et al. 2021). The anticancer impact was detected in MDA-MB-231 cells, showing antiproliferative and antimetastatic activities of fig extract.

4.6 *Nigella sativa*

Nigella Sativa (NS) is a member of the family Ranunculaceae. It is considered a miraculous herb. Researchers have discovered many pharmacological characteristics, including anti-inflammatory (Ikhsan et al. 2018; Mokhtari-Zaer et al. 2020), antihypertensive (Lokeswara et al. 2019), antioxidant (Bordoni et al. 2019), antidiabetic (Bensiameur-Touati et al. 2017; El Rabey et al. 2017), antimicrobial (Bakal et al. 2017; Randhawa et al. 2017) and anti-cancerous properties (Czajkowska et al. 2017; Tabassum et al. 2018). Anti-cancerous action was demonstrated by the extra-expression of caspase-3, which is related to apoptosis in malignant tissue. The main active ingredients of this plant are monoterpenes, i.e., cumin aldehyde, 2-ethoxy-3-isopropylpyrazine, 3-sec-butylpyrazine, 2-methoxy-3-methylpyrazine, pinene, cuminic alcohol, pyrazines, 2-methoxy, terpinene, safranal, p-cymene, and thymoquinone, (Figure 3). The aqueous and crude extract of *N. Sativa* prevented the growth of MCF cell lines as efficiently as cisplatin (Elkady et al. 2015; Reddy et al. 2015). The histological examination of *N. Sativa* seed accompanied DMBA rats showed breast cell activation and inhibition of developing breast cancer cell proliferation. Thymoquinone was the main compound in *N. Sativa* seed oil, it decreased tumor volume, LDH, and MDA levels, and the activity of ALP and AST and this Thymoquinone (TQ) was found more effective than TT in suppressing the gene expression of Brca1 and Brca2 (Linjawi et al. 2015). Moreover, it significantly elevates the P53 gene expression (Dastjerdi et al. 2016). It has been established that the ultrasonic nanoemulsion formulation of *N. Sativa* essential oil induces apoptosis in MCF-7 cells and exhibits anticancer activity (Ma et al. 2008). According to Bumidin et al. (2018) *N. Sativa* extracts may decrease the integrity of MCF-7 cell membranes, inhibiting the development and viability of MCF-7 cells. Thymoquinone induces apoptosis via coordinating pro and anti-apoptotic gene expression. It suppresses metastatic development by activating JNK and p38 and lowering NF-kB and IKK/αβ phosphorylation (Imran et al. 2018). By regulating the development of Bax, Bcl-2, and COX-2, aqueous *N. Sativa* seed extract-derived silver nanoparticles (AgNPs) induce programmed cell death in MCF-7 cell lines (Rohini et al. 2019). Rafati et al. (2019) found that applying *N. sativa* gel, such as a prophylactic intervention, considerably prolonged and lowered the prevalence of ARD and moist desquamation in cancer tumor cases. Hydroalcoholic extract of *N. Sativa* suppressed breast cancer cell proliferation (MCF 7). A reduction in NF-kB and IKK mRNA expression levels demonstrated the anti-inflammatory effects of *N. Sativa* (Kordestani et al. 2020; Khurshid et al. 2020). *N. Sativa* extracted proteins have anti-proliferative and apoptotic activities on the human breast cancer cell line MCF-7. Further, H1047R and H1047L mutations can impair breast cancer's PIK3CA kinase domain regulation, resulting in increased PI3K/Akt1 pathway activation (Khurshid et al. 2020). Through suppressing autophagy, thymoquinone was found to limit the proliferation and metastasis of MDA-MB-231 cells (Zhou et al. 2022). The binding of *N. Sativa* isolated thymoquinone to the kinase domain of PI3CA mutants inhibits

TQ-mediated activation of the PI3K/Akt1 pathway. Further, *N. Sativa* seed oil drastically decreased cell proliferation and viability and worked as an anticancer and antiproliferative agent (Baig et al. 2022; Hussain et al. 2022).

4.7 Curcuma longa

Turmeric (*Curcuma longa*) is a plant of the Zingiberaceae family, and dried rhizomes of this plant are edible (Huseini et al. 2010). The cytotoxic activities of turmeric have been studied in liver cancer cells (Hep-2). It has been discovered that dose-dependent cytotoxicity of curcumin leads to cancer cell death via the mitochondrial route (Ayyadurai et al. 2013). In breast cancer, Ranjbari et al. (2014) research findings evaluated the impacts of turmeric extract on telomerase activity and found that telomerase has antiproliferative and inhibitory properties. Mohammad et al. (2010) have also discovered the cytotoxic impacts of turmeric on lung cancer cells by suppressing telomerase activity. Curcumin, a key component of turmeric, is vital in preventing and treating cervical cancer (Reda et al. 2020; Alagawany et al. 2021).

4.8 *Rosmarinus officinalis*

Rosmarinus officinalis, also known as rosemary, belongs to the family Lamiaceae. Hassani et al. (2016) reported that the seed oil of *R. officinalis* drastically decreases cell proliferation and viability so that it can act as an anticancer and antiproliferative agent. The pharmacological qualities of this plant included antioxidant and antibacterial (Takayama et al. 2016; Bajalan et al. 2017), anti-cancerous (Soundararajan et al. 2017), anti-inflammatory (Harris et al. 2019; Rocha et al. 2015) and antidiabetic properties (Ahamad et al. 2019; Belmouhoub et al. 2018). The anti-cancerous activity of this plant is associated with various phenolic acids such as quinic acid, caffeic acid, rosmarinic acid, and caffeoylquinic acids (Moore et al. 2016). *R. officinalis* essential oil reduced the viability of the MCF-7 cell line at a dose of 400 g/ml (IC50 = 48.01 0.94), as shown by the elevated concentrations of ADP-ribosyl polymerase (PARP) cleavage, a well-known marker for apoptosis (Tabatabaei et al. 2018). Farshchi et al. (2018) evaluated the cytotoxic impacts of *R. officinalis* aqueous extract green iron nanoparticles and reported an anti-proliferative effect with bleomycin medication (Mrdjanovic et al. 2019). Using 4T1 and MCF-7 cell lines, *R. officinalis* inhibited the growth and survival of MDA-MB-231 cells at low doses (0.5-20 µg/mL). It drastically decreased the protein kinase concentrations of Akt & mTOR, which are crucial growth and survival factors for cancer cells (Jaglanian 2019; Jaglanian and Tsiani 2020). RO ethanolic extract showed an antiproliferative impact against MCF-7 cancer cell lines (Shen et al. 2020). Mahmoud et al. (2021) also suggested that *R. officinalis* significantly reduced the phosphorylation/activation concentrations of Akt & mTOR at low levels (0.5-20 g/mL).

4.9 *Urtica dioica*

Urtica dioica (UD) is a widespread herb that belongs to the family Urticaceae and the genus Urtica (Badirzadeh et al. 2020). Various pharmacological activities such as anti-inflammatory (Liao et al. 2016), anticancer (Mohammadi et al. 2017), antirheumatic (Riehemann et al. 1999), cardiovascular (Saleem et al. 2002), antiaging, and antioxidant (Bourgeois et al. 2016) have been reported from this tree. The predominant flavonoids, including quercetin, 3-rutinosides, kaempferol, isoquercitrin, astragalin, rutin, isorhamnetin, and 3-glycosides were recorded from this plant (Figure 3) (Martínez-Aledo et al. 2020). DNA fragmentation and the TUNEL test revealed the harmful effect of *U. dioica* dichloromethane extract on the proliferation and spread of MDA-MB-468 cells. PCR found that apoptosis raised caspase-3 and caspase-9 mRNA expression levels while decreasing BCL-2. The research of Mansoori et al. (2017) lowered lipid peroxidation and increased the catalase enzyme activity in rat mammary carcinoma cells. Histological study showed that malignant animals treated with *U. dioica* had significant ductular proliferation and localized epithelial hyperplasia. Further, *U. dioica* extract can suppress the development and migration of mammary tumor cell lines in *in-vivo* models and regulate miR-21 gene expression of breast cancer (Telo et al. 2017). Real-Time PCR study demonstrated increased proapoptotic caspase three and caspase nine and a decrease in anti-apoptotic Bcl- 2. Akbarian et al. (2018) also reported cytotoxic impacts of *U. dioica* ZnO nanoparticles on MCF-7. Fattahi et al. (2018) analyzed ornithine decarboxylase (ODC1) and adenosine deaminase (ADA) gene expression to assess the anticancer impact of *U. dioica* aqueous extract on MCF-7 and MDA-MB-231 cell lines. They noticed that *U. dioica* stimulates apoptosis in mammary tumor cells by elevating the expression of ODC1 and ADA in MCF-7 cell lines. Further, 1200 g/ml of *U. dioica* hydroalcoholic extract reduces the number of MCF-7 cells (Soltani et al. 2021).

4.10 *Murraya koenigii*

Murraya koenigii (MK) is extensively distributed across Eastern Asia. This plant's diverse pharmacological effects are antifungal (Tripathi et al. 2018), antioxidant (Rehana et al. 2017; Tomar et al. 2017), antibacterial (Erkan et al. 2012), antidiabetic (Husna et al. 2018), anti-inflammatory (Iman et al. 2016; Mani et al. 2013) and anti-cancerous property (Yeap et al. 2015). This plant's active constituents include murrayanine, bi-koeniquinone-A, bismahanine, murrastifoline, murrayafoline-A, bismurrayaquinone, mukoenine-A,B,C, Murrayazolinol, murrayacine, and murrayazolidine (Figure 3) (Aniqa et al. 2022). *M. koenigii* aqueous extract dramatically lowered tumor size, and the histological qualities of *M. koenigii* leaf extract indicated its ability to control inflammation, reduce the number of tumor cells, and block tumor cell proliferation (Yeap et al. 2015). It also reduces

nitric oxide levels and inflammatory mediators cytokines and genes, enhancing T cell cytokine production, which aids in mitotic division reduction and delays breast cancer growth. Caspases-3 activity and TUNEL-positive cells enhanced after treatment with *M. koenigii* extract, indicating accelerated apoptosis (Noolu et al. 2016). Total alkaloid extract from *M. koenigii* reduced breast cancer cell viability (IC50 = 14.4 g/mL), altered development dynamics, arrested cells in the "S" stage, and induced cell death (MDA-MB-231) (Ismail et al. 2016). MTT assay showed the antitumor effectiveness of *M. koenigii* silver nanoparticles against breast cancer cell types (MDAMB-231). In rats with DMBA-generated breast cancers, the ethanolic extract of *M. koenigii* demonstrated anticancer activity (Vijapur et al. 2019). Significant reductions were seen in tumor volume, the number of polymorphonuclear leukocytes, multilayered cuboid epithelium, & the proliferation of solid collagen fibers following therapy with *M. koenigii* extracts (Aisyah et al. 2020). Additionally, Aisyah et al. (2021) discovered the elevation of caspase-3, which is related to apoptosis in malignant cells and has anticancer action. Mahanimbine, the active component of *M. koenigii*, has shown apoptotic and anti-angiogenic activity against breast cancer cells (Hobani 2022).

5 Plant Metabolites against cancer cell line

5.1 Colchicine

Colchicine is the secondary plant metabolite produced by *Gloriosa superba* and *Colchicum autumnale*. It induces mitotic binding during the cell cycle, making it a solid anti-mitotic medication in both *in-vitro* and *in-vivo* conditions. Colchicine extracts, such as 3-dimethyl colchicine, colchicoside, and thiocolchicocide, were created due to the severe toxic effects and demonstrated better effectiveness against some leukemic cells solid tumors (Sadooghi et al. 2013).

5.2 Podophyllotoxin

The roots of two *Podophyllum* species, *Podophyllum peltatum* L. and *Podophyllum emodi* Wallich contain podophyllotoxin. In the 1880s, it was dismantled, and its structure was explained in 1950. Epipodophyllotoxin is a podophyllotoxin isomer. Etoposide and Teniposide, two key therapeutic analogs derived from Epipodophyllotoxin, are highly successful in curing bronchial lymphomas, testicular cancer, leukemia, and ovarian cancer (Shoeb et al. 2006).

5.3 Taxanes

The Pacific Yew, *Taxus brevifolia* Nutt, contains paclitaxel (Taxol®) (Taxaceae). Their structure was initially discovered in 1971 and has been sold since the 1990s. *Taxus baccata*, an Ayurvedic medication from India, was also utilized to cure cancer (Kingston 2007). Because paclitaxel is insoluble in water and poisonous, another taxel, i.e., Docetaxel, a water-soluble molecule, was developed. Docetaxel (Taxotere®), a paclitaxel semi-synthetic product, is more effective. Docetaxel can be utilized for individuals resistant to paclitaxel (Kingston 2007). These medications are now accessible and can treat lung, prostate tumors, and lymphoid malignancies.

Conclusion and Further Perspectives

A rational and cost-effective solution for preventing a terrible illness like cancer exists. Daily use of herbs in infusions or meals may protect tissues from oxidative stress and prevent cancer development. Recent studies suggest that medicinal plants addressed in this study have anticancer properties. These plants can inhibit tumor volume and cell proliferation, enhance histoarchitecture, stimulate apoptosis, and interrupt cell-cycle in preclinical *in-vivo* and *in-vitro* models of breast cancer. To bring innovative commodities to market as either a chemopreventive drug or an anticancer treatment, however, clinical trials are essential to assess the impact of these herbs on humans.

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Journal of Experimental Biology and Agricultural Sciences, February - 2023; Volume – 11(1) page 41 – 53

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Plant RNA-binding proteins as key players in abiotic stress physiology

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Tolerance

ABSTRACT

Abiotic stress has a major effect on global crop production. Hence, plants have evolved and developed several response mechanisms to survive and grow under abiotic stresses. Plant cells can sense and respond to changes in different environmental stresses due to the specific modifications observed in gene expression, metabolism, and physiology. Only a few recognized sensors have been found due to the difficulty of functional redundancy in genes that code for sensor proteins. A defect in one gene causes no remarkable phenotypic changes in stress responses. Recent research has identified crucial RNA-binding proteins (RBPs) important for stimulus-specific responses. RBPs play a crucial part in plants' growth and development, post-transcriptional gene regulation, and RNA metabolism induced during stress responses. Among the currently identified over 200 different RBPs, the majority of which are plant-specific and carry out plant-specific functions. As an essential component of plants' adaptive process in different environmental conditions, RBPs regulate the following processes: RNA stability, RNA export, pre-mRNA splicing, polyadenylation, and chromatin modification. Plants have also developed different defense responses or molecular mechanisms to combat stress *via* genotypic and phenotypic expressions. With a unique understanding of RBPs in other organisms, RBPs functions in a plant are still limited. Hence, this review discusses the latest developments in RBPs function during the development and growth of plants, primarily under abiotic stress circumstances.

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1 Introduction

Plants are immobile organisms that adapt to various physiological changes and environmental stresses. Stress can be biotic and abiotic, which impacts plant productivity and fertility. Global warming and increased plant productivity are impacting the global population where agricultural products are being threatened by several factors such as temperature, precipitation changes due to climate change, and increasing concentrations of atmospheric carbon dioxide (CO_2) (Zhao et al. 2017; Singh and Thakur 2018). Abiotic stress like pH, temperature, salinity, drought, and climate change challenge the growth and development of plants (Singh and Thakur 2018; Dresselhaus and Hückelhoven 2018). Drought and salinity are considered major abiotic stresses that minimize plant productivity and challenge global food security (Munns et al. 2020; Téllez et al. 2020). These two stressors raise ion toxicity, oxidative stress, evapotranspiration, water, and nutrient deficiencies in plants (Téllez et al. 2020). Thus, developing stress-resistant plants is among the most significant challenges in agrobiotechnology research (Dresselhaus and Hückelhoven 2018).

Combating the abiotic stress requires analyzing the plant's functional metabolites with translational research and enhances resources for genetic studies. Increasing the limited gene pool of wild-type plants and conducting extensive molecular studies with the omics approach is essential. These studies will clarify the mechanisms underlying abiotic stress and their responses. Various translational approaches with next-generation sequencing, transcriptomics, metabolomics, and reprogramming techniques are used to enable the plants to overcome or tolerate abiotic stresses. Also, further knowledge of plant stress physiology and its complexities is explored by developing innovative computational tools (Dresselhaus and Hückelhoven 2018).

Gene expression and its regulation occur at transcriptional and post-transcriptional levels, which is critical for plant growth and development. Plant response and adaptation to different external stimuli depend specifically on post-transcriptional regulation. Regulation of RNA metabolism, among others, is an essential modification that involves RNA-binding proteins (RBPs) directly or indirectly (Lee and Kang 2016). Research is focused on elucidating the molecular mechanisms underlying stress responses. Plants have diversified RBPs in different cellular and physiological processes. Translational investigations on RBPs and RNA-protein interactions have been done that led to the identification of numerous conserved protein motifs and domains in organisms, including plants. These conserved portions are RNA-recognition motifs (RRMs), zinc-finger motifs, K-homology domain (KH), arginine- and glycine-rich domains, and SR repeats (Jung et al. 2013). RBPs are widely recognized as functional modulators in major abiotic stresses involving the ones mentioned earlier (Marondedze 2020). To deal with abiotic stress, plants reprogrammed their gene expression rapidly, which requires transcriptional and post-transcriptional modifications. An essential molecular response by plants with an increased focus on post-transcriptional gene regulation along with RNA granules' discovery as stress granules have led to the recognition of crucial RBPs due to environmental stresses (Yan et al. 2022).

The identification of several plant RBPs is essential for stimulus-specific responses. Plants can also respond against abiotic stress stimuli because of these RBPs. They are now widely acknowledged as a regulating element of post-transcriptional gene expression (Marondedze et al. 2019). The binding of RBPs to mRNA occurs *via* RNA-binding domains (RBDs). This binding determines the amount of RNA accessible for translation, stability, turnover, and other critical elements for stimulus-specific responses (Marondedze et al. 2019). External conditions resulting from sudden environmental changes, such as climatic changes, salinity, temperature, pH, and desertification, significantly affecting plant growth, development, and productivity, is the main focus of this review.

Numerous abiotic stresses like high temperature, salinity, drought, heavy metals, submergence, and nutrient insufficiencies harm a plant's development and growth. This is attributable to the emerging ecological effects of climate change on plant growth and development (Bellard et al. 2012). These ravaging effects of climate change (abiotic stresses) have thus initiated research on developing climate change-resilient plants (Rosenzweig et al. 2014). Hence, this review discusses the latest developments in RBPs function during a plant's development and growth, primarily in abiotic stress conditions.

2 Plant Stress Physiology and its repercussions on abiotic stresses

The concept of plant stress introduces the biotic and abiotic stresses which impact plant growth, development, and productivity. The abiotic stresses play an adverse role as the external conditions are the most stressful environments that affect plant growth. Any abiotic stress factor leads to less productivity and affects global food production. Hence, it is imperative to study the various factors that affect plant physiology and how plants respond to these abiotic stress (Shabala and Munns 2017).

Stress response depends on several factors, such as the duration and severity of stress, tissue specification, and genotype of the plant. The physiological responses to abiotic stress may have three possibilities i.e. tolerance, susceptibility, and avoidance (Figure 1). Tolerance mechanisms allow plants to survive either by tolerating or by avoiding stress. The ability to tolerate a particular stress over time made these plants stress-resistant, as they can adjust or acclimate to the stress. The tolerance mechanism also allows for

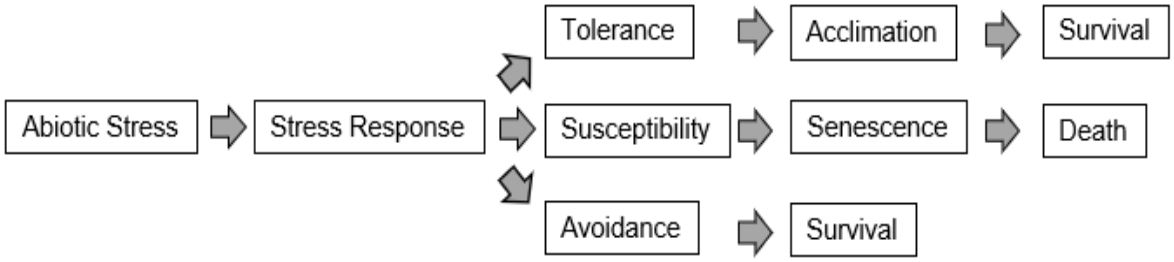

Figure 1 Schematic representation of the effect of environmental stress and plants' response

maintaining of high metabolic activity under moderate stress and reduced metabolic activity under severe stress. But, avoidance reduces metabolic activity during extreme stress, which results in a dormant state (Choudhury et al. 2017; Gururani et al. 2015a). Plants display stress resistance or tolerance because of their genetic ability to acclimate to stress and develop a new state of homeostasis over time.

In plant stress physiology, acclimatization and adaptation are essential. While acclimatization does not require genetic modification but rather changes in plant physiology (phenotypic response) to accommodate shifting environmental conditions, but adaptation takes place at the genetic level where favorable genes that are adapted to stress are acquired over several generations. For example, plants become resistant after prolonged exposure to cold or freezing temperatures over a longer duration by adjusting their growth and metabolism to suit the low temperature.

Numerous biotic and abiotic stresses affect plants that trigger variable plant responses, like altered gene expression, modified growth rates, and cellular metabolism. However, plants have also developed different defense responses or molecular mechanisms to combat these stresses *via* genotypic and phenotypic expressions (Abuqamar et al. 2009). One such mechanism is by reactive oxygen species (ROS)(H_2O_2 and superoxide $^{\bullet}O^{-}_2$) generated during oxidative stresses that cause major cellular damage (Allan and Fluhr 2001; Bartoli et al. 2013). Hence, plants remove ROS rapidly *via* its anti-oxidative mechanisms it can also be minimized by stress and further tissue damage (Allan and Fluhr 2001; Kimotho et al. 2019). Also, to induce a particular response to environmental and developmental cues, ROS interacts with some other cell signaling pathway components including hormones, RNS, and intracellular Ca^{2+} fluxes (Farooq et al. 2019).

Through the mitogen-activated protein kinase (MAPK) cascades, plants also react to abiotic stress, which is activated by even a mild sense of stress (Wurzinger et al. 2011). These are in charge of signal transduction for a variety of biotic and abiotic stress reactions in many cellular functions. Due to their involvement in various stress responses, MAPKs are crucial in the combined biotic and abiotic stresses (Danquah et al. 2014). Further, hormone signaling is also essential for mitigating biotic and abiotic stress reaction effects. The H_2O_2 signaling involves the MAPK pathway regulating gene expression during defense and hypersensitive responses (Farooq et al. 2019). Among these, the main hormone responsible for the coordinated abiotic stress response in plants is abscisic acid (ABA), by tackling decreased moisture availability in these plants (Figure 2) (Raghavendra et al. 2010; Kimotho et al. 2019). ABA-dependent pathways for gene activation, which influence stress tolerance are achieved by two regulons: the myelocytomatosis oncogene (MYC)/myeloblastosis oncogene (MYB) regulon and the ABA-binding factor/ABA-responsive element binding protein (ABF/AREB) regulon (Saibo et al. 2009). Rapamycin (TOR) is an atypical Ser/Thr protein kinase that regulates energy maintenance and metabolic homeostasis in plant stress responses and adaptation (Fu et al. 2020)

Temperature is a key element in the metabolism and growth of plants. It is shown that rapamycin (TOR) activity in *Arabidopsis* is diminished rapidly by cold stress at different time points and recovers back after 2 hours of treatment. TOR activity is also in extreme temperature tolerances (Fu et al. 2020). Many studies have examined drought and heat stress's effects on plants. According to Nadeem et al. (2018), a plant's growth stages may be impacted by heat stress, and in response, plants evolve defense mechanisms to protect against damage to membranes and control transpiration and photosynthesis. Heat stress induces molecular responses such as NO, ROS, Ca^{2+} signaling pathways, and initiation of heat stress factor (HSFs) genes as well as other transcriptional factors.

Heat stress diminishes the photosynthetic efficiency reducing the plant life cycle and productivity. The major physiological change brought on by heat stress in plants is membrane dysfunction. Heat stress induces kinetic energy, which moves the biomolecules across membranes detaching the chemical bonds. This increases membrane fluidity (Zhao et al. 2020). Additionally, Begcy et al. (2018) stated when Australian and European wheat cultivars are exposed to moderate heat stress, reduced photosynthesis, transpiration rate, and pollen viability are observed in European cultivars (HSFs down-regulated or up-regulated) compared to

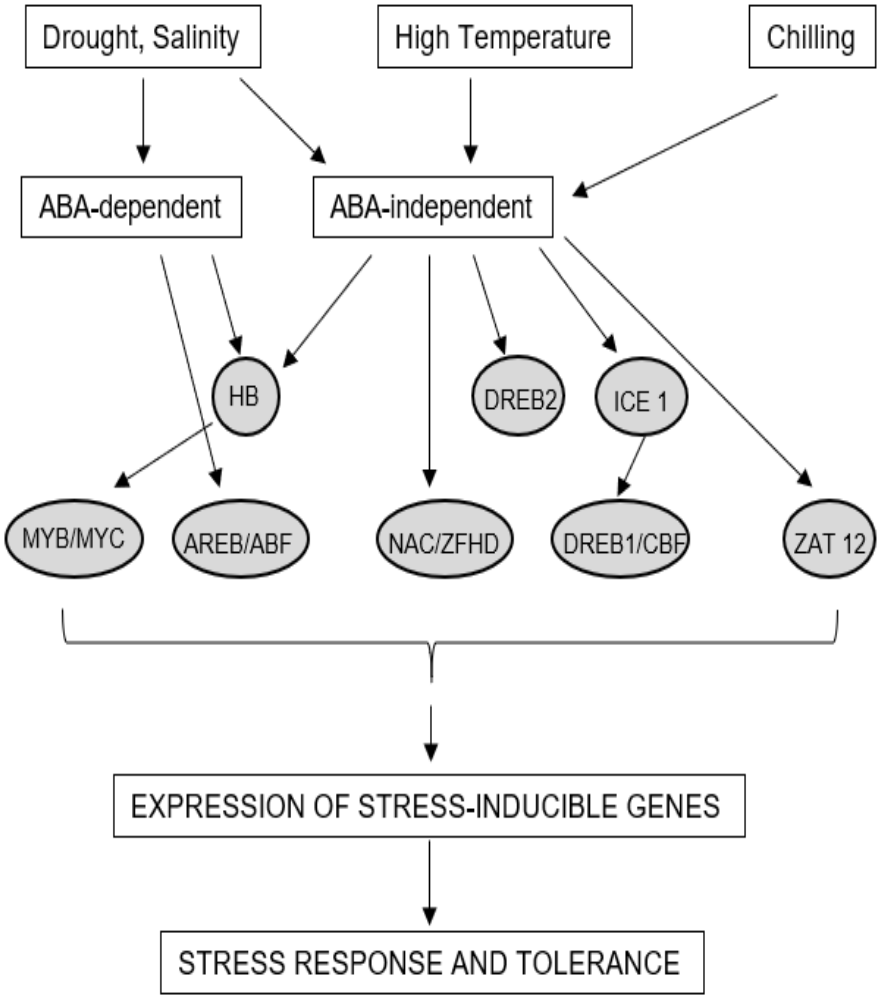

Figure 2 A schematic diagram of the cross-talk network between cis-acting components and transcription factors

Australian cultivars. This shows better adaptation to heat stress in Australian cultivars than the European cultivars. The results of a similar study utilizing wheat cultivars experiencing terminal heat stress conducted in several locations in Egypt demonstrate that heat stress had a significant detrimental effect on plant growth and resulted in nearly 40% less yield (Elbasyoni 2018).

Plant water maintenance is essential for turgor pressure, increased surface tension, and various biochemical processes. Distribution of water throughout the year will ensure proper plant production and yield. But conditions of water stress or drought are frequently unpredictable. Approximately 50% of global loss of crop yield is due to drought stress (Khalid et al. 2019). Drought stress tolerance could be achieved in *Arabidopsis* by restricting transpiration and improving water use efficiency (WUE) (Blankenagel et al. 2018). Their findings also show huge potential for improving WUE in cereals but with reduced assimilation and growth rates. Xiong et al. (2018) used the pak choi plant to study the effects of nitrogen supply and drought stress. They reported pak choi showing significant and better growth under drought stress when supplied with higher nitrogen concentrations.

Water logging is a major issue, not just in areas with heavy rainfall but also in irrigation water-used areas. In a few nations, flooding has affected 0.7 million acres and 60000 acres are permanently under water from poor drainage and water channel leakage. Water logging circumstances significantly reduce a plant's production and yield when it is still developing. However, the impact is minimal and only noticeable briefly when a plant is dormant. Flooding extensively impacts seed germination, decreases vegetative and reproductive growth and plant structure, and accelerates aging (Khalid et al. 2019).

Methods applied to improve crop resistance against floods have been extensively studied focusing on barley, maize, and soybean (Mustroph 2018). They confirmed the presence of tolerance genes

by identifying various trait loci (QTLs). Using natural salinity stress-tolerant plant quinoa, Messerer et al. (2018) emphasized next-generation sequencing technology (RNA-seq) to uncover stress-related genes, which may lead to higher salt tolerance.

3 Stress Sensing and Signaling

Plant cells can presumably sense and respond to changes in different environmental stresses due to the specific modifications observed in gene expression, metabolism, and physiology. Only a few recognized sensors have been found due to the difficulty of functional redundancy in sensor protein-coding genes. A defect in one gene causes no remarkable phenotypic changes in stress responses.*Arabidopsis* OSCA-1 gene is a potential hyperosmotic stress sensor (Yuan et al. 2014). ABA and osmotic stress factors like cold, heavy metals, heat, high salt content, and oxidative stress may elevate free cytosolic Ca^{2+}ions in plants that can be identified *via* genetically encoded aequorin. The COLD1 stress sensor is another potential sensor that mediates rice's cold stress sensing required for chilling tolerance ($0–15^0$C) in the rice subspecies *Nipponbare* (Ma et al. 2015). Transmembrane protein COLD1 controls calcium channels or senses calcium channels that sense cold as it interacts with RGA1 in plants (Ma et al. 2015). But it is still ambiguous if the chilling tolerance is due to the COLD1-mediated calcium signaling.

The fluidity of cellular membranes is modified by cold and heat stress that could be sensed by various channels, integral membrane proteins, transporters, and membrane-anchored receptor-like kinases (RLKs) (Sangwan et al. 2002). Certain molecular chaperones that bind misfolded proteins can sense the denaturation due to heat stress, which releases related transcription factors from the chaperones to initiate the heat-responsive genes (Scharf et al. 2012).

Plants with many MAP kinase family members assemble to produce many MAP kinase modules. For instance, *Arabidopsis* has 20 MAP kinases (MAPK), 10 MAP2 kinases (MAP2K), and more than 60 MAP3 kinases (MAP3K) (de Zelicourt et al. 2016). The abiotic stresses such as high salinity, drought, heat, cold, and wounds activate MAPKs in plants multiple times (de Zelicourt et al. 2016). Identification of the upstream protein sensors, MAP2Ks, and MAK3Ks, responsible for activating MAPK, and methods of connecting kinase activation to downstream effects on proteins and physiological outputs present the greatest challenges in characterizing MAPK-signaling pathways for abiotic stress (Danquah et al. 2014; de Zelicourt et al. 2016).

A drought-response photoreceptor, phytochrome C1 in *Z. mays*, has been identified for drought sensing in plants, though specific receptors have not been discovered yet (Benešová et al. 2012). Phytochrome is supposed to regulate light-responsive gene transcription by controlling numerous transcription factors' activities for biotic and abiotic stresses (Gururani et al. 2015a). In *Arabidopsis*, 3 phytochrome genes (PHYA, PHYB, and PHYE) suppress drought tolerance, which implies that phytochrome C may mediate osmotic stress (Boggs et al. 2010). Similarly, it has been shown that turf grasses can recover from salinity, heavy metal toxicity, and cold stress when a hyperactive Ser599Ala PHYA from oat is over-expressed (Gururani et al. 2015b; Gururani et al. 2016).

Osmotic regulation is essential for a plant's drought resistance. Under drought stress, various crucial osmotic homeostasis-related proteins, including betaine aldehyde dehydrogenase (BADH), dehydrin (DHN), and late embryogenesis abundant (LEA) protein, are gathered in leaves. LEA proteins have high hydrophilic proteins that aid in stabilizing cellular components due to water loss (Chakrabortee et al. 2007). Similarly, other studies have shown that DHNs (group 2 LEAproteins) have higher hydrophilicity and thermostability, which were extensively drought-accumulated among many plant species such as *Z. mays*, *T. aestivum*, *C. dactylon*, and *B. napus*. These DHNs stabilize the protein structure *via* detergent-chaperone-like properties (Hu et al. 2010; Jangpromma et al. 2010). Also, DHN in *Z. Mays* has shown a noticeably higher level of phosphorylation under drought stress (Bonhomme et al. 2012). Phosphorylation of LEA2 may increase its calcium binding since it functions as a calcium buffer and has calcium-dependent chaperone-like action similar to that of calreticulin and calnexin (Alsheikh et al. 2003). Group 3 LEA proteins also increase in *Z. Mays* and *B. napus* during specific drought conditions (Benešová et al. 2012; Koh et al. 2015). Studies have demonstrated that the LEA gene provides drought stress resistance in various plant species. For example, transgenic calli over-expressing sweet potato LEA14 (IbLEA14) increased drought stress resistance. In contrast, RNA interference (RNAi) calli showed enhanced drought stress sensitivity (Park et al. 2011). It can be concluded that LEA could be used to enhance plants' drought tolerance.

4 Regulators of Plant's Abiotic Stress Responses

Plants respond to abiotic stresses with various molecular mechanisms, such as cross-talk and interactions between several molecular pathways (Takahashi and Murata 2008; Gururani et al. 2015a). The plant signals involved in abiotic stress responses are reactive nitrogen species (RNS) and reactive oxygen species (ROS) that can alter gene regulation and enzyme activities (Molassiotis and Fotopoulos 2011; Singh and Thakur 2018; Akilan et al. 2019; Varghese et al. 2019). Further, abscisic acid (ABA) and ethylene are the most significant hormonal regulators of plant responses to abiotic stresses (Wilkinson and Davies 2010). ABA regulates osmotic stresses through transcriptional activities by

regulating these plants' ion and water transport processes (Pettigrew et al. 2015). Ethylene is also involved in stress responses such as wounding, drought, flooding, chilling, heat, ozone, and UV-B light (Goda et al. 2008; Stepanova and Alonso 2009; Wilkinson and Davies 2010; Pettigrew et al. 2015). MicroRNAs (miRNAs) have demonstrated abnormal expression induced due to abiotic stress, which implies miRNAs be specific targets for developing genetically modified stress-resilient plants (Banerjee 2020). It is now known that epigenetic mechanisms like histone modifications, DNA methylation, and chromatin remodeling are involved across all abiotic stress responses. Along with such modifications, long non-coding RNAs and small RNAs regulate the abiotic stress response and RNA silencing (Chang et al. 2020).

Similarly, C_2H_2-type zinc finger proteins play a crucial part in the growth of plants, development, and resistance to abiotic stress. Studies on the functional roles of these proteins in different stress-resilient plants like halophytes and xerophytes are being explored to identify certain regulated genes. Rapid advancements in sequencing technologies will help in plants' epigenomic profiling, which may help us study further mechanisms of stress adaptation (Chang et al. 2020).

5 RNA-Binding Protein (RBP)

Ribonucleoprotein (RNP) complexes are produced when RBPs bind to RNAs. These complexes are essential for all aspects of post-transcriptional gene regulation (Glisovic et al. 2008). Currently, more than 1000 RNPs are known that participate in plants' adaptation to various environmental conditions. RBPs are therefore essential for all organisms in regulating cellular physiology and gene expression. A few RBPs have been identified in plants; some of which are involved in the plant's innate immunity and its responses (like GaPR10, tcI14, PRP-BP, GRP7, etc.) (Fedoroff 2002; Woloshen et al. 2011).

5.1 RNA binding domains (RBDs)

According to Lunde et al. (2007), each RBP have a specific RNA binding domain (RBD) to bind on RNA. These RBDs must be able to identify particular RNA sequences (Figure 3). The double-stranded RNA binding domain (ds-RBD), RNA recognition motif (RRM), zinc finger binding domain (ZnF), DEAD box helicase domains and K-homology domain (KHD) are some of the most significant RBDs out of more than 400 already identified ones (Cléry et al. 2008; Valverde et al. 2008; Linder and Jankowsky 2011).

RNA binding is reliant on recognizing RNA structures and specific nucleotide sequences. However, RBPs utilize multiple instances of the same RBD to improve RNA binding affinity and specificity by enhancing the binding space (Lunde et al. 2007). The most frequent RBD in eukaryotes is RRM, found in 0.5–1% of genes (Cléry et al. 2008). Each RRM can recognize only 2-8 nucleotides, but the presence of more RRMs (4 or more) can recognize nucleotides at different sites within the RNA, thus increasing RNA restructuring rates (Sawicka et al. 2008).

RNA binding specificity is enhanced when certain RBPs interact with several types of RBDs (Afroz et al. 2014). The RNA-binding proteome or "RBPome" is essential to cell function, tightly

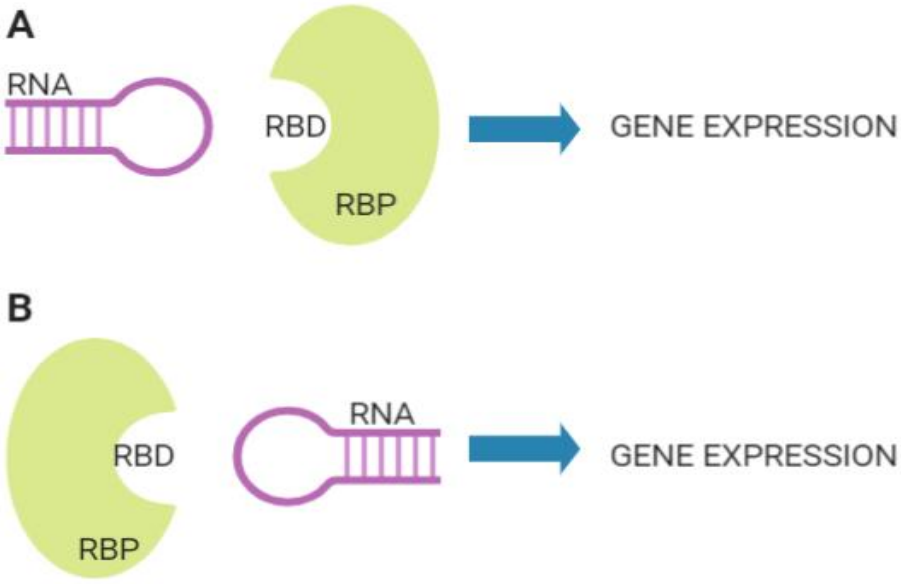

Figure 3 Cross-talk between RNA and proteins: A: RNA and RNA-binding protein (RBP) interact via a defined RNA-binding domain (RBD) and regulate RNA metabolism and functions. B: RNA can interact with RBP and regulate its functions (Adapted from Hentze et al. 2018)

regulated, and shows altered responses with varying environmental changes (Sysoev et al. 2016; Perez-Perri et al. 2018; Garcia-Moreno et al. 2019; Trendel et al. 2019).

A comprehensive strategy involving the RNA interactome capture (RIC) technique exposes ultraviolet (UV) irradiation of cells to enhance RNA-to-protein crosslinks. The RIC approach has enabled the identification of proteins that bind to polyadenylated RNAs in living cells (Baltz et al. 2012; Castello et al. 2013). The RIC approach enables the identification of proteins that are in close contact with RNA, exposes RBPs acting in their natural environment, and can be used in comparative studies to uncover RBP dynamics (Sysoev et al. 2016; Perez-Perri et al. 2018; Garcia-Moreno et al. 2019).

RIC technique has been utilized on different organisms, including *Arabidopsis thaliana* and plant leaves since 2012 (Bunnik et al. 2016; Lueong et al. 2016; Bach-Pages et al. 2017). Only 27 RBPs in the leaf were identified in one study to compare approximately 226–372 RBPs produced in the other plant species (Marondedze et al. 2016; Hentze et al. 2018). It is difficult to apply RIC in plant leaves due to the cell walls, chlorophyll (UV-absorbing pigments), secondary metabolites, and reduced UV-crosslinking efficiency due to leaf thickness (Köster et al. 2020).

Eukaryotic gene expression is regulated at the transcriptional and post-transcriptional levels. At the post-transcriptional level, small nuclear ribonucleoprotein particle (snRNP) proteins, poly(A)-binding proteins (PABPs) for mRNA stability, SR proteins for RNA splicing, heterogeneous nuclear ribonucleoprotein particle (hnRNP) proteins for RNA transport are important regulated proteins (Suzuki et al. 2000). RBP binding to target RNAs is required to regulate RNA metabolism. RBPs consist of several conserved motifs and domains like K-homology (KH) domain, RNA-recognition motif (RRM), zinc finger motif, RD-repeats, glycine/arginine-rich regions, and SR-repeats (Lee and Kang 2016).

6 Role of RNA-Binding Protein in Abiotic Stress Responses

RBPs are remarkably conserved and diverse. RBPs with one or more RNA-binding domains (RBDs) recognize RNA-protein interactions forming ribonucleoprotein complexes (RNPs). RBPs are categorized as cold-shock domain proteins (CSDP), glycine-rich RNA-binding proteins (GR-RBP), zinc finger glycine-rich proteins (RZ), S1 domain-containing proteins (SDP), pentatricopeptide repeat proteins (PPR), DEAD-box RNA helicases (RH), and chloroplast RNA splicing and ribosome maturation domain (CRM). RBPs encompass some classic proteins, such as RBPs with K-homology domain (KH), RNA recognition motif (RRM), and arginine-glycine repeats (RGG) (Lee and Kang 2020). The essential functions of some RBPs in abiotic stress response are discussed below.

6.1 RNA Recognition Motif

RNA recognition motif (RRM) is the best-known RNA binding motif which comprises a maximum of RBPs (Lee and Kang 2016).

6.2 K-homology domain

After RRM, the heterogeneous nuclear ribonucleoprotein K (hnRNP K) homology (KH) domain protein is the RNA-binding domain that is most frequently observed. Every KH domain contains a highly conserved consensus sequence (VIGXXGXXI) at the center of a 60 AA long chain with a typical hydrophobic residue pattern. Proteins can contain several copies of KH domains (up to 15). A protein with a KH domain is capable of binding single-stranded DNA or RNA to control genes' transcriptional and post-transcriptional regulation. By changing numerous genes' expression regulated by abiotic and biotic stimuli, the *Arabidopsis* KH-Domain RNA-Binding Protein ESR1 insertional knockout mutants' esr1-1 and esr1-2 confer enhanced heat tolerance (Muthusamy et al. 2021).

6.3 Cold-shock domain proteins (CSDP)

The cold shock domain (CSD) is found in the eukaryotic Y-box proteins that may bind RNA and single-stranded and double-stranded DNAs. In contrast to bacterial cold shock protein (CSP), which only has the CSD, typical plant CSDPs have CSD at the N-terminus, and at the C-terminus, a glycine-rich region is found that is interspersed with multiple zinc fingers of the CCHC type.

When exposed to cold, *AtCSP2* overexpression markedly reduced freezing tolerance, but the *atcsp2* mutant dramatically increased freezing tolerance by up-regulating the transcription factors of CBF and downstream genes in the cold stress pathway. With cold-sensitive bacterial strains, rice's *OsCSP1* and *OsCSP2* were examined for their ability to adapt to the cold. Both genes were found to be capable of compensating for the loss of bacterial CSP genes, indicating their significance in plants' ability to adapt to cold stress (Muthusamy et al. 2021).

6.4 Glycine-rich RNA-binding proteins (GR-RBP)

Glycine-rich RBPs (GRPs) are among those RBPs that have been extensively studied in plants, and the genomes of rice (*Oryza sativa*) and *Arabidopsis thaliana* each contain eight and six GRP genes, respectively. GRPs possess a C-terminal region that is rich in glycine and a conventional RRM at the N-terminus (Lee and Kang, 2016). It is a class IV GRP. The four subgroups IVa (RRM motif), IVc (CSD and two or more zinc-finger motifs), IVb (RRM and a CCHC zinc-finger motif), and IVd (two RRMs) of glycine-rich RBPs are distinguishable based on their domain features. Generally, GR-RBPs are functionally conserved across plant species (Muthusamy et al. 2021).

For instance, the rice genes *OsGRP1* and *OsGRP4* successfully rescued the cold-sensitive phenotypes of *atgrp7*, whereas *OsGRP6* was a different gene that gave the *atgrp7* plants freezing tolerance. By raising the amounts of indole-3-acetic acid in transgenic lines, *AtGRDP2* overexpression increased *Arabidopsis*' ability to withstand salt stress and enhanced growth. In contrast, over-expression of *AtGRP7* improved freezing tolerance while producing phenotypes in *Arabidopsis* that were vulnerable to salinity and drought. When exposed to cold stress, *AtRZ1a*serves as a RNA chaperone and helps *Arabidopsis* tolerate cold.

6.5 Serine/Arginine-Rich (SR) Domain

Serine/arginine-rich (SR) proteins function as RNA-binding proteins (RNA-BPs) and play significant roles in processing and regulating the splicing of precursor-mRNA (pre-mRNA). SR proteins contribute markedly to the process of alternative splicing by acting on the splice site. The highest quantity of SR proteins are found in flowering plants when compared to different eukaryotes, e.g., 24 in rice; 17 in *Brachypodium*; 18 in *Arabidopsis*; 12 in humans, and 7 in *C. elegans* (Iida and Go 2006; Longman et al. 2000; Manley and Krainer 2010; Barta et al. 2010; Vogel et al. 2010). Hence, SR proteins are considered the key regulators of the gene regulation mechanism (Duque 2011).

A broad analysis of SR gene expression in *Arabidopsis* was done by reverse transcriptase-polymerase chain reaction (RT-PCR). It did not show any changes in the overall transcript levels that are influenced by stress, but changes in temperature and salt condition repressed SCL33 (Palusa et al. 2007). However, under different abiotic stress circumstances, including high salinity, temperature, and UV irradiation, the alternative splicing pattern of various *Arabidopsis* SR protein family members exhibits significant changes (Lazar and Goodman 2000; Palusa et al. 2007; Tanabe et al. 2007; Filichkin et al. 2010). The splicing of downstream targets may be altered by stress-related environmental changes, like light, heat, and salt, in the SR protein gene products, leading to adaptive transcriptome modifications (Filichkin et al. 2010).

RBPs' functional roles are still being explored in the development and growth of plants concerning stress response mechanisms. The essential function of RBPs in organellar RNA metabolism under abiotic stress is being investigated. Genome-wide analysis of these RBPs will determine the fate of RNA during mutation and how these are engaged in the development and growth of plants.

Conclusion

Plants adopt a series of responses (stress resistance, avoidance, or defense) for responding to abiotic stress, an action carried out with the help of RBPs. Even though the functions of RBPs in plants are still being explored, there are some unanswered questions on the essential roles and capability of RBPs in plants' abiotic stress physiology. As an important component of plants' adaptive process in different environmental conditions, RBPs operates by regulating the splicing of pre-mRNA, RNA export, RNA stability, polyadenylation, and chromatin modification. With an outstanding understanding of RBPs in other life forms, RBPs' role in plants is still limited. Future research can be directed toward using these RNA-binding proteins as targets and understanding how RBPs recognize their substrates to regulate RNA metabolism to develop stress-resilient crops by focusing on the genomic and epigenomic mechanisms during abiotic stress conditions.

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Journal of Experimental Biology and Agricultural Sciences, February - 2023; Volume – 11(1) page 54 – 61

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Factors Influencing Suicidal Behaviour among University Students: A Cross-Sectional Study from North India

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ABSTRACT

In the majority of the affected nations, suicidal behavior against COVID-19 leads to various concerns. This study aimed to analyze determinants affecting suicidal behaviour among university students in Uttarakhand. An online cross-sectional survey of 18-year-old university students in Uttarakhand was conducted between April 2 and May 13, 2022. The questionnaire comprised socio-demographic information, the Suicidal Behaviors' Questionnaire-Revised (SBQ-R) scale, and elements related to the physical and psychological health of COVID-19 (CRPPF). The statistical study included demographic information, basic statistics in terms of frequency and percentage, and logistic regression. In comparison to students with fewer than seven family members, students with more than seven family members were less likely to participate in suicide behaviour (AOR = 2.21; 95% CI: 1.79 to 2.67) and vice versa (AOR = 0.81; 95% CI: 0.56 to 0.97). According to the study, a substantial majority of students (76.35%) claimed that the lockdown implemented to stop the spread of COVID-19 was extremely upsetting for them and that the pandemic had caused them to miss their graduation (73.90%). Adjusted multivariate logistic regression shows that feelings of a burden on family, (AOR= 1.98, 95% CI: 1.09 to 2.82), distancing from family or friends, (AOR =1.66; 95% CI: 1.26 to 2.01), having relationship dilemmas,

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(AOR= 2.31; 95% CI: 1.84 to 2.97), and being anxious during the lockdown, (AOR= 1.84; 95% CI: 1.08 to 2.27), are significant factors among participants that are linked to higher risk of engaging in suicidal behaviour. The possibility of university students engaging in suicide behaviour was significantly affected by numerous factors. In addition to defending the students' mental health, the concerned authorities should devise and implement strategies to safeguard the students' physical health.

1 Introduction

COVID-19 epidemic was declared a public health emergency of global concern by the World Health Organization on January 30, 2020 (Harpan et al. 2020). As of the 13th of May 2022, India had 4,26,17,810 confirmed COVID-19 cases, with approximately 5,24,636 deaths (MOFHW n.d.). According to new findings, the 2019 coronavirus disease pandemic (COVID-19) will have major psychological and social implications (Sher 2020). Suicidal behaviour is a serious mental health condition and there are significant risk factors related to gender, age, location, and socioeconomic position (Turecki and Brent 2016). Over 30% of the 5572 university students from 12 different nations who participated in the survey have thought about suicide, while 7% have attempted suicide (Eskin et al. 2016). It was discovered in earlier empirical research that approximately 18% of university students were suicidal (Britton et al. 2014). Suicidal behaviour among graduate students is marked by sorrow, pessimism, desperation, and a sense of powerlessness and additionally, suicide ideation and behaviours have been connected to bullying victimisation (Garcia-Williams et al. 2014; Holt et al. 2015). Among Chinese female students, sadness was found to be connected to suicidal behaviour (Tang et al. 2018). In a previous study among Chinese university students, it was found that the factors depression, anxiety, and stress to be the major risk factors for suicide (Lew et al. 2019). An earlier study in Greece indicated that university student suicidal intentions increased by 63.3 percent during the period of the COVID-19 pandemic (Kaparounaki et al. 2020). Also, university students in Poland during the COVID-19 outbreak were reported higher suicidal symptoms (Debowska et al. 2020). As part of the COVID-19 pandemic, suicidal behaviour is associated with fear of the disease, unstable economic conditions, an inability to access healthcare, mental illness, and social isolation (Raj et al. 2021).

Three Indian students, who were getting medical education in Wuhan, had returned to three different locations in Kerala on January 30, 2020, and discovered the first cases of COVID-19 in India (Narasimhan 2020). The second wave, which started in March 2021, was significantly worse than the first, with shortages of vaccines, hospital beds, oxygen tanks, and other medical supplies plaguing the majority of the nation's regions. By the end of April, 2021 India had the most recent and active cases worldwide. More than 400,000 new cases were added on April 30, 2021, setting new records (The Hindu 2021; BBC News 2021). The COVID-19 epidemic, which had substantial psychological repercussions and emphasized the occurrence of suicidal thinking and attempt had a particularly severe impact on Uttarakhand, a North Indian States. Studies on suicide behaviour and its causes among university students in Uttarakhand are few, especially in the aftermath of the COVID-19 outbreak, which is profoundly worrying for our nation. The proposed research objective was to identify the risk factors connected with suicidal behaviour in order to close a significant knowledge gap and to educate the proper authorities about suicidal behaviours among university students in Uttarakhand.

2 Materials and Methods

2.1 Study design, participants, and data collection

A cross-sectional online survey between the university students of Uttarakhand was carried out between April 2 and May 13, 2022. The authors started data collection from April 02 and received the required responses by May 13, 2022. Being an undergraduate student at a Uttarakhand university, being at least 18 years old, having access to internet, and residing in Uttarakhand for the duration of the study year were all taken into account as inclusion criteria. People who didn't match the qualifications couldn't take part in the study.

Data were gathered using the Google survey tool's online structured questionnaire and the convenience and snowball sampling techniques (Google Forms). The URL to the Google form was disseminated and shared over a number of social media platforms, including a Whatsapp group and the student’s email addresses. After thoroughly explaining the survey's objectives, the questionnaire's format, and its confidentially to each participant, their informed consent was obtained. All participants have been informed that their identity will be kept private and that the only use of the results would be for research. A total of 421 students filled the form, among these, 35 forms were incomplete, and therefore, 389 responses were considered for analysis. Responses were exported in MS Excel file and the same is exported in SPSS for statistical analysis.

2.2 Questionnaire and Measures

A self-designed questionnaire with four sections was made to collect data on the participants' demographics, COVID-19-related

physical and psychosocial factors (CRPPF), a precautionary mechanism to psychological strain, and the Suicidal Behaviours Questionnaire-Revised (SBQ-R) scale, which was utilised to analyse the factors linked to suicidal behaviour among the study participants. After the questionnaire had been developed and distributed to experts for their validation, a small pilot research was carried out to evaluate its usability and degree of difficulty. The results of the pilot study, however, were absent from the actual study samples.

Demographic information regarding the participants was given in the questionnaire's first section. Age, gender, education, household income, marital status, the number of children, and present location were sociodemographic traits.

The second half comprises physical alongside psychosocial aspects of the scholars like internet bullying, detachment from friends and family, relationship issues (breakup/family fights), experiencing like a load to family, stressed about getting of imprisonment, restricted accessibility to medical treatment facilities, the expertise of COVID-19 signs, loosing members of the family, friends or relatives because of COVID-19, and postponing in graduation because of COVID-19 with "Yes" or "No" response categories likewise because the COVID-19 disease status was addressed as "Tested negative", "Tested Positive" and "Did not test" response choices.

In the third section, there are questions about reducing students' mental stress that include doing nothing, chatting with friends or family, doing meditation, exercising, or engaging in leisure activities (such as watching TV or playing video games).

The final section employs the Suicidal Behaviours Questionnaire-Revised (SBQ-R) scale, a condensed self-report tool for evaluating suicidal behaviours (Osman et al. 2001). The scale has already been confirmed to be trustworthy and valid (Rueda-Jaimes et al. 2017; Amini-Tehrani et al. 2020). There are four items in the SBQ-R. The first question evaluates past attempts and ideas of suicide. The frequency of suicidal thoughts during the past 12 months is evaluated in the second question. The threat of a suicide attempt is evaluated in the third point. The fourth and last question evaluates the self-reported likelihood of engaging in suicidal behaviour in the future. The Suicide Behaviours Questionnaire-Revised (SBQ-R) has a total score range of three to eighteen, with a score of seven or lower indicating a significant risk of engaging in suicidal conduct (Osman et al. 2001). Cronbach's alpha coefficient was utilized to evaluate internal reliability, and it was found 0.83 showing that the collected data was sufficiently reliable.

2.3 Statistical analysis

BS Statistical package named SPSS was used to conduct the analysis. The information was summarised using frequency and percentage. In order to compare various participant subgroups, logistic regression was performed. The odds ratio (OR) along with a 95% confidence interval (CI) was used to summarize the results of data analyses, and also the adjusted odds ratio (AOR) is calculated for each research variable. When the p-value of the test statistics was less than the used level of significance (5%), the statistical significance was taken into account.

3 Results

Frequency (N) and percentage (%) of all the demographic characteristics like gender, age, educational background etc. are represented in Table 1. Approximately half of the students (51.15%) were between the age range from 22-24 while 32.57% and 16.62% were aged below 22 and more than 24 respectively. According to bivariate logistic regression, female students who lived in Uttarakhand had a considerably increased chance of committing suicide. According to the adjusted multivariate logistic regression (AOR), students between the ages of 22-24 had a lower probability of engaging in suicide behaviours than students aged 21 and below (AOR= 0.80; 95% CI: 0.61 to 0.98); and those between those ages (AOR= 0.87; 95% CI: 0.62 to 0.97). Additionally, students with families that included more than seven people showed lower suicidal behaviour (AOR =0.81; 95% CI: 0.56 to 0.97) than students with fewer than seven family members (AOR =2.21; 95% CI: 1.79 to 2.67) and female students demonstrated higher rates of suicidal behaviour than male students (Table 1).

The results of the correlation of COVID-19 associated physical and physiological factors (CRPPF) with suicidal behaviour are presented in Table 2. The problem of the financial crisis was high (61.18%) among the students and they were in stress (76.35%) during the lockdown. Also, students were facing problems of social media (internet) bullying (17.74%), relationship related problems/issues like break-up, or family conflicts (46.03%), etc. Approximately one-fourth of the students experienced the loss of family/relatives because of COVID-19 epidemic. It has been observed that 9.77% of students tested positive for COVID-19 although 44.73% of students did not test positive against COVID-19.

The survey showed a significant part of the participants (80.19%) said that the lockdown adopted to reduce COVID-19 transmission was extremely distressing for them and that the epidemic had delayed their graduation (73.90%). Students were also dealing with issues like financial difficulties (59.48%), cyberbullying (16.71%), interpersonal issues including breakups or family conflicts (44.95%), etc. Over 26% of the students reported physical symptoms quite similar to COVID-19, such as fever, dry cough, breathing problems, exhaustion, etc. However, almost half of the students (47.95%) did not have their COVID-

Table 1 Summary of association of socio-demographic information with suicidal behaviour

Variables	Category	N (%)	OR (95% CI)	AOR (95% CI)
Age	≤21	134 (34.44%)	RC[a]	RC[a]
	22–24	199 (51.15%)	0.86(0.69–1.03)	0.80* (0.61-0.98)
	≥25	56 (14.39%)	0.91(0.70–1.12)	0.87* (0.62-0.97)
Gender	Male	229(58.86%)	RC[a]	RC[a]
	Female	160 (41.14%)	2.26** (2.01–2.74)	2.21** (1.79–2.67)
Educational background	Science & Technology	85 (21.85%)	RC[a]	RC[a]
	Art	128 (32.90%)	0.99 (0.67–1.27)	0.94 (0.71–1.21)
	Commerce	105 (26.99%)	0.82 (0.61–1.19)	0.79 (0.63–1.09)
	Others	71 (18.25%)	0.84 (0.64–1.14)	0.78 (0.61–0.99)
Marital status	Unmarried	356 (91.51%)	RC[a]	RC[a]
	Married	191 (9.10%)	1.27 (0.98–1.71)	1.19 (0.84–1.69)
	Others	9 (0.02%)	0.77 (0.44–1.39)	0.68 (0.39–1.34)
Family monthly income	<18, 497	31 (7.96%)	1.11 (0.81-1.21)	1.17 (0.80-1.59)
	18498-30830	64 (16.45%)	1.27 (0.94-1.64)	1.21 (0.91-1.60)
	30831-44128	136 (34.96%)	1.15 (0.87-1.57)	1.21 (1.01-1.48)
	More than 44129	158 (40.61%)	1.17 (0.88-1.44)	1.13 (0.98-1.47)
Number of family members	<4	171 (43.95%)	RC[a]	RC[a]
	4-7	201 (51.67%)	0.99 (0.81-1.14)	0.94 (0.74–1.12)
	>7	17 (4.37%)	0.84 (0.57-1.17)	0.81* (0.56–0.97)
Location (at the time of study)	Inside Uttarakhand	269 (69.15%)	RC[a]	RC[a].
	Outside Uttarakhand	120 (30.84%)	1.23* (1.04–1.47)	1.03 (0.84–1.27)

[a]RC: Reference Category

19 contamination state checked. Additionally, it should be mentioned that 8.05% of the pupils screened positive against COVID-19 (Table 2).

The financial crisis, disconnection from friends and family, relationship issues (family strife, breakup, etc.), burden on family, feeling stressed during COVID-19, social media bullying victimization, Lack of health care facilities, experiencing COVID-19 symptoms, and being COVID-19 positive (infection status) were some of the factors strongly linked with an elevated risk of suicidal behaviour (Table 2).

Additionally, adjusted multivariate logistic regression shows that stress during lockdown (AOR= 1.84; 95% CI: 1.08 to 2.27), feeling like a burden to family (AOR= 1.98; 95% CI: 1.09 to 2.82), turning away from friends or family (AOR =1.66; 95% CI: 1.26 to 2.01), having issues with relationships (AOR= 2.31; 95% CI: 1.84 to 2.97) are all significant factors substantially associated with an increased risk of suicidal behaviour (Table 2).

Table 3 shows that a high percentage of the students were not doing physical exercise (87.33%) and meditation (89.33) during COVID-19 however, the majority of students (62.24%) reported that they have done recreational activities during the lockdown. Additionally, bi-variate logistic regression revealed that undergraduates who engaged in activities like a workout, leisure, and talking to family or friends to relieve stress were less likely to engage in suicidal conduct than those who did not. The risk of suicide behaviors was actually higher among students who did not participate in any activities. However, adjusted multi-variate logistic regression also showed that students without any mental stress-relieving activities or events were more likely to commit suicide (AOR=1.79; 95% CI: 1.12 to 2.76) (Table 3).

Table 2 Summary of association of COVID-19 related physical & psychosocial factors (CRPPF) with suicidal behaviour

Variables	Category	N (%)	OR (95% CI)	AOR (95% CI)
Financial crisis	Yes	238 (61.18%)	1.37** (1.19-1.67)	1.03 (0.76-1.21)
	No	151 (38.82%)	RC[a]	RC[a]
Victim of social media bullying	Yes	69 (17.74%)	1.64** (1.37-2.07)	1.27 (1.01-1.69)
	No	320 (82.26%)	RC[a]	RC[a]
Distancing from friends/family	Yes	147 (37.79%)	2.41** (209-3.01)	1.66** (1.26-2.01)
	No	242 (62.21%)	RC[a]	RC[a]
Relationship problems (e.g. Family conflicts/Break up)	Yes	179 (46.02%)	3.37** (2.98-4.01)	2.31** (1.84-2.97)
	No	210 (53.98%)	RC[a]	RC[a]
Feeling own self as a burden to family	Yes	161 (41.39%)	2.84** (2.09-3.41)	1.98** (1.09-2.82)
	No	228 (58.61%)	RC[a]	RC[a]
Being stressed of the lockdown	Yes	297 (76.35%)	2.59** (1.87-3.11)	1.84** (1.08-2.27)
	No	92 (23.65%)	RC[a]	RC[a]
Having limited access to healthcare facilities	Yes	201 (51.67%)	1.19* (1.04-1.41)	1.04 (0.86-1.18)
	No	188 (48.33%)	RC[a]	RC[a]
Experienced physical symptoms similar to COVID-19	Yes	291 (74.81%)	1.67** (1.14-1.98)	1.37** (1.05-1.72)
	No	1548 (25.19%)	RC[a]	RC[a]
COVID-19 infection status	Tested negative	174 (44.73%)	1.86** (1.39-2.17)	1.43** (1.21-1.66)
	Tested positive	38 (9.77%)	1.21* (1.04-1.38)	1.09 (0.91-1.28)
	Did not test	177 (45.50%)	RC[a]	RC[a]
Experienced loss of family/relatives due to COVID-19	Yes	98 (25.19%)	1.36** (1.14-1.61)	1.01 (0.78-1.18)
	No	291 (74.81%)	RC[a]	RC[a]
Delayed graduation due to COVID-19	Yes	91 (23.39%)	0.77 (0.61-1.93)	0.69 (0.59-0.79)
	No	298 (76.61%)	RC[a]	RC[a]

[a]RC: Reference Category

Table 3 Summary of association of preventive response to psychological stress with suicidal behaviour

Variables	Category	N (%)	OR (95% CI)	AOR (95% CI)
Physical exercise	Yes	82 (21.07%)	0.69* (0.52–0.91)	0.72 (0.64–1.12)
	No	307 (78.92%)	RC[a]	RC[a]
Meditation	Yes	54 (13.88%)	0.81 (0.64–1.11)	0.89 (0.69–1.31)
	No	335 (86.11%)	RC[a]	RC[a]
Recreational activities	Yes	256 (65.80%)	0.66** (0.59–0.79)	0.89 (0.72–1.16)
	No	133 (34.19%)	RC[a]	RC[a]
Talk to friends or family	Yes	174 (44.73%)	0.66** (0.51–0.82)	0.99 (0.82–1.21)
	No	215 (55.26%)	RC[a]	RC[a]
Do nothing	Yes	81 (20.82%)	2.44** (1.89–3.37)	1.79** (1.12–2.56)
	No	308 (79.17%)	RC[a]	RC[a]

[a]RC: Reference Category

4 Discussion

The present research looked into a number of variables that affect university students' suicide behaviour in Uttarakhand. The study found that students between the ages of 22-24 had a lower risk of acting suicidal than students aged 21 or below and students aged 25 and above. In a recent study, similar tendency was observed among Bangladeshi university students (Rahman et al. 2022). This study found a higher incidence of suicidal behaviour among female students than male students (Rahman et al. 2022; Rahman et al. 2021). It was determined that when compared to male pupils, female students exhibit suicidal behaviour more frequently. The gender differences in vulnerability to psychopathology and psychosocial pressures may have an impact on the study's findings (Vijakumar 2015).

The present study found that students who live in big families had a lower chance of suicidal behaviour. This may be due to the opportunity to receive the necessary support that comes with having a large family, which helps with coping with mental stress. Another study also found that having a big family was linked to a lower chance of suicide (Rahman et al. 2022). Limited access to medical services was mentioned as a significant contributing reason for suicide behaviour during this outbreak by the majority of respondents (69.52%), which is consistent with findings from earlier studies (Raj et al. 2021). According to additional research, the COVID-19 outbreak was brought on by a lack of healthcare facilities, and people also complained about the quality of the current medical services (Pervez et al. 2021; Rahman et al. 2020). In addition, our study found that bullying on social media increased the likelihood of suicidal conduct, and a prior study found a link between bullying victimization and suicidal behaviour (Garcia-Williams et al. 2014). In earlier studies, it was discovered that being cut off from family, friends or relatives was a significant risk factor for developing suicidal behaviour. It was also discovered that the prevalence of suicide thinking increased with the level of isolation (either living alone or having no friends) (Stravynski and Boyer 2001). People who maintain their distance from their families are more likely to experience absence of parental support, which has also been associated to a greater risk of suicidal thoughts in previous research (Chang et al. 2017). According to this study, a higher rate of suicide conduct was caused by relationships that were problematic, such as family disputes or breakups.

According to a prior study, individuals with mental conditions who committed suicide typically experienced interpersonal difficulties (Judd et al. 2012). A previous study found that students who sensed a load to their families were more likely to act suicidally (Bell et al. 2018; Klonoff-Cohen 2022). The perceived burden may be a more substantial predictor of suicidality. The results of this study indicate a strong association between lockdown anxiety and a student body that is more inclined to act suicidally (Priya et al. 2016; Yu 2022). Additionally, our study found that adolescents who engaged in sports and extracurricular activities had a decreased probability of committing suicide. This result is in line with past studies that were more focused on the notion that social interaction and physical activity reduce the risk of suicidal behaviour (Vancampfort et al. 2018; Oyama et al. 2005). Students who engaged in conversation with friends or family members had a lower risk of engaging in suicide behaviour because they were more likely to receive social and familial support while chatting or sharing with friends and family. In a previous study, it was also discovered that social and familial support were inversely connected to a history of suicide (Klonoff-Cohen 2022; Martinez et al. 2022). Additionally, family, friends, members of society, and governmental and private organizations should step up to help them and address their risky behaviour to adopt effective suicidal prevention techniques.

Conclusion and Limitations of the study

The COVID-19 pandemic's one-year impact and the consequent lockdown operations have had an adverse impression on people’s mental health, specifically the university students in north India. One of the characteristics strongly linked with a greater risk of suicidal behaviour among participants, according to the study's findings, is being separated from family and friends. Other characteristics includes having issues related to relationship, feeling like a burden to one's family, and going through periods of acute stress. The appropriate authorities, including researchers, and governmental and private organizations, must create and implement efficient preventative policies treating suicidal behaviour among university students in order to minimize the risk of suicide.

There are certain shortcomings in the current empirical research. The data collected from the survey using the online data collection tool may lead to bias, and this survey was unable to account for participants from junior socioeconomic categories that do not have access to connectivity in terms of Wi-Fi and others. Second, due to the non-availability of the sampling frame of the survey participant, the simple random sampling technique is constrained by selection bias. Third, as they may be linked to suicidal thoughts or conduct, other risk factors including subpar academic performance, depression signs, or substance abuse required to be countered. Finally, investigating causality is challenging due to the study's cross-sectional nature. Large-scale research using a mixed-method approach should be conducted to better examine these difficulties. Despite a number of limitations, we think the study offers significant information on university students' suicide behavior after a year of dealing with the pandemic.

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Conflict of Interest

None

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Optimizing the culture conditions for L-Asparaginase production from endophytic fungus *Curvularia* sp. LCJ413 through conventional and statistical approach

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ABSTRACT

L-Asparaginase (L-ASNase) is a crucial anti-tumour drug used to cure acute lymphocytic leukaemia. The current study aimed to enhance the production medium for the endophytic fungus *Curvularia* sp. LCJ413 that showed significant L-ASNase activity. L-ASNase production from *Curvularia* sp. LCJ413 was examined in six different media to select an appropriate liquid medium. Among the various media tested, Modified Czapek Dox broth (MCDB) exhibited the maximum L-ASNase activity (8.81 ± 0.52 U/mL). Physical (pH and temperature) and nutritional (carbon, nitrogen, inducer, and their concentrations) parameters were also optimized to boost L-ASNase production. Results of the study suggested a temperature of 28°C at pH 7 with 2 g/L maltose, 10 g/L L-Asparagine, and 25 g/L ammonium sulphate as the optimal carbon, inducer, and nitrogen source resulted in a high L-ASNase activity of 18.9 ± 0.40 U/mL. The statistical enhancement of L-ASNase by Response Surface Methodology (RSM) produced 20.11 U/mL of L-ASNase, which was 2.2 fold higher than the non-optimized medium. This is the first study on L-ASNase production from the endophytic *Curvularia* sp. LCJ413 isolated from *Vitex negundo* medicinal plant. Continuous fermentation with the medium composition provided in the study can produce L-ASNase on a large scale.

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1 Introduction

L-ASNase is an amidase enzyme that catalyzes the breakdown of L-Asparagine into L-Aspartate and ammonia. For more than four decades, this enzyme has been used to treat leukaemia and lymphoid system carcinomas (da Cunha et al. 2018). Aside from its medicinal properties, L-ASNase is widely utilized in the food sector to combat acrylamide (human carcinogen) formed once starch-rich foods are processed at extreme temperatures (Muneer et al. 2020). L-ASNases occurs in a broad spectrum of microorganisms, animals, and plants. Microbes are used for L-ASNase production because they are easier to process upstream and downstream than all other sources (Jia et al. 2021). Currently available L-ASNase products are made by bacteria and often induce toxicity and anaphylactic responses in patients (Moguel et al. 2020). As a result, L-ASNase synthesis by eukaryotic organisms received a lot of attention. The side effects of fungal L-ASNases are mild compared to bacterial L-ASNases (Meghavarnam et al. 2022).

L-ASNases from fungi have gained prominence as they are extracellular, simple to extract, and can be processed downstream (da Cunha et al. 2019). Plant-derived endophytic fungi have the ability to synthesize various exogenous enzymes such as proteases, lipases, xylanases, amylases, chitinases, cellulases, laccases, and asparaginases (Raghav et al. 2022). However, their use in enzyme synthesis for the food, pharmaceutical, and biotechnological industries and human welfare is limited (Mishra et al. 2019). Several strategies for producing L-ASNase from fungal endophytes have been investigated, such as solid-state (Supriya et al. 2015; Krishnapura and Belur 2020; Singh and Sao 2021) and submerged fermentations (Uzma et al. 2016; Jenila and Gnanadoss 2018; Priya and Subashini 2022). Sarquis et al. (2004) reported that submerged fermentation is a highly efficient method that demands less energy and poses little risk of contamination.

The extracellular synthesis of L-ASNase primarily relies on the enhancement of culture parameters to produce it in large quantities using a low-cost method. L-ASNase production is based mainly on optimizing nitrogen, carbon sources, and other parameters such as inoculum size, pH, and temperature (Moubasher et al. 2022). Enhancement of bioprocess using one factor at a time (OFAT) is an accepted technique. However, there are still a few drawbacks, such as increased time consumption and experimental runs, and a lack of information about the interaction between the process variables (Abdel-Fattah and Olama 2002). The utilization of a statistical approach for monitoring and optimizing process parameters is ideally suited to analyze the interactive impact of factors on the desired outcome (Ghosh et al. 2013). RSM is a statistical approach for optimizing organism growth conditions to enhance total biomass and metabolite synthesis (Abhini et al. 2022).

Many studies on L-ASNase production from numerical microbes have been reported, but L-ASNase production from endophytic fungi has rarely been examined. In this regard, this study was to select an appropriate liquid medium and enhance its culture parameters for the production of L-ASNase by the endophytic *Curvularia* sp. LCJ413 through conventional (OFAT) and statistical (RSM) methods.

2 Materials and Methods

2.1 Isolation and screening of *Curvularia* sp. LCJ413 for L-ASNase activity

The endophytic *Curvularia* sp. LCJ413 was isolated from *Vitex negundo* L. medicinal plant collected from Loyola College, Chennai (Kathiravan and Gnanadoss 2022). *Curvularia* sp. LCJ413 exhibited high L-ASNase activity on modified Czapek Dox agar medium added with phenol red (2.5 %) as substrate. Later, the isolate was cultured on the potato dextrose agar slants for further experiments at 4 ˚C. This suggests that large-scale production of the L-ASNase enzyme may be feasible.

2.2 Selection of suitable medium for L-ASNase Production

Submerged fermentation requires a liquid medium for L-ASNase production. Hence, L-ASNase production was investigated in six different basal media namely modified Czapek Dox broth (Gulati et al. 1997), modified M9 medium (Moorthy et al. 2010), modified ISP-2 broth (Mangamuri et al. 2017), Asparagine dextrose salts broth (El-Naggar et al. 2015), Glucose asparagine broth (Patro et al. 2014) and Glycerol asparagine broth (Palaniappan et al. 2013) respectively. For this, 100 mL of different media was prepared in separate conical flasks and autoclaved at 121ºC for 15 min. To avoid bacterial contamination streptomycin (100 µg/mL) was added to each liquid medium. Under sterile conditions, three mycelial discs (5mm) of *Curvularia* sp. LCJ413 cultured in different liquid media and maintained for about 8 days at 120 rpm. After every 24 hours, 0.5 mL of culture filtrate was pipetted and centrifuged for 10 min at 10,000 rpm. After centrifugation, supernatants were collected to determine the L-ASNase activity.

2.3 L-ASNase activity

Imada et al. (1973) proposed that the volume of NH_3 released from L-Asparagine was used to determine L-ASNase activity using Nessler's reagent. In brief, the reaction mixture consisting of culture filtrate (0.5 mL), 0.5 M tris HCl buffer (0.5 mL), 0.04 M L-Asparagine (0.5 mL), and distilled water (0.5 mL) was incubated for 30 min. Following incubation, 1.5 M trichloroacetic acid (0.5 mL) was added to terminate the reaction. Then the reaction solution was spun at 10,000 rpm for 15 minutes. A collection of 0.1 mL supernatant followed by 3.7 mL distilled water and 0.2 mL

Nessler's reagent were mixed and incubated for about 20 minutes. Absorbance was recorded at 450 nm. One unit of asparaginase is the quantity of enzyme necessary to produce 1 µmol of NH_3 per minute under specific conditions. The L-ASNase activity was determined using the formula below (Balbool and Abdel-Azeem 2020).

$$\text{Enzyme activity } \left(\frac{\text{U}}{\text{mL}}\right) = \frac{NH_3 \text{ liberated } (\mu M) * \text{Reaction mixture (initial volume)}}{\text{Reaction mixture (final volume)} * \text{Volume of enzyme used} * \text{time of incubation}}$$

2.4 Protein estimation

Protein concentrations were determined using the method by Lowry et al. (1951) using bovine serum albumin as standard. Briefly, 1000 µL of the crude enzyme (diluted) was added to 5 mL of alkaline copper solution and maintained at room temperature for 10 minutes. Then the reaction solution was mixed with Folin's phenol reagent (0.5 mL) followed by 30 minutes of incubation. After incubation, the intensity of the formed blue colour was measured at 660 nm.

2.5 Optimization studies for L-ASNase production by submerged fermentation

The primary objective of medium optimization is to investigate the operational parameters that enhance enzyme production. Inducer, nitrogen, and carbon sources are important factors when optimizing the medium to improve L-ASNase production. They are also essential for synthesizing key nutrients in a liquid medium for organism development. The nutritional parameters were optimized by OFAT (Jenila and Gnanadoss 2018). The L-ASNase production from endophytic *Curvularia* sp. LCJ413 was examined in MCDB consisting of 2 g/L glucose,1.52 g/L KH_2PO_4, 10 g/L L-Asparagine, 0.52 g/L $MgSO_4$, 0.52 g/L KCl, 0.0001 g/L of $FeSO_4$, $CuNO_3$, $ZnSO_4$ and distilled water (1L).

2.5.1 Influence of carbon source and its concentration on L-ASNase production

The influence of various carbon sources on L-ASNase production from *Curvularia* sp. LCJ413 was investigated in MCDB. Lactose, dextrose, maltose, sucrose, and galactose were used as carbon sources. In separate sets of experiments, the original carbon source in the medium (glucose) was replaced by various carbon sources at 2 g/L concentration, while the standard MCDB medium was treated as a control. After selecting the optimal carbon source, the concentration of the selected carbon source was optimized (1-6 g/L). Protein and L-ASNase activities were measured. The optimal carbon source was chosen for subsequent experiments.

2.5.2 Influence of nitrogen source and its concentration on L-ASNase production

The L-ASNase production from *Curvularia* sp. LCJ413 was examined using different organic (yeast extract, urea, and peptone) and inorganic (ammonium sulphate and potassium nitrate) nitrogen sources at 10 g/L concentration in MCDB and the standard MCDB medium was employed as control. After selecting the optimal nitrogen source, the concentration of the chosen nitrogen source was optimized at various concentrations (5 to 30 g/L).

2.5.3 Influence of inducer and its concentration on L-ASNase production

Various inducers influence on L-ASNase production from *Curvularia* sp. LCJ413 was investigated. In separate experiments, MCDB was treated with inducers such as L-Arginine, L-Tryptophan, L-Tyrosine, L-Asparagine, and L-Glutamic acid at 10 g/L concentration. After selecting the best inducer, the concentration of the inducer was optimized (5 to 30 g/L).

2.5.4 Influence of pH on L-ASNase production

The pH of the MCDB was varied from 4 to 9 for the L-ASNase production from *Curvularia* sp. LCJ413 using 0.1N HCL and 0.1N NaOH. The L-ASNase and protein activities were measured every day.

2.5.5 Influence of temperature on L-ASNase production

Different incubation temperatures (28 ºC to 36 ºC) on L-ASNase production from the isolate *Curvularia* sp. LCJ413 in MCDB was investigated.

2.6 Optimizing the L-ASNase production by RSM

Central Composite Design (CCD) was employed in RSM to investigate the optimized medium parameters for enzyme production. Face Centre Central Composite Design (FCCCD) was adopted to identify the values of critical parameters (maltose, ammonium sulphate, L-Asparagine, and pH) for investigation. The Design Expert 13 software was used to estimate test results. Table 1 shows the four variables investigated at three levels (-1, 0, and +1).

To predict the pure error, 30 trials were designed, and four factors in a minimum and maximum range were investigated using CCD. A polynomial function used to describe the experimental result revealed the response (L-ASNase production).

$$Y= \beta_0 + \beta_1A+ \beta_2B+ \beta_3C+ \beta_4D+ \beta_{11}A^2+ \beta_{22}B^2+ \beta_{33}C^2+ + \beta_{44}D^2+ \beta_{12}AB+ \beta_{13}AC+ \beta_{14}AD+ \beta_{23}BC+ \beta_{24}BD+ \beta_{34}CD$$

Table 1 Experimental range and levels of four independent variables for L-ASNase production from *Curvularia* sp. LCJ413

		Range of levels					
Variables	Symbol	Actual	coded	Actual	coded	Actual	Coded
Maltose (g/L)	A	1	-1	2	0	3	+1
Ammonium sulphate (g/L)	B	20	-1	25	0	30	+1
pH	C	6	-1	7	0	8	+1
L-Asparagine (g/L)	D	5	-1	10	0	15	+1

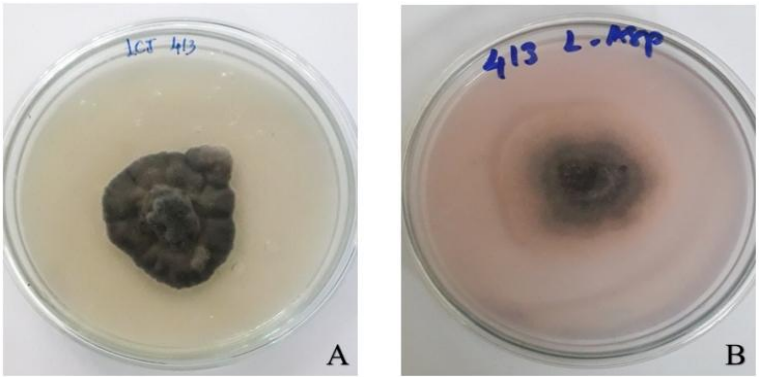

Figure 1 A) Pure culture of endophytic fungus *Curvularia* sp. LCJ413
B) *Curvularia* sp. LCJ413 showing L-ASNase activity on modified Czapek Dox agar

Here, Y - L-ASNase production (predicted response), β_0 – Intercept, Squared coefficients (β_{11}, β_{22}, β_{33}, β_{44}), Linear coefficients (β_1, β_2, β_3, β_4), and Interaction coefficients (β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34})

To support the statistical model, regression analysis was used to determine coefficients and significance levels. The analysis of variance (ANOVA) by design expert 13 was employed for the L-ASNase production in the experimental design. The response surface graphs were computed to determine the ideal parameter levels for increased L-ASNase production.

2.7 Comparative study of original and optimized medium for L-ASNase production

The effectiveness of the improved medium (MCDB) on L-ASNase production from *CurvIularia* sp. LCJ413 was demonstrated by comparing the original (standard MCDB) and optimized medium. *Curvularia* sp. LCJ413 was cultured in 1L of standard MCDB as well as optimized medium and maintained for 8 days as separate experiments, and the L-ASNase activity was estimated every day.

3 Results and Discussion

3.1 Isolation and screening of *Curvularia* sp. LCJ413 for L-ASNase activity

The endophytic *Curvularia* sp. LCJ413 was isolated from *V. negundo* medicinal plant. The culture showed high activity for the L-ASNase enzyme in plate assay (modified Czapek Dox agar) (Figure 1). The 18S rRNA sequence of *Curvularia* sp. LCJ413 was submitted, and the accession number MZ646132 was obtained from Genbank. Jenila and Gnanadoss (2018) reported L-ASNase producing endophytic *Fusarium* sp. from *Adhatoda vasica* medicinal plant. L-ASNase has been revealed to be used therapeutically as an antileukaemic drug, industrially as a biosensor, and in amino acid biosynthesis (Balbool and Abdel-Azeem 2020). Fungal endophytes have the potential to produce a wide array of secondary metabolites and enzymes with a diverse range of biological properties. Nevertheless, endophytic fungi are sparingly exploited as a reservoir of industrially essential enzymes (Correa et al. 2014).

3.2 Selection of Liquid medium for L-ASNase production

The L-ASNase and protein was produced in six different basal media for about 8 days. The L-ASNase production significantly varied across the selected media. Among the six studied media, MCDB showed maximum enzyme production of 8.81 ± 0.52 U/mL, while the lowest L-ASNase production was reported in modified ISP-2 media, i.e., 6.14 ± 0.346 U/mL on the 6th day. The results from this study demonstrated that other media also exhibited L-ASNase activity from *Curvularia* sp. LCJ413 but relatively less enzyme activity when compared with MCDB (Figure 2). Solid-state or submerged fermentation can produce L-ASNase (Doriya and Kumar 2016). The L-ASNase production was improved mainly by the fermentation medium, particularly the nitrogen and carbon sources, as well as physical parameters like

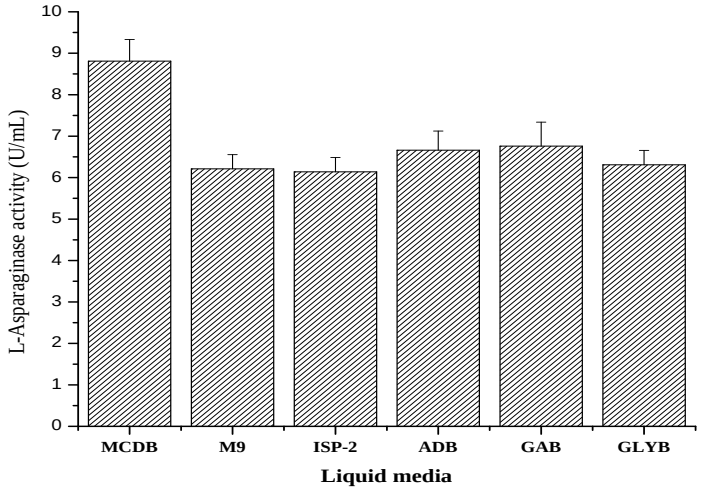

Figure 2 Selection of suitable liquid media for L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day (*Modified Czapek Dox broth – MCDB, M9 - modified M9 medium, ISP-2 – Modified ISP-2 medium, ADB - Asparagine Dextrose Salts Broth, GAB - Glucose Asparagine Broth, GLYB - Glycerol Asparagine Broth)

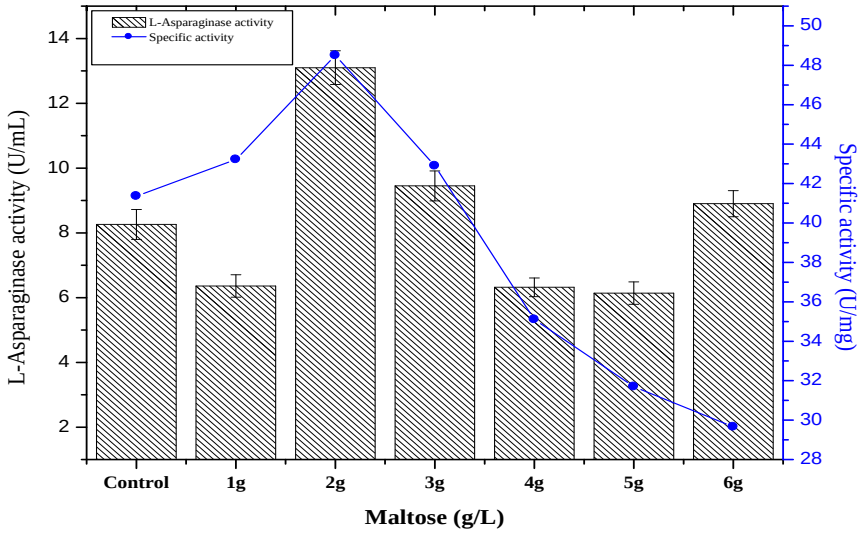

Figure 3 Influence of maltose concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

inoculum size, incubation time, temperature, and pH (da Cunha et al. 2019). Several other research works on L-ASNase production from different endophytic fungi were reported by Araujo-Magalhaes et al. (2021), Chow and Ting (2021), and Jenila and Gnanadoss (2018).

3.3 Influence of carbon source and its concentration

In this study, glucose was substituted with various carbon sources like maltose, lactose, sucrose, dextrose, and galactose in the fermentation medium. The findings showed that maltose displayed the highest L-ASNase production (11.3 ± 0.231 U/mL) compared to other carbon sources tested on the sixth day of incubation from *Curvularia* sp. LCJ413. Subsequently, the L-ASNase production decreased when galactose was used as a carbon source (4.98 ± 0.115 U/mL) (Table 2). The influence of maltose (1 to 6 g/L) was also investigated in the MCDB. The optimal L-ASNase production was observed at maltose (2 g/L), i.e., 13.1 ± 0.52 U/mL (Figure 3). The increased L-ASNase production from the endophytic fungus *Talaromyces pinophilus* was achieved using starch 10 g/L as a carbon source (Krishnapura and Belur 2016). Incorporating glucose as the carbon source in the MCDB medium resulted in the maximum L-ASNase production by the endophytic *Aspergillus niger* (El-said et al. 2016). *Trichoderma viridae*, a marine soil fungus, exhibited L-ASNase activity of 759.5 U/mL, with maltose as the carbon source (Lincoln et al. 2015).

Table 2 Influence of different carbon, nitrogen, and inducer sources on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

Sources	L-ASNase activity (U/mL)
Carbon source (2 g/L)	
Dextrose	8.45 ± 0.289
Maltose	11.3 ± 0.231
Sucrose	7.98 ± 0.462
Lactose	8.58 ± 0.52
Galactose	4.98 ± 0.115
Nitrogen source (10 g/L)	
Potassium nitrate	3.63 ± 0.231
Peptone	5.95 ± 0.404
Ammonium sulphate	14.5 ± 0.346
Urea	5.15 ± 0.173
Yeast extract	6.06 ± 0.289
Inducer source (10 g/L)	
L-Arginine	14.5 ± 0.462
L-Tryptophan	15 ± 0.115
L-Tyrosine	14.9 ± 0.462
L-Asparagine	17.7 ± 0.52
L-Glutamic acid	14.7 ± 0.173

3.4 Influence of nitrogen source and its concentration

In the MCDB, the effects of different nitrogen sources (potassium nitrate, peptone, urea, yeast extract, and ammonium sulphate) on L-ASNase production were investigated. The results proved that better production of L-ASNase (14.5 ± 0.346 U/mL) was attained with the addition of ammonium sulphate. Potassium nitrate as a nitrogen source yielded the lowest L-ASNase activity in a medium (Table 2). The optimal nitrogen source was found to be ammonium sulphate which was further investigated at various concentrations (5-30 g/L). The optimal L-ASNase production was 16.1 ± 0.289 U/mL at 25 g/L (Figure 4). The results from this study follow the results of Jenila and Gnanadoss (2018), where ammonium sulphate at 20 g/L improved the L-ASNase production by the endophytic fungus *Fusarium* sp. According to Baskar and Renganathan (2009), peptone was utilized as an additional nitrogen source by *Aspergillus terreus* to enhance L-ASNase production. Many reports suggest that L-Asparagine is the sole source of nitrogen for the increased L-ASNase production (Benchamin et al. 2019; Prihanto et al. 2019; Doriya and Kumar 2016; Abbas Ahmed et al. 2015). The nitrogen source is critical for L-ASNase production. Microorganisms can utilize organic or inorganic nitrogen sources (Elshafei and El-Ghonemy 2015).

3.5 Influence of inducer and its concentration

Different amino acid inducers were studied for their effect on L-ASNase production. However, the L-Asparagine with enzyme activity of 17.7 ± 0.52 U/mL served as a favourable inducer source for the L-ASNase production. Subsequently, the inducers such as

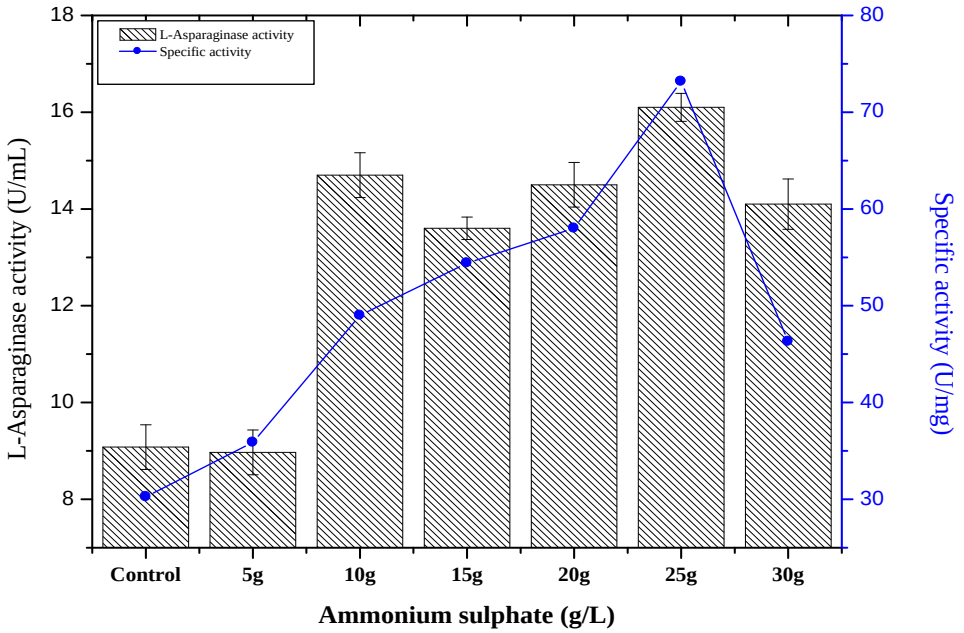

Figure 4 Influence of ammonium sulphate concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

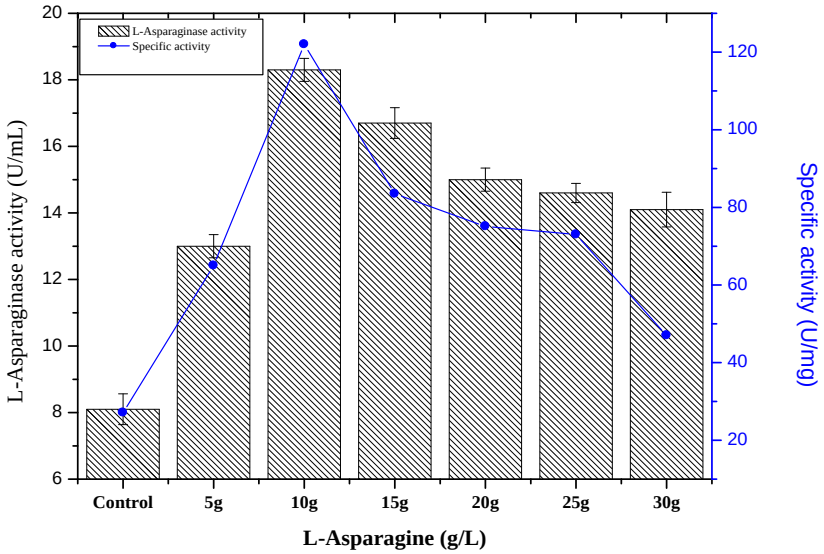

Figure 5 Influence of L-Asparagine concentrations on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

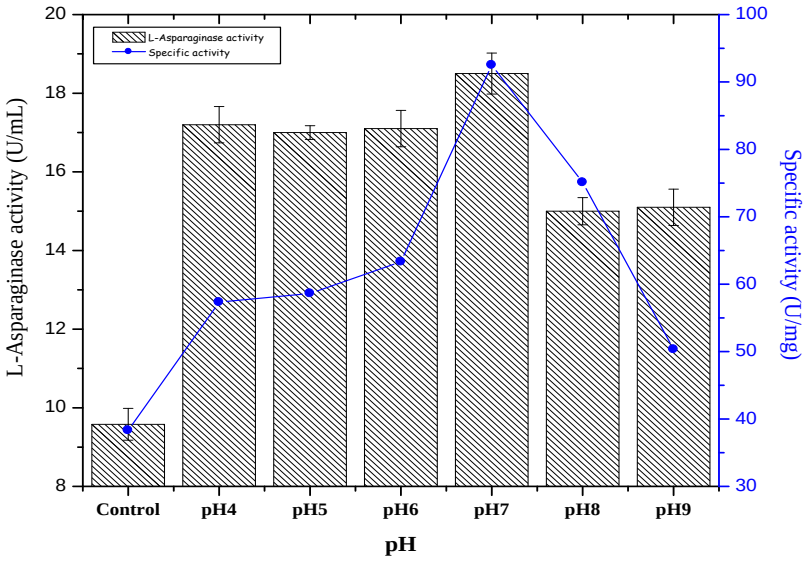

Figure 6 Influence of pH on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

L-Arginine, L-Tryptophan, L-Glutamic acid, and L-Tyrosine recorded L-ASNase production of about 14.5 ± 0.462 U/mL, 15 ± 0.115 U/mL, 14.7 ± 0.173 U/mL, and 14.9 ± 0.462 U/mL respectively (Table 2). L-ASNase production was also studied at various L-Asparagine concentrations (5-30 g/L). 10 g/L showed optimum L-ASNase production of 18.3 ± 0.346 U/mL for *Curvularia* sp. LCJ413 (Figure 5). L-Asparagine has been reported to be used as an inducer for increasing L-ASNase production (Elshafei and El-Ghonemy 2015). Abbas Ahmed et al. (2015) reported high L-ASNase production of 35.16 U/mL from marine endophytic fungus *Aspergillus* sp. ALLA2000 was achieved using the amino acid arginine as an inducer.

3.6 Influence of pH

The effect of different pH (4 - 9) on L-ASNase production was evaluated in MCDB. At pH 7, the highest L-ASNase activity of 18.5 ± 0.52 U/mL was recorded. L-ASNase production reduced as the pH increased (Figure 6). Kalyanasundaram et al. (2015) reported endophytic *Aspergillus terreus* produced high L-ASNase activity at pH 7 (32.25 U/mL). The pH of the growth medium is critical for nutrient transfer across the cellular membranes and the increase in L-ASNase production (Farag et al. 2015). For optimal L-ASNase production, a pH range of 6.3 to 9.0 culture medium was used (da Cunha et al. 2019).

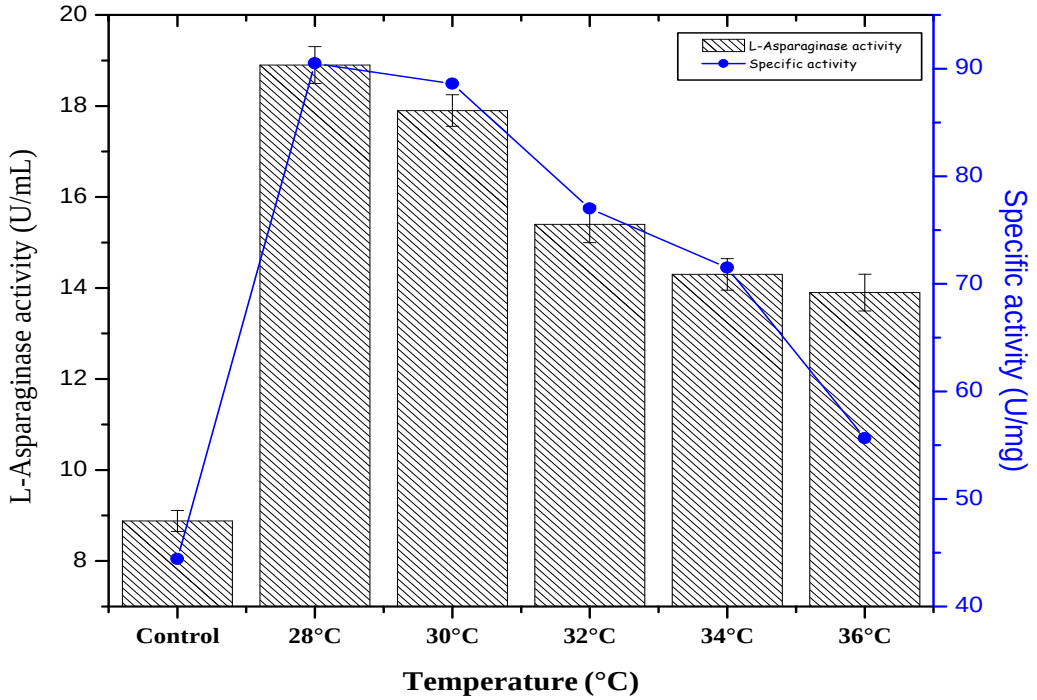

Figure 7 Influence of temperature on L-ASNase production from *Curvularia* sp. LCJ413 on the 6th day

3.7 Influence of temperature

Curvularia sp. LCJ413 could thrive and produce L-ASNase at various temperatures (28°C - 36°C). At 28°C, L-ASNase production reached a maximum of 18.9 ± 0.404 U/mL. Higher temperatures were found to reduce L-ASNase activity. The lowest level of L-ASNase production was 13.9 ± 0.404 U/mL at 36°C (Figure 7). The reduction in L-ASNase production might be due to heat generation inside the medium, which causes enzyme denaturation. Temperature influences the stability and rate of catalysis of the enzyme. Temperature tolerance and stability of L-ASNase vary between the isolated fungal species (Chand et al. 2020), and it was reported that 27°C was the ideal temperature for L-ASNase production from *Fusarium* sp. (Thirunavukarasu et al. 2011), 30.5°C for *Fusarium proliferatum* (Yap et al. 2021), and 35°C for *Aspergillus terreus* (Farag et al. 2015). The L-ASNase enzyme was most active between 25°C and 45°C (Castro et al. 2021).

3.8 Optimizing the L-ASNase production using RSM

The effect of four variables such as maltose, ammonium sulphate, pH, and L-Asparagine were studied by FCCCD using RSM. Additional trials were investigated in triplicate at the shake flask level with the optimized medium to validate the model's prediction. The FCCCD results examine the influence of four independent variables, namely maltose, ammonium sulphate, pH, and L-Asparagine, on L-Asparagine production, together with the experimental and predicted response (Table 3).

The p-value in the model was 0.0001 based on the ANOVA test, which is <0.005, representing that the model terms are substantial. The R^2 value of this study was 0.99, indicating that the model describes 99% of the variance in the response. The predicted sum of squares was 11.86, the adequate precision ratio was found to be 39.11, and the coefficient of variance was 2.70% (Table 4). All these responses validate that this model has the potential to initiate the design space for L-ASNase production. The FCCCD experimental findings were modelled with the following polynomial equation that predicted L-ASNase activity;

$$Y= 19.18 + 1.58A + 1.64B – 0.5989C + 0.7267D – 2.04A2 – 0.7090B2 – 1.68C2 – 0.4490D2 + 0.5456AB + 0.2744AC – 0.4494AD – 0.2069BC – 0.5356BD – 0.0794CD$$

Where Y is the activity of L-ASNase (U/mL); A, B, C, and D are maltose, ammonium sulphate, pH, and L-Asparagine, respectively

Three-dimensional graphs were created using RSM to interpret the association of selected components and the optimal concentration necessary for maximal L-ASNase synthesis (Figure 8-10). The highest L-ASNase production of 20.11 U/mL was attained using the combination of 2 g/L maltose, 10 g/L L-Asparagine, pH 7, and 30 g/L ammonium sulphate. The highest enzyme yield in this experiment was 2.28 times higher than the unoptimized medium. At the highest levels of all four parameters, L-ASNase production decreased. The model was evaluated in a designed space, the predicted responses were similar to the experimental responses,

and the model was successfully validated. The optimization of L-ASNase using RSM was helpful to this research because it aided in quickly identifying essential factors and their interactions. It also suggested the importance of the range of factors at altered levels. In the study conducted by Baskar and Renganathan (2011), optimization of L-ASNase using RSM yielded 35.73 U/mL of the enzyme from *A. terreus* MTCC under ideal conditions. The highest L-ASNase production (15.7808 U/mL) was attained using RSM resulting in a 108.62% increase in the L-ASNase production from *A. niger* (Vala et al. 2018).

Table 3 Experimental and predicted responses for L-ASNase production using FCCCD

Run	Maltose	Ammonium sulphate	pH	L-Asparagine	L-ASNase activity (U/mL)	
					Experimental value	Predicted value
1	-1	-1	-1	-1	10.38	10.50
2	-1	1	1	-1	12.34	12.17
3	-1	-1	1	-1	9.49	9.32
4	1	-1	1	-1	12.94	12.85
5	-1	1	-1	-1	13.93	14.17
6	1	1	1	-1	17.42	17.88
7	1	1	-1	-1	19.12	18.78
8	0	0	0	-1	18.24	18.01
9	1	-1	-1	-1	12.74	12.92
10	0	0	0	0	19.21	19.18
11	0	0	0	0	19.46	19.18
12	0	0	0	0	18.86	19.18
13	0	0	-1	0	18.11	18.10
14	0	0	0	0	19.57	19.18
15	1	0	0	0	18.74	18.72
16	0	-1	0	0	16.5	16.83
17	0	0	0	0	19.36	19.18
18	0	1	0	0	20.11	20.12
19	0	0	1	0	16.56	16.90
20	-1	0	0	0	15.2	15.55
21	0	0	0	0	19.64	19.18
22	-1	1	1	1	13.24	13.29
23	-1	-1	1	1	12.57	12.59
24	1	1	-1	1	18.02	18.43
25	1	1	1	1	17.64	17.20
26	-1	1	-1	1	15.84	15.61
27	1	-1	1	1	14.32	14.31
28	1	-1	-1	1	14.86	14.71
29	0	0	0	1	18.89	19.46
30	-1	-1	-1	1	14.3	14.08

Table 4 ANOVA analysis to validate the model's adequacy

Statistics	Model's response (L-ASNase production)
Standard deviation	0.3902
Mean	16.25
R^2	0.9914
F-value (lack of fit)	2.32
Adjusted R^2	0.9834
Coefficient of Variance	2.40
Predicted R^2	0.9554
PRESS	11.86
Adequate precision	39.11

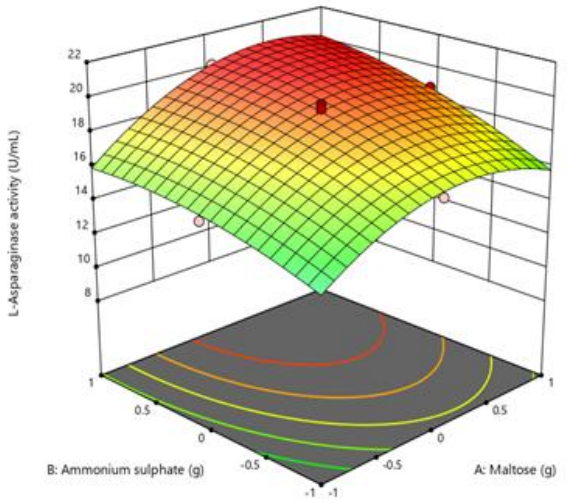

Figure 8 RSM plot representing the effect of interaction between maltose and ammonium sulphate on L-ASNase production

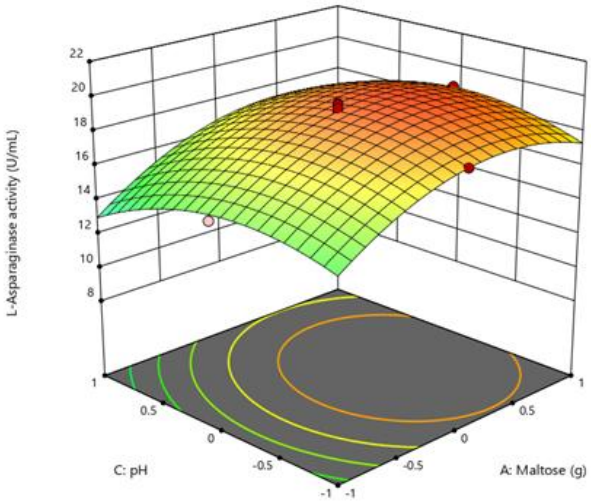

Figure 9 RSM plot representing the effect of interaction between maltose and pH on L-ASNase production

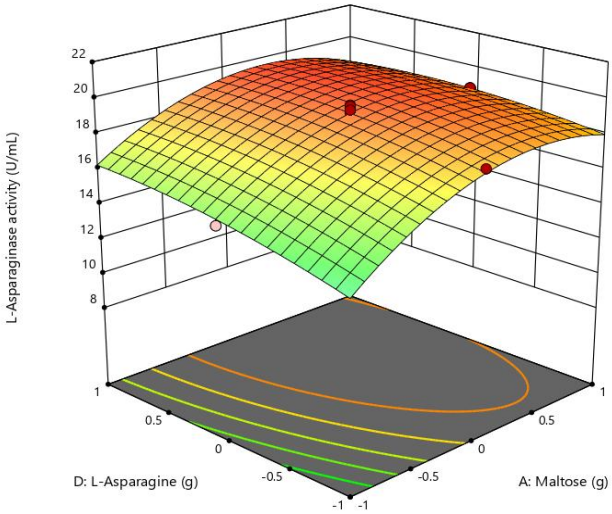

Figure 10 RSM plot representing the effect of interaction between maltose and L-Asparagine on L-ASNase production

Table 5 Comparative study of original and optimized medium for L-ASNase production

Medium	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Original medium	2.91 ± 0.231^{e}	3.86 ± 0.173^{de}	5.98 ± 0.346^{bc}	8.79 ± 0.346^{a}	6.54 ± 0.173^{b}	5.08 ± 0.289^{cd}
Optimized medium	12.7 ± 0.346^{e}	14.8 ± 0.404^{cd}	17.6 ± 0.346^{ab}	18.98 ± 0.52^{a}	16.2 ± 0.462^{bc}	14.1 ± 0.462^{de}

ANOVA (one-way) and TUKEY tests were performed. Values are in means ± SEM

3.9 Comparative study of original and optimized medium for L-ASNase production

The maximum L-ASNase production from *Curvularia* sp. LCJ413 in the original (standard MCDB) medium was 8.79 ± 0.346 U/mL. The observed L-ASNase activity in the MCDB after conventional optimization of parameters was found to be 18.98 ± 0.52 U/mL (Table 5). Thus, traditional one-factor optimization resulted in a 2.15 fold increase. Numerous studies on optimizing the process parameters for L-ASNase production were reported by Krishnapura and Belur (2016), Yap et al. (2021), Jenila and Gnanadoss (2018), and Abhini et al. (2022).

Conclusion

Endophytic fungus *Curvularia* sp. LCJ413 was an effective producer of the L-ASNase enzyme under submerged fermentation. On the 6th day, L-ASNase production in submerged fermentation was enhanced to 18.9 ± 0.404 U/mL with pH 7, an incubation temperature of 28°C, 2 g/L (maltose), 25 g/L (ammonium sulphate), and 10 g/L (L-Asparagine). Furthermore, statistical optimization with RSM raises L-ASNase production to 20.11 U/mL. A good level of resemblance was noticed with the experimental and predicted data indicating the reliability and applicability of RSM for enhancing L-ASNase production. Response surface plots also revealed the substantial interaction between four variables and their impact on L-ASNase production. The optimization approach used in this study can be implemented to upscale the yield of other enzyme production. Additional studies on the purification and characterization of L-ASNase will help in establishing its application in the food (for acrylamide mitigation) and pharmaceutical (as a chemotherapeutic agent) industries. The application of cutting-edge recombinant technology may increase the overall yield of this therapeutic enzyme L-ASNase.

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Conflicts of interest

The authors have no conflicts of interest.

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Antioxidant Potential of *Chloranthus erectus* (Chloranthaceae) from various solvents extract

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ABSTRACT

Chloranthus erectus is a herbaceous plant that has been used as a medicinal plant in several regions such as China and Southeast Asia. Although it possesses valuable medicinal properties, till now there is not much research has been carried out on the medicinal properties of this plant and the knowledge of this plant is limited among the research fertility. Therefore, this study aimed to identify the phytochemicals, total phenolic content (TPC), and antioxidant activity of leaf and twig of *C. erectus* in various solvents extract (hexane, petroleum ether, chloroform, ethyl acetate, and methanol). Phytochemical screening of extracts showed the presence of alkaloids, flavonoids, terpenoids, saponins, quinones, glycosides, and steroids. The highest phenolic content for leaf and twig samples was determined from the methanolic (9.64 ± 0.15 µg GAE/g) and hexanoic extract (7.39 ± 0.27 µg GAE/g), respectively. Meanwhile, the highest antioxidant activity was reported from the methanolic extract of both leaf (88.36 ± 0.24%) and twig (91.25 ± 0.10%) samples. Hence, the results of the study can be concluded that *C. erectus* has the potential to become a good natural antioxidant and the information from this study can be utilized by the communities as well as other researchers.

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1 Introduction

The consumption of herbal medicinal products by the public has increased significantly. Many medicinal practitioners have been focusing on the use of natural antioxidants instead of synthetic antioxidants. Synthetic antioxidants, such as Tempol, Probucol, and Transcrocetinate have various negative effects on human health and causing cancer and inducing premature senescence (Kornienko et al. 2019). Therefore, researchers have turned their attention toward medicinal plants and herbs and produced a safer and more natural antioxidant.

C. erectus belong to the family of Chloranthaceae, locally known as Sambau Paya in Peninsular Malaysia, Langut Langut in Sarawak, and Totol in Sabah has a long history of use as a medicinal plant. This plant has an average height of 3 m with broad shining dark green leaves (20 cm x 8 cm) and produces white bud-like flowers (Kiew et al. 2010). This shrub is found in tropical climate regions such as China, Eastern Himalayas, and Southeast Asia. Its habitat is primarily in a mountain forest at a lower altitude, where the plant can grow under the shade of trees and nearby a river that has moist soils (Kiew et al. 2019).

Although it is highly used as an herbal cure by folk traditional healers and modern herbalists in many indigenous communities of Asian countries, it is generally a lesser-known medicinal herb in the region, which is almost entirely unknown in other countries. A few studies have been done on different species from the Chloranthaceae family. For example, Zhang et al. (2016) have reported that *C. henryi* has been used as an alternative supplement in improving blood circulation. However, insufficient scientific research has been made to discover *C. erectus* chemical constituents and to know its pharmacological actions. Therefore, the present study was conducted to identify phytochemicals, total phenolic content (TPC), and antioxidant activity of leaf and twig of *C. erectus* from various solvents extract (hexane, petroleum ether, chloroform, ethyl acetate, and methanol). The expected output from this study is information about the potential of unexplored valuable local medicinal plant of *C. erectus* collected from Taman Negara Ledang, Johor for the early stage of drug discovery.

2 Materials and methods

2.1 Plant collection

Fresh *C. erectus* plant parts were collected from Taman Negara Ledang, Johor (2.3312589, 102.6125548). The collected plant samples were sent to Forest Research Institute Malaysia (FRIM) in Kepong, Malaysia for authentication (PID 160820-12).

2.2 Preparation of plant extract

The leaf and twigs of the plant were separated and gently washed with tap water and air-dried for a week. Dried samples were ground to a fine powder by using a mixer grinder. The dried powdered sample of the leaf (200 g) and twig (80 g) was extracted sequentially with five different solvents namely hexane, petroleum ether, chloroform, ethyl acetate, and methanol by maceration technique for 72 hours, respectively. The extracts were then gravitationally filtered using Whatman No. 1 filter paper and the filtrates were evaporated to dryness using a rotatory evaporator. The percentage yield of each extract was calculated and recorded by using the below equation and sample extracts were kept refrigerated at -20^{o}C until further use (Balasubramaniam et al. 2020).

% Yield of extract =

$$\frac{\text{Weight of extract residue after solvent removal (g)}}{\text{Weight of dried sample powder (g)}} \text{X } 100$$

2.3 Phytochemical screening

The presence of phytochemicals from each solvent extract of leaf and twig was carried out using the standard procedure of Keshav et al. (2019) with slide modification to identify the presence of flavonoids, terpenoids, and saponins. While for the identification of quinones, steroids, and glycosides, a method by Shaikh and Patil (2020) was used.

2.4 Determination of total phenolic content (TPC)

The TPC for *C. erectus* leaf and twig extracts was determined by using the Folin-Ciocalteu method given by Madiha et al. (2016) with a slight modification. For this, a total of 0.5 ml of sample was mixed with Folin-Ciocalteu reagent and left in dark for 5 minutes. This was followed by the addition of 1.5 ml of 7.5% of sodium carbonate and then left incubated for 30 minutes in the dark. Finally, the absorbance was read by using UV-Vis spectrophotometer at 725 nm. The same process was repeated for gallic acid with various concentrations (10, 25, 50, 75, and 100 µg/ml) to construct a calibration curve. The results were expressed as µg gallic acid equivalent (GAE)/g dry weight of extract (Romes et al. 2019).

2.5 DPPH scavenging activity

Exactly 0.2 ml with a concentration of 50 µg/ml of the sample was pipetted out into a test tube. To this tube, 3 ml of 0.1 mM DPPH solution was added, mixed well, and incubated in a dark room for 30 minutes. The absorbance was read at 517 nm against a blank solution which is distilled water. Ascorbic acid was used as a standard reference. The percentage inhibition was calculated for the sample and standard (Masuku et al. 2020). Using the below-mentioned equation the percentage inhibition was calculated:

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \text{ X } 100$$

Where Abs is the absorbance

2.6 Statistical analysis

The data were expressed as a mean value ± standard deviation of triplicates (n=3). The statistical analysis used a one-way ANOVA test, while the significance of differences between means was determined by using the Games-Howell test at $p \leq 0.05$ by using Statistical Package for Social Science (SPSS) software.

3 Results and discussions

3.1 Extraction of *C. erectus* plant extract

In this study, the maceration extraction technique was applied, in which the sample was first extracted with a non-polar solvent and then continue with more polar solvents successively. This is because a single solvent would not be able to extract all phytochemical and antioxidant compounds from the plant material due to the chemical nature of the compounds. Therefore, by using different solvents and increasing the polarity of the solvent from non-polar to polar, a wide range of compounds can be extracted (Nawaz et al. 2019). Results presented in Table 1 revealed the summary of the extraction of *C. erectus* leaf and twigs.

Results presented in Table 1 suggested that among the tested five extracts, methanolic extract yielded the highest phytochemical compound consistently compared to the other four extracts from both leaf and twig samples which were 4.29% and 14.79%, respectively. The differences in yield percentage among the solvents were due to the polarity of the solvent used during extraction. According to Do et al. (2014), the polarity of the solvent affects the yield of crude extracts. This also indicates that the chemical compounds in this plant are mostly polar, thus, producing a high yield percentage in the polar solvent. Table 1 also shows that the extracts produced are mostly semi-solid and viscous.

3.2 Phytochemical screening

Phytochemical screening tests were conducted to detect the presence of phytochemical compounds in five different solvents. Each extract may consist of different types of compounds therefore some of the extracts showed negative results on certain tests. Table 2 summarises the results of phytochemical screening that had been conducted on *C. erectus* leaf and twig extracts demonstrating the presence of various phytochemicals such as alkaloids, flavonoids, terpenoids, saponins, quinones, and steroids.

The phytochemical qualitative analysis displayed that the hexane and ethyl acetate extracts recorded the highest phytochemical compounds in both leaf and twig samples compared to other extracts. These findings can be supported by a study done by Vivi Mardina et al. (2020) in which they used both ethyl acetate and hexane solvent for extraction and recorded that ethyl acetate recovered more chemical compounds compared to hexane. This demonstrated that the polarity of solvent can affect the phytochemical compound yield. In addition, the twig sample exhibits a wide range of phytochemical compounds in phytochemical analysis. This finding was in line with a study reported by Wang et al. (2015) in which they managed to recover various phytochemical compounds from the same part of the plant in other different species of Chloranthus plant.

The presence of flavonoids indicated that this species has the potential to be used as remedies and as a natural antioxidant agent. Zhang et al. (2016) studied the constituents of many plant species belonging to the Chloranthaceae family. In the study, they managed to isolate flavonoids from another *Chloranthrus* plant namely *C. multistachys*. A study done by Xu et al. (2020) also discovered flavonoids from another *Chloranthus* species

Table 1 The extraction summary of leaf and twig extracts from *C.erectus* on various solvents

Plant Part	Solvent	Weight of Dried Leaf Powder (g)	Physical Properties	Weight of Extract Residue After Solvent Removal (g)	Percentage Yield (%)
Leaf	Methanol	200	Semi-solid	8.76	4.29
	Ethyl Acetate		Semi-solid	3.49	1.71
	Chloroform		Semi-solid	3.12	1.53
	Petroleum Ether		Semi-solid	3.21	1.57
	Hexane		Semi-solid	7.17	3.51
Twig	Methanol	80	Jelly-like	11.83	14.79
	Ethyl acetate		Semi-solid	0.69	0.86
	Chloroform		Semi-solid	3.35	4.19
	Petroleum ether		Semi-Solid	0.83	1.04
	Hexane		Semi-solid	1.14	1.43

*Semi-solid physical properties represent a sticky and viscous-like texture; Jelly like physical properties represent a squishy cube-like structure

Table 2 Phytochemical screening of *C.erectus* leaf and twig extract

Test	Methanol		Ethyl acetate		Chloroform		Petroleum ether		Hexane	
	Leaf	Twig	Leaf	Twig	Leaf	Twig	Leaf	Twig	Leaf	Twig
Alkaloid	+	-	-	-	-	-	-	-	-	-
Flavonoids	-	-	-	+	-	+	-	+	+	+
Terpenoids	-	+	-	+	-	+	-	+	-	+
Saponins	+	+	-	+	-	+	-	-	-	+
Quinones	-	-	+	+	+	-	+	+	+	+
Steroids	-	+	+	+	-	+	+	+	-	+

(+) = Presence, (-) = Absence

named *C. henryi*. These findings, therefore, strongly suggest high antioxidant activity in *Chloranthus* spp, which is commonly associated with the presence of polar phenolic and flavonoid compounds.

Presence of the terpenoids in plant extract also plays an important role in antioxidant activity. A study conducted by Mohandas and Kumaraswamy (2018) stated that the presence of terpenoids in a considerable amount could contribute to high antioxidant activity. However, based on Table 2, there were no terpenoids present in both leaves or twig samples. This could be due to the method used in this study which is qualitative analysis. Compared to quantitative analysis, the qualitative analysis only detects the compound's presence and appeared to lack sensitivity and specificity, which could impact the result obtained (Tzima et al. 2018). Therefore, further analysis is recommended to quantify the density/number of terpenoids in this plant.

3.3 Total phenolic content (TPC)

In this study, the total phenolic content (TPC) for *C. erectus* leaf and twig samples is tabulated in Table 3. The TPC was expressed in terms of gallic acid equivalent (µg GAE/g dry weight) by using the equation based on the calibration curve where $y = 0.0109x + 0.0377$, $R^2 = 0.9801$.

Based on the results obtained in Table 3, the highest TPC was recorded for a methanolic extract for the leaf sample by 9.64 ± 0.15µg GAE/g dry weight while for the twig sample, hexane extract managed to record 7.39 ± 0.27µg GAE/g dry weight. Due to its polar nature, the amount of phenolic compound in methanolic leaf samples was influenced by the solvent polarity thus giving a higher value. However, the hexanoic twig sample managed to achieve the highest total phenolic content compared to other extracts. This could be because of the presence of other compounds in the hexane extract that could influence the TPC of the twig extract. A study carried out by Gema et al. (2020) reported that different compounds such as terpenes and saponin can interact with the complex phenol structure which in turn interferes with the phenolic content quantification. These results can also conclude that this plant has the potential as a good natural antioxidant agent. This is because phenolic compound possesses redox properties that could help in neutralizing free radicle molecules (Zheng and Wang 2001; Huyut et al. 2017). As of today, there is no study has been reported on the total phenolic content of *C. erectus* or any other Chloranthus genus plants except for one study done by Xu et al. (2020) in which they managed to obtain TPC content ranging from 4.36 – 19.64 mg GAE/g dry weight from the n-butanol extract of *C. henryi*.

Table 3 Total phenolic content of *C. erectus* leaf and twig extracts

Extract	Total phenolic content (µg GAE/g dry weight)	
	Leaf	Twig
Methanol	9.64 ± 0.15	6.07 ± 0.03
Ethyl acetate	4.92 ± 0.04	6.36 ± 0.15
Chloroform	2.34 ± 0.08	2.83 ± 0.07
Petroleum ether	3.00 ± 0.02	0.94 ± 0.38
Hexane	0.47 ± 0.02	7.39 ± 0.27
Standard 2 (Gallic Acid)	99.60 ± 0.86	

*Values are represented as mean value ± standard deviation

Table 4 Antioxidant activity of *C. erectus* leaf and twig extracts

Extract	Concentration (µg/ml)	DPPH Scavenging Activity (%)	
		Leaf	Twig
Methanol	50	88.36 ± 0.24	91.25 ± 0.10
Ethyl acetate		55.43 ± 0.20	13.32 ± 0.45
Chloroform		31.87 ± 0.77	13.27 ± 0.38
Petroleum ether		11.00 ± 3.37	33.40 ± 1.33
Hexane		12.65 ± 4.01	30.34 ± 0.52
Standard (Ascorbic Acid)		95.10 ± 0.21	

*Values are represented as mean value ± standard deviation

3.4 DPPH scavenging activity

In the case of the antioxidant activity of *C. erectus*, the results presented in table 4 summarized the result of DPPH scavenging activity in percentage.

Results presented in Table 4 revealed that the highest antioxidant activity can be observed from the methanol extract of both leaves (88.36 ± 0.24%) and twig (91.25 ± 0.10%). Few studies suggested that antioxidant activity is associated with the maturity of the plant itself. A study done by Kuntorini et al. (2022) on the maturity effect and antioxidant activity of leaves and fruits of *Rhodomyrtus tomentosa* suggested that young leaves have high antioxidant activity compared to old leaves. Some studies suggest that plant antioxidant activity could be affected by the presence of chlorophyll in the sample. According to Simao et al. (2013), the antioxidant level is high when there is a low presence of chlorophyll in a sample. This could be supported by the results shown in Table 4 and when the methanolic extract of the twig sample managed to record the highest antioxidant activity compared to the methanolic leaf sample due to the reason twigs have less chlorophyll compared to the leaf. It is also noted that the methanol extract of twig showed the highest antioxidant activity and it is as good as ascorbic acid (95.10 ± 0.21%). Xu et al. (2020) reported that the antioxidant activity of another different Chloranthus species suggested the presence of a phenolic compound that could show a good antioxidant property. Other than that, there are not many studies that have reported on antioxidant activity for this plant or any associated species. In short, *C. erectus* has the potential and can be applied as an antioxidant agent.

Conclusion

Recent findings exhibited that both leaf and twigs extracts of *C. erectus* possess various phytochemicals such as alkaloids, flavonoids, saponins, quinones, and steroids. Among the tested various extracts, the methanolic extract showed the highest TPC for the leaf sample while for the twig sample, hexane extract displayed the highest TPC compared to other extracts. In addition, both samples of methanolic extracts displayed the highest antioxidant activity.

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Metal Accumulation in Ekiti State's Three Major Dams' Water and Sediments, the Ecological Hazards Assessment and Consequences on Human Health

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ABSTRACT

Water is indispensable to life. Consequently, water and sediment contamination poses severe ecological threats to life. Thus, this investigation aimed to evaluate metal deposition in the sediments and surface water in Ekiti State's three dams and to analyze its potential ecological effects on man's bodily, social, and mental well-being. Metal levels in sediments and dam water were determined using Atomic Absorption Spectroscopy (AAS). Average values of the metals in Egbe, Ero, and Ureje dams, except for K, Mn, and Pb (in Ureje dam), were lower than the acceptable boundaries of local and foreign establishments. The values of the risk quotient (HQ) on the skin and consumption contacts with all metals (except Mn for ingestion exposure for children) were less than one in the Egbe, Ero, and Ureje dams for both adults and children. Consumption HQ values were higher than skin HQ values in the three dams for children and grown-ups. The total hazard index (HI) posed adverse non-carcinogenic risk to children in the catchment area of the dams while the adults were not affected by the non-carcinogenic hazard. The highest cancer hazard was found in the Ureje dam, while the lowest was in the Ero dam. Further, adults were prone to higher cancer risk than children. Using multiple pollution indices revealed that the sediments in Egbe, Ero, and Ureje dams were less contaminated by harmful metals in dry and wet periods. There is a need to reduce current polluting anthropogenic activities around the dams.

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1 Introduction

Contamination of freshwater and sediment poses a severe ecological risk to countless water bodies in the developing world and toxicity to the ecosystem (Olayinka-olagunju et al. 2021). Water is indispensable to life, and potable drinking water is essential for human existence. As a result, potable drinking water is not supposed to pose any significant threat to human life or well-being. Metal contamination in aquatic environments has become a global issue. It is worsening at an alarming rate (Al-Afify and Abdel-Satar 2022), with consequent threats to the food chain of animals (Olayinka-olagunju 2021). Metals are non-biodegradable substances that naturally occur in the upper part of the lithosphere and are constantly deposited through anthropogenic activities and become integrated into the sediment, water, and aquatic biota, causing aquatic ecosystem contagion (Briffa et al. 2020). Metals occur in small amounts in nature and are formed primarily by rock and soil weathering (Masindi and Muedi 2018; Obasi and Akudinobi 2020). Thus, sources of metals in our environments are natural and anthropogenic (Ali et al. 2019).

However, anthropogenic sources are the primary donors of metal to the environment. Metals such as Cu, Mn, Ni, Mn, Fe, and Zn are required in minimal sums as microelements by plants and animals. In contrast, metals without useful functions are exceedingly hazardous in the minutest quantities (Khalef et al. 2022). Metals in significant amounts in the environment could be dangerous and detrimental to the effective operations of natural ecosystems and human health (Velma and Tchounwou 2010; Vieira et al. 2012) as a result of their toxic effects, extended persistence, bio-accumulative properties, and bio-magnification in the food chain. Aquatic ecosystems are an essential feature of our environment because they serve as reservoirs for such resources as minerals, fisheries, and portable water. Thus, their protection as a sensitive resource is critical for long-term development. The entrance of metals into water ecosystems follows different pathways, either from point or non-point sources (Mustapha and Getso 2014). In water, metals can inflict considerable harm to biological environs and, consequently, the well-being of human populations (Kawser et al. 2016; Dehghani et al. 2017). The two most expected ways humans can be exposed to metals in an aquatic environment are through ingestion and dermal absorption (Li and Zhang 2010[a], Benoit et al. 2019). Sustainable utilization of water environments contributes to man's comprehensive state of bodily, social, and mental well-being and existence.

Sediments are an essential and dynamic element in aquatic ecosystems and have been frequently used as environmental indices for metal pollution assessment in natural water (Morillo et al. 2004; Islam et al. 2015; Tao et al. 2012; Proshad et al. 2019; Radomirović et al. 2021). Sediments have become sinks for heavy metals and sometimes act as carriers and environmental sources of heavy metals (Algül and Beyhan 2020). Increased anthropogenic and agricultural activities in Ekiti State contribute to increased discharge of chemical pollution into the ecosystem, which may likely result in higher metal levels in water sources and sediments. The contaminant concentrations in sediments are functional ecological hazard indices related to contamination in water environments (Stamatis et al. 2019). Metal levels in water are occasionally below detectable levels; hence sediments are helpful in the estimation of the degree of metal pollution in water ecosystems (Tunde and Oluwagbemiga 2020). The necessity to evaluate the bottom sediment quality concerning metal pollution prompted the development of several geochemical and ecotoxicological indicators, the application of which is advantageous for environmental pollution hazard assessment and water source safety public policy (Cymes et al. 2012). These indices include metal levels in the water, Geo-accumulation index (Igeo), index of metal pollution (MPI), possible ecological hazard index, contamination factor, degree of contamination (Cd), and sediment quality criteria (SQGs). In addition, these indicators are helpful in the environmental hazards assessment, evaluation of the decline in physicochemical and biological soil quality, creation of opportunities for societal and ecological awareness, and future ecosystems sustainability prediction (Kowalska et al. 2018, Radomirović et al. 2021).

Further, these indices have been used by numerous researchers in measuring the ecological risks of pollution by metals in water environments (Cymes et al. 2012; Likuku et al. 2013; Harikrishnan et al. 2016; Yang et al. 2016; Bubu et al. 2017; Samuel et al. 2019; Radomirović et al. 2021). Despite their widespread use, there is no report on any of these indices on the population living in the catchment area of the Egbe, Ero, or Ureje dams. Therefore, this research was designed to (1) assess the level of metal accretion in the surface water and sediments of Egbe, Ero, and Ureje dams, (2) analyze seasonal fluctuations, and assess any ecological, environmental, and health problems that may be connected with them, and (3) contribute to future management and study of metals in the State's dams.

2 Materials and Methods

2.1 Area of Study

Ekiti State is situated in the eastern part of the Greenwich Meridian in the north of the Equator between longitudes 4° 45' - 5° 45' east and latitudes 7° 15' - 8° 5'. Ekiti State shares a border with Kwara, Kogi, and Osun States in the north, east, and south, respectively. The studied area has several metamorphic and igneous rocks which are situated underneath stratified rocks in southwestern Nigeria. This investigation was carried out in Egbe, Ero and Ureje dams, the three major dams in the Ekiti State. The locations of the dams in the Ekiti State map are shown in Figure 1. Fishing, recreational,

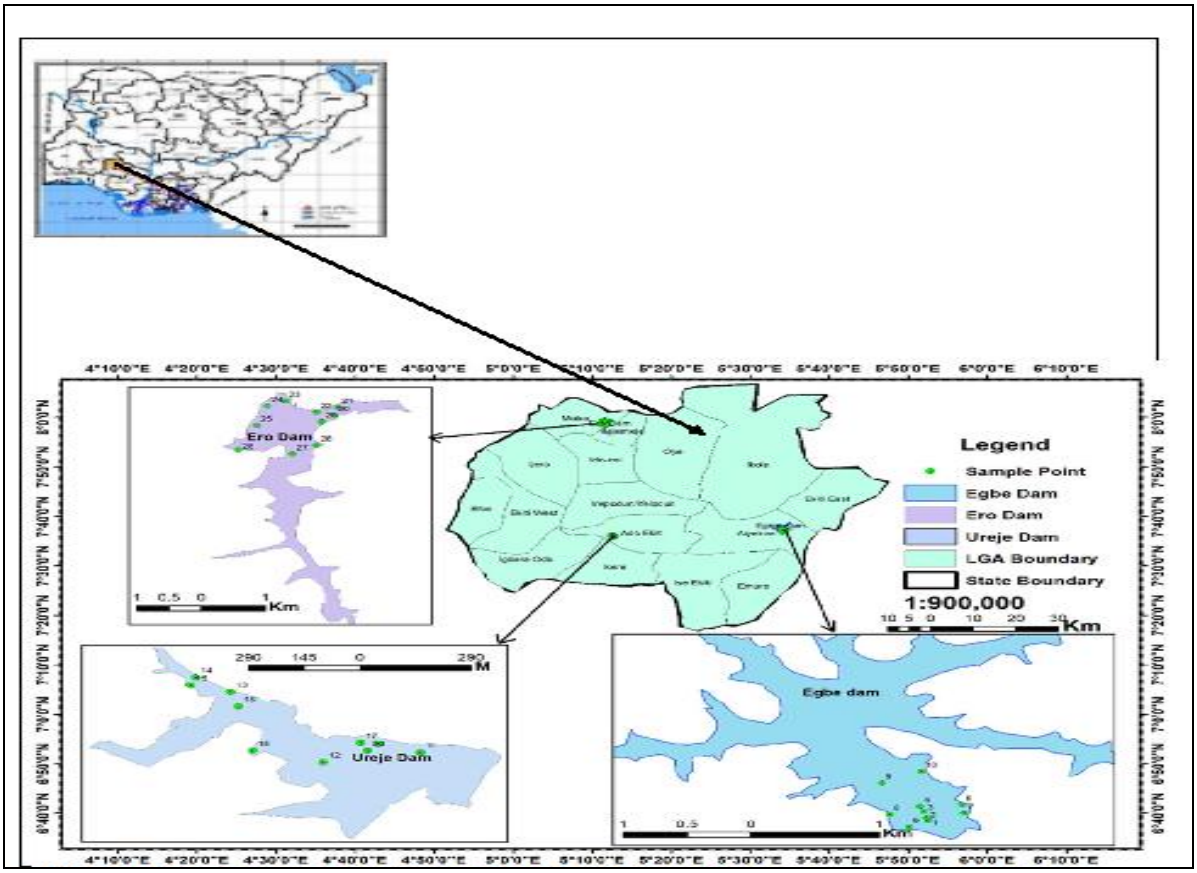

Figure 1 Ekiti State map showing the dams (Egbe, Ero, and Ureje) and the sampling sites in Ekiti state, Southwest Nigeria.

agricultural, domestic activities, and sewage disposal are all common occurrences, particularly in human communities around the dams. Insecticides and agricultural-based chemicals are used regularly in the dams' catchment areas for various agricultural and domestic operations. There is also the dumping of household solids and liquid wastes directly into the dams without proper treatment. In addition, runoff from farms is also offloaded directly into the dams.

2.2 Water and Sediment Sample Collection

Ten sites were selected from each dam based on discussions with fishermen because they operate adjacent to the residential and farming regions alongside high pollution levels and increased fishing activities. Water samples were taken from about 30 cm beneath the water surface from the ten sites into 2-liter polypropylene containers between 8 a.m. and 10 a.m. The GPS of sampling sites was taken using Garmin hand-held GPS. The containers were cleaned with distilled water and washed with 10% nitric acid before utilization for water collection. Dams water was used to rinse the containers thrice before being submerged in the dam to collect water samples during the sampling period. A composite subdivision of water was formed monthly by blending the collected samples from multiple sites to obtain a representative sample (Prakash et al. 2011; Said et al. 2012; Ndimele and Kumolu-Johnson 2012; Olafisoye et al. 2016; Tichkule and Bakare 2017). The containers of the samples were correctly registered to designate the period and site of sampling. After collecting the water sample, 2 mL of strong nitric acid was added to inhibit the adherence of metals to the sides of the container and to stop microbiological growth in the water samples.

With the aid of the Eckman clutch sampler, sediment samples were collected from various sites at a depth of 20-50cm for 24 months. Each of the three dams had a composite sediment sample deposited into a polythene bag, which was then correctly registered to denote the sampling period and location. The appropriately stored samples were conveyed in a container, which was thoroughly insulated and packed with ice cubes into the research laboratory and preserved at -20 °C in a freezer, pending heavy metal analysis.

2.3 Water Sample Preparation

For water sample preparation, 2 ml of undiluted nitric acid and 4 ml of undiluted Hydrochloric acid were added to the 100 ml water sample. The sample was enclosed and heated to 95 ^{o}C to decrease

the volume by 20 ml. After cooling, the interior of the beaker was rinsed down with distilled water, and silicates and other soluble components were filtered out using Whatman paper. The sample filtrate was increased to 100 mL and used for metal/mineral analysis.

2.4 Sediment Sample Preparation

The sediments were heated at 105°C, homogenized by lightly crushing them in a mortar, and then prepared for evaluation of metal content. Sediment digestion was carried out according to APHA (2005). A carefully weighed gram of the sample was added 5 ml of strong and undiluted nitric acid. Samples were dried by heating them to a temperature of 120 °C. The acid addition and heating method was repeated three times. This was followed by the addition of 25 ml of distilled water to boost the filtrate volume derived from adding of roughly 50 ml of water to the remaining material and filtering with a 0.45 m Whitman filter.

2.5 Metal Content Analysis in Water and Sediment Samples

AAS (Buck Scientific Model 211 VGP) and a Flame Photometer FP902 PG was used in metal content appraisal in water and silt. Solutions of each element in known concentrations of 1.0, 0.8, 0.6, 0.4, and 0.2 mgL^{-1} were prepared from a highly concentrated solution. AAS was utilized to analyze each processed metal's filtrate and a series of reference solutions. The metal discovery limits in each sample were 0.0001 using the AAS model. Magnesium, manganese, iron, copper, zinc, calcium, lead and cadmium ions were measured in the sample filtrate and standard solutions using cathode lamps at wavelengths of 285.21, 279.48, 248.00, 324.75, 213.86, 422.67, 217.00 and 228.80 for the metals respectively. The AAS was auto-zeroed with distilled water before standard solutions were added from the lowest to the highest concentrations to assess each element. The AAS gave the matching absorbance to the concentrations of the various solutions, and the graph of different concentrations against the absorbance was designed. The metal concentrations in the sample were calculated in parts per million using the standard graph as a guide (Greenberg et al. 1985).

2.6 Human Health Hazard Appraisal Indicators

Toxicology indicators were used to evaluate the health hazard effects of non-carcinogenic and cancer-causing metal on the dams (Dorme et al. 2011; Wongsasuluk et al. 2014). Quantification of pollution in metal, probable cancer-causing, and non-carcinogenic health hazards induced by heavy metal ingestion and skin absorption in the Egbe, Ero, and Ureje dams was accomplished by Risk Quotients, Risk Index, and the incremental lifespan tumor hazard. Health consequences due to metals in the dams' surface were evaluated through ingestion and skin contact according to USEPA (2004) and Li and Zhang (2010^{b}). Below in equations 1 and 2 are the formulae for the computations of the mean diurnal dose for consumption and cutaneous contact, respectively.

$$(Cn \times Ef \times Ed \times Ir \times) / (Bwt \times At) = ADDing \quad (1)$$

$$(Cn \times Et \times Ef \times Sa \times Pc \times Ed \times Cf) / (Bwt \times At) = ADDderm \quad (2)$$

Here ADDing is the mean diurnal dose from water consumption (g/kg/d); ADDderm is the mean diurnal dose from skin contact (g/kg/d); Cn is the level of the metal in the dam (g/L). For adults, Ir, which is the rate of water consumption, was taken to be 2.2 L/d; Ef is the contact rate is 365 days/year; Ed is the contact period of 70 years; Bwt is the mean body weight i.e. 70 kg; At is the averaging time (Ed x Ef). A year is taken to be 365 days, averaging time (25,550 days). Sa is the exposed dermal area of 18,000 cm^2; Et is the contact time of 0.58 h/d. Cf is the entity change factor (0.001 L/cm^3); and pc is the coefficient of skin permeability (cm/h), which are 0.0006, 0.001, 0.001, 0.001, 0.001 and 0.0001 for Zn, Cu, Mn, Fe, Cd, and Pb respectively (Iqbal and Shah 2013; Liang et al. 2011; Hadzi et al., 2015).

For children, Ir = 1.8, Ed = 6yrs, Bwt = 15kg, At = 2190, Et = 1, Sa = 6,600 (Tay et al. 2019).

Risk Quotient through consumption or skin (HQ) = ADD/RfD (3)

Where RfD is the ingestion/dermal reference measure (mg/L/day), procurement of ingestion reference measures (RfDi) of metals was according to Li and Zhang (2010^{b}) while skin absorption reference measure (RfDd) were computed from the following equation 4.

$$RfD_d = RfD_i \text{ x } ABS_g \quad (4)$$

Here, ABSg is the digestive system assimilation factor of metals, for this, USEPA (2004) recommended values were applied.

The potential damage to man's health from several metals was determined from the hazard index (summation of all hazard quotients of the metals). A hazard index < 1, signifies no substantial non-carcinogenic hazard

To assess Cancer-causing hazards, (S.F. is multiplied by ADD) in mg/kg/day (Wongsasuluk et al. 2014; Adamu et al. 2015). Therefore,

$$CRing = ADDing \text{ x Slope factor} \quad (5)$$

$$CRderm = ADDderm \text{ x Slope factor} \quad (6)$$

Here CRing is the cancer-causing hazard due to consumption, and CRderm is the cancer-causing hazard due to skin contact. The slope factor in mg/kg/day is 0.0085 for Pb and 6.3 for Cd (Bamuwamye et al. 2017; Ayenuddin Haque et al. 2018). The cancer-causing acceptable hazard range is between 1×10^{-6} and

Table 1 Risk grade, value and assessment standard

Hazard Rankings	Variety of hazard value	Acceptability
Rank I (Exceptionally small hazard)	$<10^{-6}$	Total admit
Rank II (Small hazard)	10^{-6}, 10^{-5}	unwillingness to be carefulness about the threat
Rank III (Small moderate hazard)	10^{-5}, 5×10^{-5}	Care less about the threat
Rank IV (Moderate hazard)	5×10^{-5}, 10^{-4}	Caution about the threat
Rank v (Moderate high hazard)	10^{-4}, 5×10^{-4}	Caution concerning the threat willing to spend
Rank VI (Great hazard)	5×10^{-4}, 10^{-3}	Be responsiveness to the threat and proffer solution
Rank VII (Exceedingly great threat)	$>10^{-3}$	Refuse the hazard and resolve it.

Source: Li et al. (2017).

1×10^{-4} (Li et al. 2017). The hazard grade and assessment standard are shown in Table 1.

2.7 Appraisal of Metal Pollution in Sediments

The following pollution indices determined sediment contamination and the degree of pollution:

2.7.1 Index of Metal pollution (MPI)

The formula of Usero et al. (1997) was employed to determine the index of metal contamination (MPI).

$$MPI = (Cm1 \times Cm2 \times \ldots\ldots\ldots\ldots\ldots\ldots\ldots Cmn)^{\frac{1}{n}} \quad (7)$$

In which n = total number of metals studied; Cm1= concentration value of metal no. 1; Cm2 = concentration of metal no. 2, Cmn is the concentration of nth metal.

2.7.2 Pollution factor and degree of pollution

Contamination or pollution factor is the proportion of the sediment's metal content to the metal's background value. In contrast, contamination degree is the addition of the contamination factors (C.F.) of all metals in the sediment divided by the number of metals investigated.

$$\text{Contamination Factor} = \frac{\text{Evaluated of metal level in sediments}}{\text{Background value of the metal}} \quad (8)$$

Where the background metal value is equivalent to the average world's surface rock

The degree of pollution = the summation of the metal contamination factors (9)

2.7.3 Index of Pollution Load (PLI)

This can be obtained from the contamination factor of metals concerning the background rate in sediment. It gives an aggregate measure of the total level of metal harmfulness in specific sediment and is evaluated as the n^{th} root of the multiplication of the pollution factors. Tomlinson et al. (1980) proposed an index of pollution.

$$PLI= (CF1x\ CF2\ x\ CF3\ x\ldots\ldots\ldots x\ CFn)^{1/n} \quad (10)$$

in which, n = total no. of metals; CF = pollution factor.

2.7.4 Geo-accumulation Index (Igeo)

Geo-accumulation Index is useful in evaluating sediment pollution by metal by relating current metal concentration with pre-industrial concentrations. The following formula computed the geo-accumulation index (Mùller 1979)

$$Igeo=\log 2\left[\frac{Cn}{1.5 \times Bn}\right] \quad (11)$$

in which Cn is the level of metal "n" in sediment, Bn is the metal "n" background value (Turekian and Wedepohl, 1961), 1.5 is to make up for differences in the background data due to lithological discrepancies.

Table 2 Indicators and corresponding degrees of Pollution factor and Degree of Contamination

Pollution factor (C_f^i)	Degree of single-metal pollution	Degree of pollution (Cd)	Degree of multiple-metal pollution
$C_f^i < 1$	Factor of pollution is low	Cd <*6*	Degree of pollution is low
$1 < C_f^i < 3$	Factor of pollution is reasonable	*6* ≤ Cd < 12	Degree of pollution is reasonable
$3 < C_f^i < 6$	Factor of pollution is substantial	12≤ Cd < 24	Degree of pollution is substantial
$C_f^i > 6$	Factor of pollution is very great	Cd > 24	Degree of pollution is very high

Source: Hakanson (1980).

Table 3 Indices and corresponding Geo-accumulation Ranks

Index of geo-accumulation (*I*geo)	Ranks
Igeo < 1	Rank 0: un8contaminated
0 <Igeo≤ 1	Rank 1: uncontaminated - reasonably contaminated
1 <Igeo≤ 2	Rank 2: reasonably contaminated
2 <Igeo≤ 3	Rank 3: reasonably-powerfully contaminated
3 <Igeo ≤ 4	Rank 4: powerfully contaminated
4 <Igeo ≤ 5	Rank 5 powerfully to exceptionally contaminated
Igeo > 5	Rank 6: exceptionally contaminated

Source: Mùller (1979).

2.7.5 The ecological hazard index (ERI)

The ecological hazard index (E.R.), together with the potential ecological hazard index (E_{RI}) were derived from the contamination Factor (C.F.) and toxicity factor (*Tr*).

Contamination factor, $C.F._f^i = C_D^i/C_R^i$ (Hakanson, 1980) (12)

where C_D^i is the metal level in the sediment of each location; C_R^i is the background metal level in the sediment of each site; $C.F._f^i = C_D^i/C_R^i$ is the pollution of a metal.

Monomial Probable Ecological Hazard Factor, $E_r^i = T_r^i \times \frac{C^i}{C_o^i}$

Here E_r^i is the monomial probable ecological hazard factor of metal *i*; C^i and $C_0^i$are the concentration of metal "*i*" and its reference concentration in sediment respectively; T_r^i is the toxicity factor of the metal "*i*".

The Ecological Hazard Index, $E_{RI} = \sum_{i=1}^{n} E_r^i$ (13)

Here E_{RI} is the summation of the probable ecological hazard index for the elements in the sediments.

2.7.6 Ecotoxicological metals appraisal by sediment quality guidelines

The levels of metal obtained in sediment in an ecotoxicological context and the obtained results were related to the consensus-based sediment quality guiding principle. The effect range medium quotient (ERMQ) and the probable effect level quotient (PELQ) were used (Soliman et al. 2015).

$$\text{ERM-Q or PEL-Q} = \sum \left[\frac{Ci}{ERMi \text{ or } PELi}\right]/n \quad (14)$$

Here, *Ci* is the level of metal "*i*" in sediments, *ERMi*, *PELi* are the reference values for metal "*i*", and n is the total number of metals in the sediment.

Table 4 Indices and Corresponding Classes of Potential Ecological Hazard

Ecological hazard factor (E_r^i)	Ecological risk level of single-factor pollution	Potential ecological risk index (ERI)	Potential ecological risk of multiple-factor pollution
$E_r^i < 40$	Little hazard	ERI < 150	Little hazard
$40 \le E_r^i < 80$	Reasonable hazard	150 ≤ ERI <300	Reasonable hazard
$80 \le E_r^i < 160$	Great hazard	300 ≤ ERI < 600	Great hazard
$160 \le E_r^i < 320$	Great hazard	ERI ≥ 600	Significantly high risk
$E_r^i \ge 320$	Serious risk		

Source: Hakanson (1980).

Table 5 Indicators of toxic possibility for aquatic organisms

Mean-effect range quotient (*m*-ERM-q)	Harmfulness to aquatic biota	Mean-effect range quotient (*m*-PEL-q)	Harmfulness to aquatic biota
mean-PELQ < 0.1	12% possibility of toxicity	mean-ERMQ < 0.1	10% possibility of toxicity
mean-PELQ = 0.11–0.5	30% possibility of toxicity	mean-ERMQ = 0.11–1.5	25.5% possibility of toxicity
mean-PELQ = 0.5–1.5	46% possibility of toxicity	mean-ERMQ = 1.51–2.3	50% possibility of toxicity
mean-PELQ > 1.5	74% possibility of toxicity	mean-ERMQ > 2.3	76% possibility of toxicity

Source: Long et al. (1995).

2.8 Statistical Analysis

The means of metal contents in water and sediment and the determination of significant differences between different dams were resolved using descriptive statistics and analysis of variance, respectively. The variations in various parameters between the two seasons were shown by *T*-test.

3 Results

3.1 Metals in Water

The dam's water contained detectable amounts of sodium, calcium, potassium, iron, manganese, zinc, magnesium, copper, lead, and cadmium. In water samples, seasonal levels of metals differ among the dams in dry and wet seasons (Table 6). The sequence of seasonal metal concentration in Egbe dam during the dry season was: Ca > Na > K > Mg > Zn > Mn > Fe > Cu > Pb > Cd, and in the sequence of Ca > Na > K > Mg > Zn > Mn > Fe > Cu > Pb > Cd during the rainy season. Ero dam exhibited seasonal metal sequence: Ca > Na > K > Mg > Zn > Mn > Fe > Cu > Pb > Cd during the dry season and in the pattern: Ca > Na > K > Mg > Zn > Mn > Fe > Cu > Pb > Cd in the rainy season. In Ureje dam, the sequence of the seasonal concentration of metals was: Ca > Na > K > Mg > Fe > Zn > Mn > Cu > Pb, Cd during the dry season and in the sequence: Ca > Na > K > Mg > Zn > Mn, Fe > Cu > Pb, Cd during the wet season. Except for values of Na, K, and Cd for Egbe dam; Na, Mg, Ca, Mn, Fe, and Zn for Ero dam; and Na, Ca, Fe, and Cd for Ureje dam, the mean metal concentrations in the three dams during the dry period were not substantially different at $P < 0.05$ from that of the rainy seasons. The mean values of metals except for K, Mn, and Pb (in Ureje dam) were lower than the allowable boundaries of local and international establishments.

3.2 Health Hazard Assessment

The metal's health hazards in water from the three dams were valued through consumption and skin exposure. The results presented in Tables 7 and 8 evaluate the health hazards of non-carcinogenic metals exposure in adults and children in Egbe, Ero, and Ureje dams, Ekiti State. The study revealed that the risk quotient (HQ) values for ingestion and skin contact with metals were < 1 in the three dams for both grown-ups and children, except the HQ value for consumption exposure to Mn for children. Consumption HQ values were more outstanding than skin HQ values in children and grown-ups in all three dams. The computed risk index (HI) for consumption of metals for adults in the three dams was 0.65, 0.52, and 0.79 for Egbe, Ero and Ureje dams, respectively (Table 7). In contrast, the calculated values of HI for skin contact were 0.048, 0.044, and 0.049 for Egbe, Ero, and Ureje dams, respectively. The total HI (ingestion and dermal) was 0.70, 0.57, and 0.84 for Egbe, Ero, and Ureje dams, respectively.

The HI values for the consumption of metals for children in the dams were shown in Table 8. It was reported as 2.48 (Egbe dam), 2.00 (Ero dam), and 3.01 (Ureje dam), while the computed values of HI for dermal exposure were 8.15×10^{-2} (Egbe dam), 7.60×10^{-2} (Ero dam) and 8.49×10^{-2} (Ureje dam). Further, the total HI (ingestion and dermal) for children was 2.56 (Egbe dam), 2.08 (Ero dam), and 3.09 (Ureje dam), thus, indicating non-carcinogenic risk to children's health.

Table 6 Mean seasonal metals levels in the water of Egbe, Ero, and Ureje dams and their allowable limit

	EGBE DAM		ERO DAM		UREJE DAM						
Metal (mg/L)	Arid period	Wet period	Arid period	Wet period	Arid period	Wet period	CANADA 2006	WHO 1993, 2006	NSDWQ 2007	NESREA 2009	USEPA 2018
Na	29.14 ± 1.41^{a}	24.36 ± 1.05^{b}	28.63 ± 1.09^{a}	37.47 ± 2.98^{b}	16.75 ± 0.73^{a}	26.64 ± 1.56^{b}	200	200	200	200	
Mg	5.12 ± 0.12^{a}	4.97 ± 0.24^{a}	3.65 ± 0.10^{a}	5.51 ± 0.39^{b}	4.92 ± 0.31^{a}	5.16 ± 0.08^{a}		30	20		
K	13.95 ± 0.71^{a}	18.07 ± 1.05^{b}	21.81 ± 1.66^{a}	27.84 ± 3.28^{a}	13.45 ± 1.11^{a}	22.15 ± 3.30^{a}	10	10		10	
Ca	44.43 ± 2.23^{a}	50.69 ± 3.17^{a}	41.67 ± 2.23^{a}	51.94 ± 2.66^{b}	26.93 ± 2.54^{a}	36.77 ± 2.32^{b}		75			
Mn	0.24 ± 0.02^{a}	0.28 ± 0.01^{b}	0.27 ± 0.01^{a}	0.28 ± 0.01^{a}	0.28 ± 0.02^{a}	0.27 ± 0.02^{a}	0.05	0.05	0.2	0.05	0.05
Cu	0.19 ± 0.02^{a}	0.22 ± 0.03^{a}	0.07 ± 0.01^{a}	0.13 ± 0.02^{b}	0.27 ± 0.01^{a}	0.20 ± 0.01^{b}	0.3	0.3	0.3	0.3	0.3
Fe	0.18 ± 0.01^{a}	0.19 ± 0.02^{a}	0.06 ± 0.01^{a}	.055 ± 0.00^{a}	0.15 ± 0.01^{a}	0.16 ± 0.02^{a}	1.0	2.0	1.0	1.0	1.0
Zn	0.33 ± 0.03^{a}	0.28 ± 0.03^{a}	0.18 ± 0.03^{a}	0.29 ± 0.02^{b}	0.25 ± 0.02^{a}	0.28 ± 0.01^{a}			3.0		
Cd	0.001 ± 0.00^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.001 ± 0.00^{a}	0.00 ± 0.00^{b}	0.005	0.003	0.003	0.003	
Pb	0.005 ± 0.00^{a}	0.003 ± 0.00^{a}	0.002 ± 0.00^{a}	0.006 ± 0.00^{a}	0.001 ± 0.00^{a}	0.02 ± 0.01^{a}	0.01	0.01	0.01	0.01	

*Metal concentrations in dry and wet periods with similar superscription are not significantly different.
USEPA – United States Environmental Protection Agency; WHO, World Health Organization; NSDWQ - Drinking water quality standard for Niger; NESREA - National Environmental Standards and Regulations Enforcement Agency

Table 7 Risk Quotient and cancer hazard of metals for adults in Egbe, Ero, and Ureje dams' water, Ekiti State, Southwest Nigeria

Metal	Mn	Fe	Cu	Zn	Cd	Pb	HI
RfDi (mg/kg/d)	2.40×10^{-2}	7.00×10^{-1}	4.00×10^{-2}	3.00×10^{-1}	5.00×10^{-4}	1.40×10^{-3}	
RfDd (mg/kg/d)	9.60×10^{-4}	1.40×10^{-1}	8.00×10^{-3}	6.00×10^{-2}	2.50×10^{-5}	4.20×10^{-4}	
Egbe dam							
ADDing	8.17×10^{-3}	6.44×10^{-3}	5.81×10^{-3}	9.59×10^{-3}	1.57×10^{-5}	1.26×10^{-4}	
ADDderm	3.88×10^{-5}	3.06×10^{-5}	2.76×10^{-5}	2.73×10^{-5}	7.46×10^{-8}	5.97×10^{-8}	
HQing	3.40×10^{-1}	9.20×10^{-3}	1.45×10^{-1}	3.20×10^{-2}	3.14×10^{-2}	8.98×10^{-2}	6.47×10^{-1}
HQderm	4.04×10^{-2}	2.18×10^{-4}	3.45×10^{-3}	4.55×10^{-5}	2.98×10^{-3}	1.42×10^{-4}	4.72×10^{-2}
Ero dam							
ADDing	8.64×10^{-3}	3.14×10^{-3}	1.81×10^{-3}	7.39×10^{-3}	0.00	1.26×10^{-4}	
ADDderm	4.10×10^{-5}	1.49×10^{-5}	8.58×10^{-6}	2.10×10^{-5}	0.00	5.97×10^{-8}	
HQing	3.60×10^{-1}	4.49×10^{-3}	4.52×10^{-2}	2.46×10^{-2}	0.00	8.98×10^{-2}	5.24×10^{-1}
HQderm	4.27×10^{-2}	1.07×10^{-4}	1.07×10^{-3}	3.51×10^{-4}	0.00	1.42×10^{-4}	4.44×10^{-2}
Ureje dam							
ADDing	8.64×10^{-3}	7.39×10^{-3}	4.87×10^{-3}	8.33×10^{-3}	1.57×10^{-5}	3.30×10^{-4}	
ADDderm	4.10×10^{-5}	3.51×10^{-5}	2.31×10^{-5}	2.37×10^{-5}	7.46×10^{-8}	1.57×10^{-7}	
HQing	3.60×10^{-1}	1.06×10^{-2}	1.22×10^{-1}	2.78×10^{-2}	3.14×10^{-2}	2.36×10^{-1}	7.88×10^{-1}
HQderm	4.27×10^{-2}	2.50×10^{-4}	2.89×10^{-3}	3.95×10^{-4}	2.98×10^{-3}	3.73×10^{-4}	4.96×10^{-2}

Here: ADDing is the mean diurnal dose through ingestion; ADDderm is the mean diurnal dose through skin contact; HQing is the risk quotient for consumption; HQderm is the risk quotient for skin exposure; CDI is the chronic daily intake; *RfDing* is the reference dose for consumption; *RfDderm*, is the reference dose due to skin exposure; CRing is the carcinogenic risk due to consumption; CRderm is the carcinogenic hazard due to skin exposure; HI, Risk index.

Table 8 Hazard Quotient and cancer-causing hazard of metals for children in surface water from Egbe, Ero, and Ureje dams, Ekiti State, southwest Nigeria

Metal	Mn	Fe	Cu	Zn	Cd	Pb	HI
RfDi (mg/kg/d)	2.40×10^{-2}	7.00×10^{-1}	4.00×10^{-2}	3.00×10^{-1}	5.00×10^{-4}	1.40×10^{-3}	
RfDd (mg/kg/d)	9.60×10^{-4}	1.40×10^{-1}	8.00×10^{-3}	6.00×10^{-2}	2.50×10^{-5}	4.20×10^{-4}	
Egbe dam							
ADDing	3.12×10^{-2}	2.46×10^{-2}	2.22×10^{-2}	3.66×10^{-2}	6.00×10^{-5}	4.80×10^{-5}	
ADDderm	6.64×10^{-5}	5.23×10^{-5}	4.72×10^{-5}	4.67×10^{-5}	1.28×10^{-7}	1.02×10^{-7}	
HQing	1.30	3.51×10^{-2}	5.55×10^{-1}	1.22×10^{-1}	1.20×10^{-1}	3.43×10^{-1}	2.48
HQderm	6.91×10^{-2}	3.74×10^{-4}	5.90×10^{-3}	7.78×10^{-4}	5.10×10^{-3}	2.43×10^{-4}	8.15×10^{-2}
Ero dam							
ADDing	3.30×10^{-2}	1.20×10^{-2}	6.90×10^{-3}	2.82×10^{-2}	0.00	4.80×10^{-5}	
ADDderm	7.00×10^{-5}	2.55×10^{-5}	1.47×10^{-5}	3.60×10^{-5}	0.00	1.02×10^{-7}	
HQing	1.38	1.71×10^{-2}	1.73×10^{-1}	9.40×10^{-2}	0.00	3.43×10^{-1}	2.00
HQderm	7.31×10^{-2}	1.82×10^{-4}	1.83×10^{-3}	6.00×10^{-4}	0.00	2.43×10^{-4}	7.60×10^{-2}
Ureje dam							
ADDing	3.30×10^{-2}	2.82×10^{-2}	1.86×10^{-2}	3.18×10^{-2}	6.00×10^{-5}	1.26×10^{-3}	
ADDderm	7.00×10^{-5}	6.00×10^{-5}	3.96×10^{-5}	4.06×10^{-5}	1.28×10^{-7}	2.68×10^{-7}	
HQing	1.38	4.03×10^{-2}	4.65×10^{-1}	1.06×10^{-1}	1.20×10^{-1}	9.00×10^{-1}	3.01
HQderm	7.31×10^{-2}	4.28×10^{-4}	4.94×10^{-3}	6.76×10^{-4}	5.10×10^{-3}	6.38×10^{-4}	8.49×10^{-2}

Here: ADDing, mean diurnal dose through consumption; ADDderm, mean diurnal dose through skin exposure; HQing, hazard quotient for consumption; HQderm, hazard quotient for skin exposure; CDI, chronic daily intake; *RfDing*, reference dose for ingestion; *RfDderm*, reference dose as a result of skin exposure; CRing, cancer-causing hazard due to consumption; CRderm, carcinogenic risk due to skin contact.

Table 9 Incremental life tumor hazards in children and grown-ups via consumption and skin contact to the water of the dams

		Egbe dam		Ero dam		Ureje dam	
Element	Medium	Children	Adult	Children	Adult	Children	Adult
Cd	Ingestion	3.24×10^{-5}	9.89×10^{-5}	0.00	0.00	3.24×10^{-5}	9.89×10^{-5}
	Dermal	6.87×10^{-5}	4.07×10^{-7}	0.00	0.00	3.49×10^{-7}	2.81×10^{-6}
Pb	Ingestion	3.49×10^{-7}	1.07×10^{-6}	3.49×10^{-7}	1.07×10^{-6}	6.87×10^{-8}	4.70×10^{-7}
	Dermal	7.44×10^{-11}	5.07×10^{-10}	7.44×10^{-11}	5.07×10^{-10}	1.95×10^{-10}	1.33×10^{-10}
∑ILCR		3.24×10^{-5}	1.04×10^{-4}	3.49×10^{-7}	1.07×10^{-6}	3.28×10^{-5}	1.02×10^{-4}

ILCR- Incremental lifetime cancer risk

Table 9 summarizes the cancer-causing health hazard evaluation of Cd and Pb for adults and children in Egbe, Ero, and Ureje dams, Ekiti State. In Egbe dam, lifespan tumor hazard computed through consumption of Cd and Pb was 9.89×10^{-5} and 1.07×10^{-6} for adults and 3.24×10^{-5} and 3.49×10^{-7} for the children, respectively. Cancer hazards computed via skin contact of Cd and Pb were 4.70×10^{-7} and 5.07×10^{-10} for adults and 6.87×10^{-5} and 7.44×10^{-11} for the children respectively. In Ero dam, lifespan tumor hazard computed through consumption of Cd and Pb was nil and 1.07×10^{-6} for adults (Table 9), while it was nil and 3.49×10^{-7} for the children, respectively. Cancer risk calculated through dermal contact of Cd and Pb was nil and 5.07×10^{-10} for adults and nil and 7.44×10^{-11} for children, respectively. Further, in Ureje dam, lifespan tumor hazard computed through consumption of Cd and Pb were 9.89×10^{-5} and 2.81×10^{-6} for adults and 3.32×10^{-5} and 3.49×10^{-7} for the children, respectively. Cancer risk calculated through dermal contact of Cd and Pb were 4.70×10^{-7} and 1.33×10^{-10} for adults and 6.87×10^{-8} and 1.95×10^{-10} for the children, respectively. In Egbe dam, the aggregate tumor hazard of the investigated metals was 1.01×10^{-4} for the adult and 3.24×10^{-5} for the children; in Ero dam, the aggregate tumor hazard was 1.07×10^{-6} for the adult and 3.49×10^{-7} for the children, and in Ureje dam, the aggregate tumor hazard was 1.02×10^{-4} for the adult and 3.28×10^{-5} for the children. The results of the study showed more significant cancer hazards for adults in comparison to children. The collective cancer hazard value in Egbe, Ero, and Ureje dams was slightly above the suitable tumor hazard range of 1.00×10^{-6} to 1.00×10^{-4}.

3.3 Metals in Sediment

Seasonal levels of different metals in the dams' sediments during both seasons are given in Table 10. The average seasonal concentration of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb in the sediments during the dry season in Egbe dam was 6.00 ± 0.73, 1.98 ± 0.10, 5.51 ± 0.82, 11.02 ± 1.61, 0.50 ± 0.07, 74.32 ± 8.78, 0.44 ± 0.02, 0.31 ± 0.02, 0.05 ± 0.01 and 0.21 ± 0.02 ppm respectively. The sequence of the mean metal concentrations in the dry season in Egbe dam was Fe > Ca > Na > K > Mg > Mn > Cu > Zn > Pb > Cd.

Further, the average level of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb during the dry season in Ero dam was 10.31 ± 0.98, 1.32 ± 0.24, 10.56 ± 0.77, 14.31 ± 1.23, 0.39 ± 0.07, 61.17 ± 8.05, 0.19 ± 0.02, 0.48 ± 0.08, 0.04 ± 0.01, 0.13 ± 0.02 ppm respectively and the sequence of the mean metal concentrations in the dry season in Ero dam was Fe > Ca > K > Na > Mg > Zn > Mn > Cu > Pb > Cd. In the case of Ureje dam, the average level of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb during the dry season were 5.59 ± 1.44, 2.12 ± 0.36, 7.49 ± 2.71, 11.93 ± 3.39, 0.45 ± 0.05, 92.51 ± 18.62, 0.31 ± 0.02, 0.43 ± 0.05, 0.05 ± 0.03 and 0.14 ± 0.03 ppm respectively. The trends of mean seasonal metals concentration in the dry season in Ureje dam was Fe > Ca > K > Na > Mg > Mn > Zn > Cu > Pb > Cd.

The trends of the rainy season are similar to the dry season. During the rainy season in Egbe dam, the mean seasonal levels of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb in sediments were 7.29 ± 0.56, 2.24 ± 0.14, 9.30 ± 1.25, 10.68 ± 0.68, 0.62 ± 0.08, 88.30 ± 6.89, 0.37 ± 0.03, 0.38 ± 0.03, 0.06 ± 0.01, 0.18 ± 0.02 ppm respectively. The sequence of mean metal concentration in the rainy season in Egbe dam was Fe > Ca > K > Na > Mg > Mn > Zn > Cu > Pb > Cd. Further, in the case of Ero dam, the average levels of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb in Ero dam were 9.99 ± 0.46, 1.45 ± 0.16, 13.31 ± 0.93, 18.29 ± 1.84, 0.39 ± 0.05, 69.46 ± 10.23, 0.19 ± 0.01, 0.54 ±0.05, 0.07 ± 0.01, 0.28 ± 0.04 ppm respectively. The trend of mean metal concentration in the rainy season in Ero dam was Fe > Ca > K > Na > Mg > Zn > Mn > Cu > Pb > Cd. The average concentration of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb in Ureje dam during the rainy period was 7.72 ± 1.29, 3.30 ± 0.46, 12.49 ±2.93, 12.85 ± 1.80, 0.80 ± 0.12, 134.31 ± 23.73, 0.31 ± 0.01, 0.44 ± 0.03, 0.12 ± 0.03, 0.19 ± 0.02 ppm respectively. The trends of mean seasonal metal concentration in the rainy season in Ureje dam were Fe > Ca > K > Na > Mg > Mn> Zn > Cu > Pb > Cd.

Sediment metal levels of the dams varied widely and exhibited fluctuations between the different dams. Metals showed a similar pattern of concentrations in the dams, with the highest values recorded in Fe and Na and the lowest in Cd and Pb. Studied metals had higher levels in the wet period than the dry period except for Ca, Cu, and Pb in the Egbe dam and Na in the Ero dam.

Table 10 Seasonal metal concentrations in sediment from Egbe, Ero, and Ureje dams, Ekiti State, southwest Nigeria

	Egbe Dam		Ero Dam		Ureje Dam	
Metal (ppm)	Arid period	Wet period	Arid period	Wet period	Arid period	Wet period
Na	6.00 ± 0.73^{a}	7.29 ± 0.56	10.31 ± 0.98^{b}	9.99 ± 0.46	5.59 ± 1.44^{a}	7.72 ± 1.29
Mg	1.98 ± 0.10^{a}	2.24 ± 0.14^{a}	1.32 ± 0.24^{bc}	1.45 ± 0.16^{be}	2.12 ± 0.36^{a}	3.30 ± 0.46^{c}
K	5.51 ± *0.82	9.30 ± **1.25	10.56 ± **0.77	13.31 ± **0.93	7.49 ± 2.71	12.49 ± 2.93
Ca	11.02 ± 1.61	10.68 ± 0.68^{a}	14.31 ± 1.23	18.29 ± 1.84^{b}	11.93 ± 3.39	12.85 ± 1.80^{a}
Mn	0.50 ± 0.07	0.62 ± 0.08^{ab}	0.39 ± 0.07	0.39 ± 0.05^{a}	0.45 ± *0.05	0.80 ± **0.12^{b}
Fe	74.32 ± 8.78	88.30 ± 6.89^{a}	61.17 ± 8.05	69.46 ± 10.23^{a}	92.51 ± 18.62	134.31± 23.73^{b}
Cu	0.44 ± 0.02^{a}	0.37 ± 0.03^{a}	0.19 ± 0.02^{be}	0.19 ± 0.01^{b}	0.31 ± 0.02^{c}	0.31 ± 0.01^{ac}
Zn	0.31 ± *0.02	0.38 ± **0.03^{a}	0.48 ± 0.08	0.54 ± 0.05^{b}	0.43 ± 0.05	0.44 ± 0.03^{ab}
Cd	0.05 ± 0.01	0.06 ± 0.01^{a}	0.04 ± *0.01	0.07 ± **0.01^{ab}	0.05 ± 0.03	0.12 ± 0.03^{b}
Pb	0.21 ± 0.02^{a}	0.18 ± 0.02^{a}	0.13 ± *0.02^{b}	0.28 ± **0.04^{b}	0.14 ± 0.03^{b}	0.19 ± 0.02^{a}

Mean metal concentration during the dry and rainy seasons with different superscripts (*) show a significant difference at $P < 0.05$; Sites with similar alphabet superscripts in the same season show no significant difference at $P < 0.05$

Table 11 Contamination load index (PLI), degree of pollution, and contamination factor, of metals in the dams' sediments during dry and wet periods

	Egbe Dam		Ero Dam		Ureje Dam		
	Contamination Factor		Contamination Factor		Contamination Factor		
Metal (ppm)	Arid period	Wet period	Arid period	Wet period	Arid period	Wet period	Aver. Shales conc.
Na	6.25×10^{-4}	7.59×10^{-4}	1.07×10^{-3}	1.04×10^{-4}	5.82×10^{-4}	8.04×10^{-4}	9,600
Mg	7.30×10^{-4}	1.49×10^{-4}	8.79×10^{-5}	9.63×10^{-4}	1.41×10^{-4}	2.20×10^{-4}	15,000
K	2.07×10^{-4}	3.49×10^{-4}	3.97×10^{-4}	5.00×10^{-4}	2.81×10^{-4}	4.70×10^{-4}	26,600
Ca	4.99×10^{-4}	4.83×10^{-4}	6.48×10^{-4}	8.28×10^{-4}	5.40×10^{-4}	5.82×10^{-4}	22,100
Mn	5.90×10^{-4}	7.24×10^{-4}	4.54×10^{-4}	4.57×10^{-4}	5.32×10^{-4}	9.45×10^{-4}	850
Fe	1.57×10^{-3}	1.87×10^{-3}	1.30×10^{-3}	1.47×10^{-3}	1.96×10^{-3}	2.85×10^{-3}	47,200
Cu	9.70×10^{-3}	8.13×10^{-3}	4.14×10^{-3}	4.11×10^{-3}	6.88×10^{-3}	6.96×10^{-3}	45
Zn	3.22×10^{-3}	4.03×10^{-3}	5.03×10^{-3}	5.64×10^{-3}	4.55×10^{-3}	4.65×10^{-3}	95
Cd	1.70×10^{-1}	2.01×10^{-1}	1.20×10^{-1}	2.36×10^{-1}	1.81×10^{-1}	4.02×10^{-1}	0.3
Pb	1.07×10^{-2}	9.17×10^{-3}	6.54×10^{-3}	1.42×10^{-2}	6.78×10^{-3}	9.29×10^{-3}	20
Deg. Cont.	1.98×10^{-1}	2.27×10^{-1}	1.39×10^{-1}	2.65×10^{-1}	2.03×10^{-1}	4.28×10^{-1}	
PLI	1.84×10^{-3}	2.08×10^{-3}	1.72×10^{-3}	2.16×10^{-3}	1.86×10^{-3}	2.63×10^{-3}	

Here Aver. Shales conc. - Average shales level, PLI – Contamination load index; deg. Cont – Degree of pollution.

3.4 Sediment Pollution Evaluation of the three Dams

3.4.1 Contamination factor, Pollution Degree, and Indicator of Pollution Load

The metal contamination factors (C.F.) of Na, Mg, K, Ca, Mn, Fe, Cu, Zn, Cd, and Pb indicated low C.F. < 1 in all the dams in the dry and rainy periods. Contamination degree (mCd) was highest in the Ureje dam and lowest in the Ero dam in the arid period. During the rainy season, the Ureje dam had the highest level of contamination, and the Egbe dam had the lowest (Table 11). The contamination load index revealed that sediment pollution is highest in the Ureje dam during the rainy season.

3.4.2 Index of geo-accumulation (Igeo)

Igeo values did not change with the seasons. The Igeo values for Egbe, Ero, and Ureje area sediments vary from metal to metal and month to month. In the three dams, all the metals were in Igeo grade less than zero, thus signifying that the dams' sediments did not experience contagion by these metals during dry and rainy seasons.

3.4.3 Probable Ecological Hazard Index

The ecological hazard factor (E_i^r) sequence of the sediments' metals of the Egbe, Ero, and Ureje dams in dry and rainy periods was Cd > Pb > Zn > Cu >Mn. The ecological risk factor of Cd, Cu, Pb, Zn, and Mn in the three dams was lower than 40. The probable ecological hazard index values of the metals are < 150 in the dams during the dry and rainy periods, thus, posing a low environmental risk to the three dams.

3.4.4 Ecological Risk Assessment according to sediment quality guidelines

According to Long et al.(1995), different mean ERMQ values: < 0.1, 0.11–0.5, 0.5–1.5, and > 1.5 are related to 12 %, 30 %, 46 %, and 74 % possibility of harmfulness correspondingly. Likewise, different values of mean PELQ: < 0.1, 0.11–1.5, 1.51–2.3, and > 2.3 correspond to 10 %, 25.5, 50 %, and 76 % also show the possibility of harmfulness, respectively. The computed values of mean-ERMQ of Cu, Zn, Cd, and Pb in the dry season were 2.20×10^{-3} (Egbe dam), 1.50×10^{-3} (Ero dam), and 2.10×10^{-3} (Ureje dam) and they follow the orders of Egbe dam > Ureje dam > Ero dam. The computed values of mean-ERMQ of Cu, Zn, Cd, and Pb) in the rainy season were 2.40×10^{-3} (Egbe dam), 2.70×10^{-3} (Ero dam), and 3.90×10^{-3} (Ureje dam) and they follow the orders of Ureje dam > Ero dam > Egbe dam. Likewise, the values of mean-PELQ obtained were 5.00×10^{-3} (Egbe dam); 3.50×10^{-3} (Ero dam), and 5.00×10^{-3} (Ureje dam) during the dry season with the inclination of Egbe dam, Ureje dam > Ero dam. While in the case of the rainy season, the mean-PELQ values of 5.50 × 10-3 (Egbe dam), 6.50 × 10-3 (Ero dam), and 9.80 × 10-3 (Ureje dam) were obtained with the inclination of Ureje dam,> Ero dam > Egbe dam.

4 Discussion

Except for K and Mn in all three water bodies and Pb in Ureje dam during the rainy period, the mean metal levels in the water of the three dams during the dry and wet periods did not exceed the WHO recommendation and allowed limits for potable water. However, the K, Mn, and Pb levels in the dams were above the WHO-endorsed limit. Hence, the water from the three dams might be unsafe to drink, and the fish might not be safe to eat without proper treatment. Further, seasonal variations were reported in the metal levels in the three dams. Except for Na, Mg, Zn, Cd, and Pb in Egbe dam; Cu in Ero dam; and Mn, Fe, and Cd in Ureje dam, the rainy season showed slightly more significant amounts of heavy metals than the dry season. Therefore, draining water after precipitation is likely a primary means of carrying metals into the dams. A similar observation was made by Kiema et al. (2017) on the impacts of human activities and periods on the dissemination of metal in the sedimentary coastline of Lake Victoria, Kisumu City, Kenya.

Further, Na quantities recorded during both seasons were within the usual range for freshwater (Chapman and Kimstach 1992), and on aesthetic concern, 200 mg of sodium/L was established by WHO's (2006) water guideline. Naturally, K occurs in low concentrations in waterways because potassium-rich rocks are highly resistant to weathering, and concentrations of K in freshwater are usually below 10 mg/L (Chapman and Kimstach 1992). However, the higher values were established in the three dams compared to the WHO recommended values. It is most likely due to the use of potassium fertilizers for agriculture, which enter freshwaters via runoff from agricultural land. Frequently, magnesium accompanies calcium in various fluids, but its content is usually lesser than calcium (Qureshimatva et al. 2015), as seen in this study. The Mg concentrations in this study are within the range of natural magnesium concentrations in freshwaters, and it ranging from 1 to below 100 mg/L (Chapman and Kimstach 1992).

Manganese in the dams was higher than the recommended level. Bolaji et al. (2017) made a similar observation for the Ureje dam. Though the concentrations of Mn in the dams are higher than the recommended standard boundary of 0.2 mg/L (NSDWQ 2007), the presence of the manganese in water obtained from a faucet is obvious when its concentration is higher than 0.05 mg/L by tinting and adding taste, aroma to tap water (SCDPH 2021). But, manganese well-being consequences pose no apprehension pending when concentrations are almost 10 times greater. Thus, the present level of manganese in the dams affects their aesthetic value. The presence of cadmium and zinc and their relative concentrations were reported by Svobodová et al. (1993) that cadmium in surface waters is usually found together with zinc but at much lower concentrations. The dams in this investigation were subjected to similar observations.

In the Ureje dam catchment area, the water carried by runoffs into the dam was affected by several practical socio-economic and waste dumping activities such as car garages, automobile workshops, schools, construction works, car washing, domestic wastewater, and garbage around the dam and these activities are related to the observed higher value of Pb than the WHO allowable boundary (0.01 mg/L) during the rainy season. Leachates from lead-acid batteries, which are carelessly abandoned by battery chargers and disposed of at the garbage dumps in this urban community, are possible sources of Pb.

In the Egbe, Ero, and Ureje dams, the values of consumption HQ were more significant than the values of skin HQ in children and grown-ups. A comparable report was given by Liang et al. (2011) in their work on the water of Taihu Lake, China. In adults and children, the values of hazard quotient (HQ) for metals through consumption and skin contact were less than one in Egbe, Ero, and Ureje dams. It is signifying the contamination level in the surface water of the Egbe, Ero, and Ureje dams had little antagonistic health consequences. However, HQ values for Mn in consumption exposure for children that were greater than one in the dams had adverse health effects. The average HI is less than 1 for all three dams that's why for skin exposure and consumption of dams water does not show aggregate probable antagonistic health hazards to adult. As a result, the dams pose a non-carcinogenic risk to adults' health that can be overlooked. There is an aggregate risk of adverse health effects on children owing to uninterrupted ingesting contact. Still, there is no cumulative risk of adverse health effects through skin contact with water users among children across the three dams. For Egbe, Ero, and Ureje dams, the total HI (ingestion and dermal) for children was 2.56, 2.08, and 3.09, indicating a non-carcinogenic risk to children's health.

In adults and children, the tumor hazard associated with Cd and Pb exposure by oral intake was higher than the cutaneous exposure. Obiri et al. (2010) reported a similar observation in Ghanaian surface water, and they linked it to differences in genetic, immunologic, dietary, hormonal status, and other factors that influence the form and manner in which harmful consequences of a given chemical appear. Joseph et al. (2022) reported similar results from cancer risk associated with metals in water from a borehole in a community in Akwa Ibom State. The results of the cumulative cancer risk in Egbe, Ero, and Ureje dams showed greater tumor risk in grown-ups than in children. Cumulative cancer risk values in Egbe, Ero, and Ureje dams were slightly higher than the acceptable tumor hazard range 1.00E-06 to 1.00E-04. According to Li et al. (2017), Egbe and Ureje dams are in medium risk grade, while the Ero dam is in extremely low-risk rate. However, every carcinogenic substance, such as Pb and Cd, can develop cancer at any dose higher than nil. That's why Pb and Cd have been linked to a lifetime carcinogenic risk (IARC 2011; Cao et al. 2014).

Higher metal levels in dams' sediments than metal concentrations in dams' water reflected that sediments operate as tanks or basins to metals in these water environments (Gupta et al. 2009). It also affects water quality and bioaccumulation of metals along the trophic levels with lasting health implications on human beings and the health of aquatic ecosystems (Fernandes et al. 2007). Metal concentrations obtained from the sediments varied and exhibited fluctuations among the dams. Except for K and Zn in Egbe dam, K, Cd, and Pb in Ero dam, and Mn in Ureje dam, a significant difference did not occur in the mean value of metals in sediment between dry and wet seasons. However, a substantial number of the metals were higher in rainy than dry seasons. Wardhani et al. (2021) reported higher Cd concentrations in the sediment from Saguling reservoir, West Java Province, in the rainy season compared to the dry season. While Gunes (2021) reported greater metal levels in Bartin River in the rainy period when related to a dry period, this might be attributed to natural runoff from various sources during the rainy season that entered the aquatic systems. Asaolu and Olaofe (2005) made similar observations on the coastal areas of Ondo state, and Adefemi (2013) conducted previous investigations on the major dams in Ekiti state, Nigeria. These results are in contrast to Aladesanmi et al. (2016)'s findings on streams and adjacent fish ponds in Osun state, which found that higher metal readings during the dry season were due to a slow water circulation that allows particles to settle. The peak values of Fe out of all elements in the dams' sediments in both seasons established the widespread Fe occurrence in Nigerians' soil reported by various workers (Adefemi et al. 2007; Adeyeye and Ayoola 2013; Aladesanmi et al. 2016; Yahaya et al. 2021).

Employment of various sediment pollution indices indicated that the sediments of Egbe, Ero, and Ureje dams were less polluted by lethal metals during the dry and rainy periods. However, due to accumulative anthropogenic activities in the dams and their surroundings, the dams' sediments may still be subject to future deterioration. Furthermore, the presence of metals, particularly cadmium and lead, which are carcinogenic (IARC 2011; Cao et al. 2014; Kim et al. 2020) points to the need for proper management strategies and continuous dam monitoring for optimal fish production and the protection of the health of the aquatic biota and that of their consumers.

Conclusion

The presence of metals in the dams in quantities higher than recommended levels and the observed carcinogenic risks especially in Ureje dam necessitates appropriate action to reduce current pollution state and guard against further deterioration due to accumulative anthropogenic activities in the dams, as well as their surroundings.

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Comparative analysis of antioxidant activities of *Vitex negundo* and *Ficus carica* leaf extracts

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DPPH assay

FRAP assay

ABSTRACT

Humans have been dependent on nature for various vital supplies and resources for a long time. Most biotechnological and pharmacological industries use chemicals and active compounds to treat diseases or make medications isolated from natural resources. A variety of plants have been explored for research of which *Vitex negundo* and *Ficus carica* are also examples as they are strong candidates for their potential antioxidant properties. In the current research, the anti-oxidant activities of *V. negundo* and *F. carica* leaf extracts were evaluated. The antioxidant activities of selected plants were analyzed using DPPH and FRAP assay. The results obtained from the DPPH assay indicated that methanolic extracts of *V. negundo* showed the highest inhibition of 90.07±1.17 percent at 1000 µl with IC_{50} value of 415.98 µg/ml followed by ethyl acetate and chloroform extracts (64.05±0.89 and 54.39±0.99 percent, respectively) with IC_{50} value of 751.96 µg/ml and 896.55 µg/ml when compared to *F. carica* extracts which showed highest inhibition of 75.75±1.08 percent at 1000 µl with IC_{50} value of 475 µg/ml followed by ethyl acetate and chloroform extracts (51.94±0.79 and 44.21±0.60 percent respectively) with IC_{50} value of 967.51 µg/ml and 1092.48 µg/ml. On comparing both plants, FRAP results indicated that methanol extracts of *V. negundo* showed the highest FRAP value (1042.1±0.98 µM) followed by ethyl acetate and chloroform extracts, which shows 996.6±1.25 µM and 949.6±1.63 µM at 1000 µl whereas *F. carica* showed highest FRAP value (995.6±1.35µM) followed by ethyl acetate and chloroform extracts, which shows 987.6±1.05µM and 447.6±1.01µM at 1000 µl. The results of the study can be concluded that among the tested extracts, the best antioxidant potential was exhibited with *V. negundo* leaf extracts, especially in methanol extracts.

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1 Introduction

Oxidative stress is a normal phenomenon and one of the most concerning elements in the scientific community. Oxidative stress arises when the number of oxidants increases in the body above normal level while the amount of antioxidants in the body decreases, resulting when free radicals are produced (Rahal et al. 2014). Free radicals have received a lot of attention. As part of standard cellular function, free radicals are produced continuously in all cells. Abundant free radical production from endogenous or exogenous sources could be a factor in the development of many diseases. Antioxidants play a significant role in the maintenance of tissue health (Young and Woodside 2001).

An antioxidant prevents reactive oxygen species (ROS) formation and helps the biological system sustain improved health against numerous diseases, including inflammation, liver damage, and cardiovascular disease that can be spread by these reactive oxygen species (Liao and Yin 2000). Antioxidants are frequently used as food supplements to prevent food deterioration. Prior studies have demonstrated that foods that have high antioxidants play a critical role in lowering the chance of developing heart illnesses, as well as other chronic diseases (Mata et al. 2007). Because several lifestyle-related diseases and the aging process are intimately linked to active O_2 and LPO, anti-oxidants play a crucial role in preventing these conditions (Noguchi and Niki 2019).

Earlier, synthetic anti-oxidants made up through chemical processes were utilized in many food and pharmacology industries but had various negative effects on human health. Therefore, a natural way of antioxidant preparation was opted for through the use of natural sources such as plant extracts to reduce the toxic and negative impact of synthetic antioxidants. Plants consist of many valuable bioactive compounds and are a perfect candidate for antioxidant production. Most of the anti-oxidants are derived from plant materials such as fruits, vegetables, herbs, and leaves (Hasani et al. 2007). There have already been numerous plant species evaluated for possible anti-oxidant properties (Hussain et al. 2008). *Vitex negundo* and *Ficus carica* have been used as food products and traditional medicines for the treatment of various diseases.

V. negundo Linn. belongs to the family Verbenaceae, it has quadrangular branches and tri- or penta-foliate leaves with 5 leaflets grouped like a palm and so is also known as the 5-Leaved Chaste Tree. Further in Indian traditional medicine, *V. negundo* is known as "sarvaroganivarani" which means "the remedy for all diseases" (Sabbagh and Kim 2022). The plant prefers to moisten environments to grow that's why it preferred to grow in India, Thailand, Madagascar, Malaysia, Sri Lanka, Eastern Africa, and Pakistan. Each part of the plant is developed with medicinal value; hence this plant plays a crucial role in traditional medication systems. Since the plant has therapeutic potential in every component, it is essential in systems of traditional medicine (Tandon 2005). All parts of *V. negundo* contain several phytoconstituents like fatty acids, alkaloids, flavonoids, phenols, glycosidicirridoids, lignans, tannins, steroids, and di- and sesquiterpenes. Due to the presence of a variety of secondary metabolites, *V. negundo* is used to treat different types of diseases such as spermatorrhoea, stomachache, asthma, cold, diarrhoea, indigestion, gallstone, hernia, eye disorders, rheumatism, irritable bladder and dysmenorrhea, headache, migraine, kwashiorkor, neck gland sores, tubercular neck swelling, reddened, arthritis, jaundice, urticaria, eczema, and liver disorders. It is most widely used for curing disorders of the reproductive system like vital power, frail erection without libido, stool-containing prostatic fluid, and testicle pain (Perveen et al. 2023). In Unani medicine, the seeds of *V. negundo* are also utilized as an aphrodisiac and to treat swellings. Chinese medicine recommends consuming the fruit of the *V. negundo* plant to alleviate headaches, soreness, and swollen eyes (Liu et al. 2005).

Over 800 species of the incredibly vast pantropical genus Ficus (family Moraceae) can be found globally (Adhikari et al. 2023). *Ficus carica* Linn. also known as the "common fig" or "Anjeer," is one of them. The leaves have an oval shape, a pubescent underside, a rough top, and three to five lobes (Taviano et al. 2018). Extracts from the roots, barks, leaves, and fruits, have a variety of pharmacological properties, including anti-inflammatory, anti-diabetic, antioxidant, anti-inflammatory, anti-arthritic, anti-hyperlipidemic, and gastroprotective, effects (Adhikari et al. 2023). The biological activities of *F. carica* are mostly related to the presence of diverse phytoconstituents present in the roots, latex, leaves, and fruits including anthocyanins, organic acids, amino acids, phytosterols, aliphatic alcohols, fatty acids, and phenolics (Li et al. 2021). *F. carica* boosts total protein expression, notably for genes relevant to fertility, and possesses anti-hyperglycemic properties (Bakar et al. 2020). *F. carica* leaves are consumed as a tea or utilized as a medication (Barolo et al. 2014). According to reports, *F. carica* leaves are helpful in several conditions like pustules, hemorrhoids, diabetes, dysentery, breathing, heart, and skin problems (Taviano et al. 2018).

Both *V. negundo* and *F. carica* have been reported to possess various medicinal values but despite this, no detailed work is reported. Therefore, in this study anti-oxidant potential of methanol, ethyl acetate, as well as chloroform leaf extracts of both *V. negundo* and *F. carica* plants have been evaluated.

2 Materials and Methods

2.1 Plant materials

The experimental plant part i.e., *V. negundo* (nirgundi) and *F. carica* (Anjeer) fresh leaves were collected from the Neem Vatika

Figure 1 NeemVatika Herbal Park, Samargopalpur, Rohtak, Haryana

Herbal Park, Samargopalpur, Rohtak, Haryana, followed by their proper authentication was done.

2.2 Preparation of Extracts

Leaves of *V. negundo* and *F. carica* were properly cleaned two to three times under running water, and then air dried at 32°C to 37°C in a shady place for about 2-3 weeks. The dried plant samples were ground into powder form by using a homogenizer. After that, 50 grams of dried and coarsely powdered plant leaf samples were extracted with solvents $CHCl_3$ followed by $C_4H_8O_2$ and CH_3OH in order of their increasing polarity for the sequential extraction using the Soxhlet apparatus. These extracts were filtered individually and concentrated as dried mass for further use (Gupta 2005).

2.3 Determination of antioxidant activities

2.3.1 DPPH radical scavenging activity

The modified Brand-Williams et al. (1995) method was employed to analyze DPPH. For this, 1 mg/ml samples were prepared in methanol individually. The range of 100-1000 µl of the sample was selected and volume was maintained up to 1 ml with methanol. 1 ml of the DPPH solution (1 mg/10 ml) was added to all test tubes and vortexed. Tubes were then placed in the dark surrounding for thirty minutes. Later, the sample was replaced with methanol in the blank and absorbance was taken at 517 nm. Ascorbic acid was used as standard. To calculate the DPPH radical scavenging activity the following formula was used:

$$\% \text{ inhibition of DPPH} = \{(A_B - A_S)/A_B\} \times 100$$

Here, A_S = absorbance of samples; A_B = absorbance of blank or reference.

2.3.2 Ferric reducing antioxidant power (FRAP)

FRAP assays were conducted by using Pulido et al. (2000) method. The sample range of 100 µl to 1000 µl was fixed for FRAP estimation. During standardization, an aqueous solution with known Fe^{2+} content (10 µM) was used in the range between 100–1000 µmol/l. Methanol was used for volume make-up of up to 1 ml. In this, 1 ml FRAP reagent was added in each sample and standards tubes and vortexed properly then incubated for 30 minutes at 37°C before using. The absorbance observations were recorded at 593 nanometres. The blank was made without any sample addition and the quercetin was taken as standard. By contrasting the activities of the standard curve, the proportional activities of the samples were evaluated. The results were expressed in a micromolar.

2.4 Statistical Analysis

These results from trials that were carried out in triplicates were noted as mean ± SD.

3 Results and Discussion

3.1 DPPH assay

The ability of diverse samples, including plant extracts, to scavenge free radicals is frequently assessed using the scavenging of the stable DPPH radical. Each extract ($CHCl_3$, EtOAc, and CH_3OH) was tested for DPPH scavenging activity in the current investigation, and it was found to be rising in a dosage-dependent pattern. The highest concentration of MeOH extract (1000µl) demonstrated the highest anti-oxidant activity for both plants (90.07±1.17% in *V. negundo* and 75.75±1.08% in *F. carica*), followed by EtOAc (64.05±0.89 % and 51.94±0.79 %) and $CHCl_3$ extract (54.39±0.99% and 44.21±0.60%), as shown in Figure 2 and 3. Additionally, the MeOH extract of *V. negundo* showed the

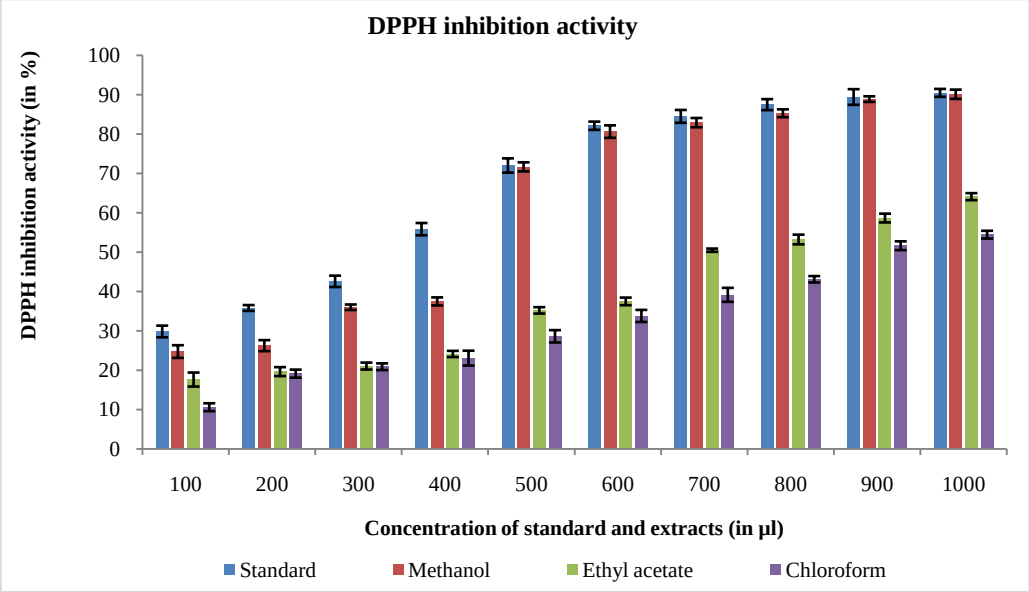

Figure 2 DPPH inhibition activity of *V. negundo*

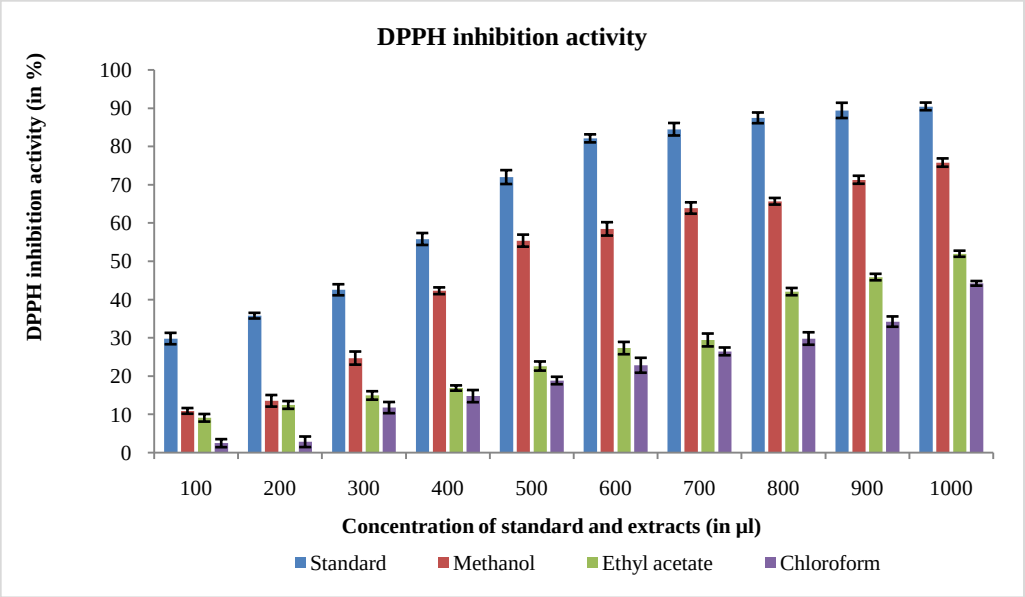

Figure 3 DPPH inhibition activity of *F. carica*

Table 1 IC_{50} values of *V. negundo* and *F. carcica*

Plant Samples	IC_{50} (µg/ml)			
	Standard (Ascorbic acid)	Methanol	Ethyl acetate	Chloroform
V. negundo	360 ± 1.58	415.98 ± 0.76	751.96 ± 1.32	896.55 ± 2.76
F. carcica		475 ± 1.78	967.51 ± 2.34	1092.48 ± 3.22

comparatively best minimum inhibition activity (IC_{50}) (Table 1). When compared to the extracts from *F. carica*, it was shown that the extracts from *V. negundo* had excellent DPPH inhibitory activity and a remarkable IC_{50} value. Similar to this, Ahmad et al. (2013) experiment showed that the anti-oxidant property of the *F. carica* leaves extract dramatically rose to the extract concentration.

At 25 µg/ml of sample, scavenging inhibition was observed 1.285 ± 0.15 % while at 250 µg/ml, it was observed 1.00 ± 0.09%. Anti-oxidants are thought to affect DPPH because of their capacity to donate hydrogen. It was clear that the extracts did exhibit some proton-donating potential and might act as free radical inhibitors or scavengers, possibly acting as major anti-oxidants, even though their DPPH radical scavenging activities were substantially lesser than those of ascorbic acid. Another investigation on the antioxidant potential of latex from unripe fruits of *F. carica* cultivar Pingo de Mel (Northeast Portugal) was carried out by Oliveira et al. (2010) who reported the IC_{25} value for the DPPH test was 1049µg/ml. Further, the latex may also inhibit the development of additional biologically significant oxidative species, such as peroxynitrite and OH radicals, as a result of the interaction between these two oxidizing agents, based on the demonstrated scavenging capacity of the latex. To assess the *V. negundo* ethanolic extract's capacity to serve as hydrogen atom or electron donors in the transformation of the DPPH radical into its reduced form DPPH-H, Kadir et al. (2013) used the DPPH test. The stable, purple-colored radical DPPH could be converted by the extract of *V. negundo* into the yellow-colored DPPH-H. When DPPH radical scavenging activities of *V. negundo* extract, gallic acid, BHT, and ascorbic acid were compared, the percentage of radical scavenging activity for *V. negundo* was 79.43 ± 1.3 %, BHT was 82.53 ± 1.7 %, gallic acid was 89.51 ± 1.14 %, and ascorbic acid was 90.65 ± 1.34% at the highest concentration. These results suggested that *V. negundo* exhibited notable DPPH inhibitory action. Pinipay et al. (2022) also studied the effect of *F. religiosa* seed extracts on the percentage of DPPH inhibitions and reported that the chloroform extract had the highest percentage of inhibition (68.97 ± 0.08), which is nearer to that of standard ascorbic acid (75.60 ± 0.03), and BHT (69.30 ± 0.15). Further, hexane, chloroform, ethyl acetate, methanol, and aqueous extracts of *F. religiosa* had IC_{50} values for DPPH activity was 140.25±11.22 g/ml, 131.17±0.41 µg/ml, 148.78±0.92 µg/ml, 143.87±10.37 µg/ml, and 116.52 ±2.74 µg/ml respectively. Teruel-Andreu et al. (2023) examined the leaves of four biferous varieties of *F. carica*: San Antonio (SA), Colar (CA), CuelloDamaNegra (CDN), and Superfig (SF) and observed DPPH assay between 72.45 - 52.54 mMTroloxdw. Furthermore, Ginting et al. (2020) assessed the percentage of DPPH scavenging activity of quercitrin, morin, myricitrin, and eleutheroside, as well as the ethanolic extract of *F. elastic* (FEE). When compared to other compounds (eleutheroside B, morin, quercitrin, and myricitrin), FEE had the least attribute at the greatest concentration (31.26 µg/ml), with a value of 62.52±0.66 %. Comparing other compounds, FEE displayed the highest IC_{50} value (13.82±0.51 µg/ml), indicating that it has the lowest DPPH scavenging activity. Similarly, Le et al. (2022) reported that 80% ethanol extracts of *V. rotundifolia* showed greater radical scavenging activity than those of 100% MeOH extracts at concentrations of 10 and 100 µg/mL. In the current investigation, leaf extracts from *V. negundo* had the highest DPPH inhibitory activity when compared to leaf extracts from *F. carica*.

3.2 Ferric reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power test evaluates the potential of an anti-oxidant compound and its reduction from Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. The result of the present study depicted that the anti-oxidant potential of MeOH extract was significantly higher than that of $C_4H_8O_2$ and $CHCl_3$ extracts respectively for leaves of *F. carica* and *V. negundo* with the former being more efficient. But as compared to the *F. carica* extracts, *V. negundo* extracts showed higher FRAP values as shown in Figures 4 and 5. In the FRAP assay conducted by Soni et al. (2014), the vivid blue color that results from the reduction of the $C_{18}H_9FeN_6$ (ferric tripyridyltriazine) to $Fe^{2+}$was observed at a wavelength of 593 nm. FRAP activity was found to be very good in *F. carica* extract (60.48 µM). Another antioxidant study (FRAP) conducted by Vijayalakshmi and Rao (2020) on *V. negundo* revealed that water extract (90.56 µM) had greater antioxidant potential than quercetin (85.162 µM) and ascorbic acid (79.647µM). The antioxidant activity of the $C_4H_8O_2$ (80.67µM) and CH_3OH (84.57µM) extracts were likewise positive and were closer to the ranges of the standards. Similarly, the result of Zargar et al. (2011) revealed that the MeOH extract of *V. negundo* has an antioxidant capacity of 44.6 ± 7.8 µM TE/g which was significantly higher than that of the plant's essential oil (11.53±1.35 µM TE/g) and hexane (11.30±1.3 µM TE/g) leaf extracts. Nevertheless, it was discovered that CH_3OH extract's antioxidant capacity was four times greater compared to essential oil as well as hexane extract. Likewise, in another study by Traore et al. (2021), the fruit extracts of *V. doniana* differed in their ability to reduce Fe^{3+}, although all results were below standard i.e. butylated hydroxytoluene and ascorbic acid. Ayoub et al. (2019) analyzed the reducing capabilities of extracts from *F. carica* as well as *O. europaea*, and the results revealed that extracts had a strong reduction power and it was dosage dependent and increased with the levels of extract. Results of the study revealed that *O. europaea* extract had a FRAP value that ranged from 0.125±0.001 to 0.683 ±0.026 µg/ml, while this value was reported from 0.113 ± 0.004 to 0.494 ± 0.008 µg/ml and 0.260± 0.014 to 2.81± 0.014 g/ml for *F. carica* and ascorbic acids respectively. Therefore a prominent statistical difference was reported in these. *F. carica* leaves of the four biferous variants i.e. San Antonio, Colar, Cuello Dama Negra, and Superfig were examined for FRAP by Teruel-Andreu et al. (2023). The cultivars were ranked from top to lowest in terms of MTroloxdw: CDN > SF > CUMH > CA > SA with values being 124.79, 115.66, 67.15, 60.70, and 56.09 mMTroloxdw respectively. The FRAP test of *V. doniana* fruit indicated a value of 600.19 ± 2.37 µM as reported by Moffo Foning et al. (2022). In the study of Ginting et

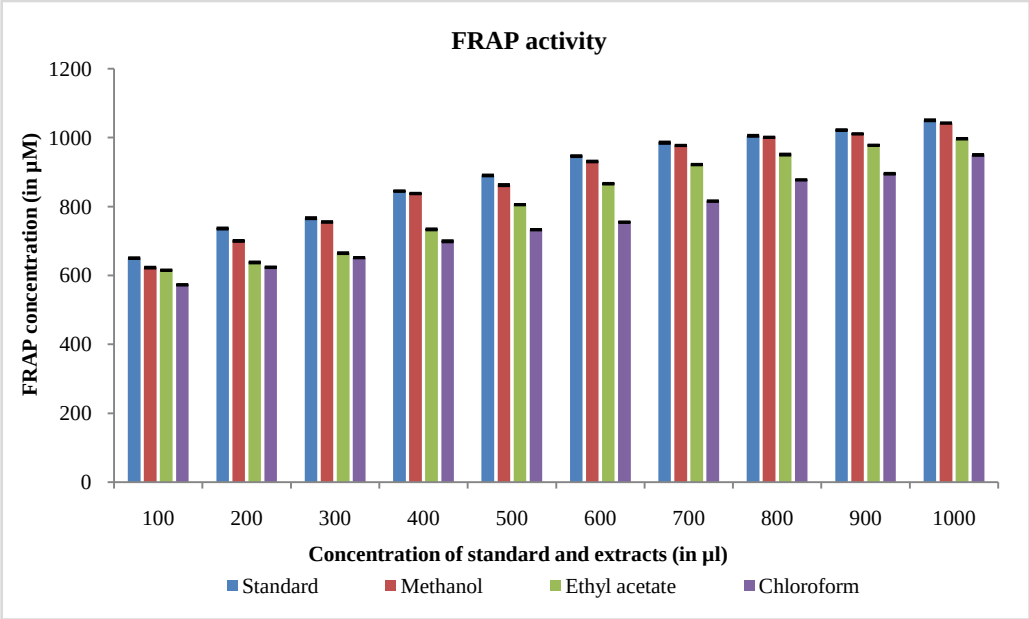

Figure 4 FRAP activity of *V. negundo*

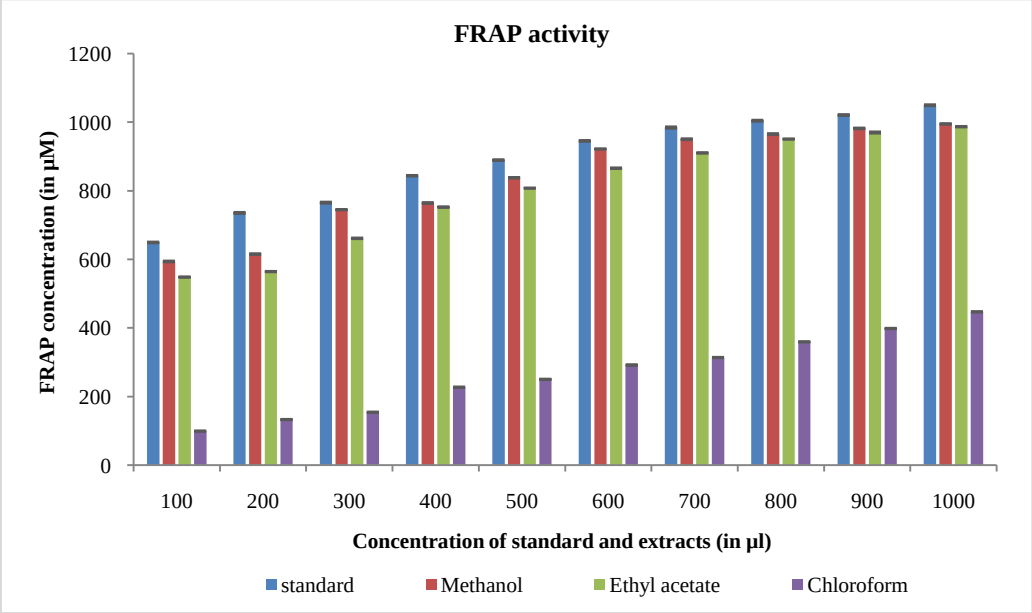

Figure 5 FRAP activity of *F. carica*

al. (2020), it was discovered that compounds, including eleutheroside B, quercitrin, morin, and the ethanolic extract of *F. elastica*, had FRAP-reducing action. At the maximum concentration (50.00 µg/mL), ethanolic extract demonstrated the least amount of FRAP-reducing action in comparison to eleutheroside B (117.08±27.35 µM Fe (II)/µg), myricitrin (456.00±13.43 µM Fe (II)/µg), quercitrin (487.58±5.59 µM Fe (II)/µg), and morin (496.58±9.25 µM Fe (II)/ µg). As a result, the ethanolic extract has a moderate level of antioxidant activity that is comparable to that of the morin molecule. Likewise, in the current study, the three *F. carica* leaf extracts gave FRAP activity, while the three *V. negundo* extracts displayed the highest FRAP activity.

Conclusion

Results of the current research revealed a comparative analysis of the antioxidant activities of *V. negundo* and *F. carica* leaves extracts. Based on the findings, it can be concluded that the

methanolic extract of *V. negundo* possesses more antioxidant activity as compared to the *F. carica* extracts. However, further investigations for potential applications and *in vivo* experiments, are needed to verify these antioxidant effects of the selected plant species.

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Conflicts of Interest

The authors declare no conflict of interest.

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Journal of Experimental Biology and Agricultural Sciences, February - 2023; Volume – 11(1) page 105 – 118

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Biogenic Synthesis and Characterization of Silver Nanoparticles (AgNPs) Produced by Indigenous Microorganisms Isolated from Banana (*Musa spp*) Soils

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Bacillus

Microorganism

Nanotechnology

Penicillium

ABSTRACT

This research focused on the screening of indigenous microorganisms isolated from banana soils for their capability to synthesize silver nanoparticles (AgNPs) extracellularly. Ninety-five isolates were screened for AgNP production. The cell-free extracts of these isolates were added to silver nitrate ($AgNO_3$) aqueous solution and were observed for color changes from original pale yellow to dark brown. Ten isolates (3 bacteria and 7 fungi) were found capable of producing AgNPs. Bacterial isolates B2, B3, and B5 were molecularly identified as *Bacillus aryabhattai*, *Priestia megaterium*, and *B. megaterium*, respectively. The AgNPs produced by these bacterial isolates were circular and showed an absorbance peak at approximately 420 nm. On the other hand, the fungal isolates F2, F3, and F43 were molecularly identified as *Penicilliumcitrinum*, *P. glaucoroseum*, and *P. oxalicum*. The AgNPs produced by the *Penicillium spp* were aggregated, circular and showed absorbance peaks at 420 nm. The other four fungal isolates, F7, F24, F29, and F40, were identified as *Aspergillus flavus*, *A. terreus*, and *A. japonicum* (F29 and F40), respectively. The AgNPs produced by the *Aspergillus* spp. were circular and showed absorbance peaks between 420 nm and 450 nm. The continuous search for novel isolates that can carry out the biogenic synthesis of AgNPs remains the focus of nanotechnological research. This study confirms microorganisms of *Bacillus*, *Penicillium*, and *Aspergillus* genera can effectively biosynthesize AgNPs.

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1 Introduction

Nanotechnology is a field of science concerned with the study and control of matter with dimensions ranging from 1 to 100 nanometers (Nasrollahzadeh et al. 2019). It covers a wide range of topics, from expanding traditional device physics to completely new methods based on molecular assembly, from synthesizing novel materials with nanoscale dimensions to whether we can directly manipulate matter at the atomic scale (Jeevanandam et al. 2018). It has the potential to generate a wide range of novel materials and products with applications in medicine, electronics, energy generation, and agriculture (Rai and Ingle 2012). Nanotechnology has the potential to provide green and environmentally friendly plant disease management options. Many microorganisms are already recognized for forming inorganic material within or outside the cell to create nanoparticles. Among the many nanoparticles, silver nanoparticles (AgNPs) stand out in various fields (Yaqoob et al. 2020; Alharbi et al. 2022; Salleh et al. 2022).

Silver nanoparticles are between 1 and 100 nm in size and have unique features that aid molecular diagnostics, treatments, and devices used in a variety of medical operations (Prabhu and Poulose 2012). High electrical and thermal conductivity, surface-enhanced Raman scattering, catalytic activity, chemical stability, and non-linear optical behavior are among the physicochemical attributes of AgNPs that distinguish them from bulk Ag. In addition, AgNPs have a high surface area-to-volume ratio, have better interaction with the microorganism, and are well-known antimicrobial agents that work against a wide range of bacteria, both Gram-positive and Gram-negative (Anjum et al. 2013).

Bacteria, fungi, and plant extracts are the three major sources of biosynthesis of silver nanoparticles and are produced mostly by reduction/oxidation reactions. Nanoparticles can be synthesized by bacteria either through extracellular mechanisms or within cells. Bacteria such as *Bacillus licheniformis* (Kalimuthu et al. 2008) use the NADH-dependent nitrate reductase- mediated reduction of silver ion (Ag^{+}) to elemental silver (Ag^{0}). However, non-enzymatic reduction also occurs, such as with *Lactobacillus* A09, where Ag^{+} reduction occurs intracellularly on the surface of the bacterial cell (Van Hullebusch et al. 2003). Fungal biosynthesis of AgNPs is possible due to their ability to secrete proteins such as those of *Aspergillus flavus,* which secretes a 32-kDa reductase protein that can reduce Ag^{+} ions (Jain et al. 2011).

The antioxidant or reducing characteristics of microbial enzymes interact with the appropriate compounds to synthesize the desired nanoparticles. The three essential considerations for the biogenic synthesis of nanoparticles are the non-toxic stabilizing agent, solvent medium for synthesis, and an environmentally friendly reducing agent. Behavior, biodistribution, safety, and efficacy are some important factors that depend on the physicochemical properties of the nanoparticle. Characterization of AgNPs is significant for evaluating the functional features of the produced particles. Parameters such as size, size distribution, surface area, solubility, aggregation, and other properties of the nanoparticle need to be evaluated (Zhang et al. 2016).

Several studies have proved the potential of microorganisms as a biofactory of silver nanoparticles (Mukherjee et al. 2008; Fayaz et al. 2010; Sunkar and Nachiyar 2012). Rhizosphere soils of healthy banana plants harbor bacteria belonging to *Trichoderma, Actinobacteria,* and *Bacillus* genera (Xue et al. 2015), and fungi belonging to *Fusarium, Aspergillus,* and *Penicillium* genera (Zhou et al. 2019). The diversity of indigenous microorganisms found in banana rhizosphere soil can be a source of novel isolates that can carry out reliable biosynthesis of silver nanoparticles with certain properties such as high stability, and having the desired size and composition. Hence, this study aims to isolate, screen, and identify indigenous microorganisms found in the banana rhizosphere for the biogenic synthesis of silver nanoparticles.

2 Materials and Methods

2.1 Sample Collection and Processing

The banana soil samples were collected from the central experimental station, Pili Drive, University of the Philippines Los Baños College, Laguna. The soil of the study area which belongs to the Lipa series was classified as fine, clayey, mixed, shallow, isohyperthermic Typic Eutrudepts formed from weathering of hard tuffaceous rocks.

Three banana plants per variety were randomly selected in the field to obtain a composite sample. Plants were uprooted and roots were carefully shaken. Soil particles less than 3 mm thick adhering to the roots were considered as rhizosphere soil and were collected by gently brushing the roots. The samples were placed in sterile plastic bags and stored under cooled conditions until preparation in the laboratory.

2.2 Isolation of fungi and bacteria from banana rhizosphere soil

Ten grams of the soil was mixed with 95 ml of sterile distilled water. Prepared soil solution was taken for serial dilution (10^{-3}, 10^{-4}, $10^{-5,}$ and 10^{-6}) and cultured by spread plate method on nutrient agar (NA) for bacterial isolation and on potato dextrose agar (PDA) for fungal isolation. Extensive colony purification was done to attain single colony cultures by repetitive inoculation of the bacterial colony on NA and point inoculation on PDA for fungal isolates. The pure cultures of the isolates were transferred in NA and PDA slants. The morphological and microscopic properties of the isolates were assessed. The pure cultures of the isolates capable of biogenic synthesis of AgNPs

were submitted to Philippine Genomic Center for sequencing. Resulting sequences were aligned and compared with those from Basic Local Alignment Search Tool (BLAST).

2.3 Extracellular Synthesis of AgNPs using Bacteria

The capability of the bacterial isolates to carry out biosynthesis of AgNPs was determined by Malarkodi et al. (2013), and Adan et al. (2018) with some modifications. The isolates were inoculated freshly in 10 ml NA broth and were kept in an orbital shaker (150 rpm) for 24 hours. The same incubation condition was applied to ten ml (10 ml) of nutrient broth only, which served as the control. The cultures were centrifuged at 2000 rpm for 15 minutes and the supernatant was obtained. The supernatants (free from any kind of precipitates) were passed through sterilized membranes using a 0.22-micron pore-size filter.

Silver nitrate ($AgNO_3$) aqueous solution was added to the vials containing 5ml of the supernatant at a final concentration of 1mM $AgNO_3$. These vials ($AgNO_3$ – supernatant mixture) were incubated in an orbital shaker (150 rpm) at 35° C for 7 days under dark conditions. Control solution ($AgNO_3$ – nutrient broth) was prepared and subjected to the same incubation condition to confirm that AgNP synthesis was mediated by extracellular agents of bacterial origin. After incubation, a visual inspection was performed relative to color changes in the control, to confirm whether AgNPs were produced. Purification of AgNPs was carried out by centrifugation at 10,000 rpm for 10 min twice and the nanoparticles were collected for characterization.

2.4 Extracellular Synthesis of AgNPs using Fungi

The capability of the fungal isolates to carry out biosynthesis of AgNPs was determined by the method described by Magdi et al. (2014) with some modifications. For this, fungal isolates were cultured on PD broth at 28 °C on a rotary shaker for 96 hours. The biomasses were obtained using Whatman filter paper No. 1 and then washed with ultrapure water to eliminate any remaining components of the medium. Then, the biomass was incubated for 24 h in separate flasks containing 100 ml water. The biomass was filtered, and the resulting cell filtrate was collected and used for AgNP biosynthesis.

For the biosynthesis of AgNPs, 50 ml of cell filtrate was mixed with 10 ml $AgNO_3$ at a final concentration of 1 mM $AgNO_3$. The control was a reaction mixture without $AgNO_3$. The prepared solutions were incubated for 7 days at 28 °C and were kept in the dark during the experiment. After incubation, a visual inspection was performed relative to color changes in the control, to confirm whether AgNPs were produced. Purification of AgNPs was carried out by centrifugation at 10,000 rpm for 10 min twice and the nanoparticles were collected for characterization.

2.5 Characterization of Produced Silver Nanoparticles

The AgNPs were subjected to optical absorbance measurements using a UV-Vis spectrophotometer (MultiSkan Sky Spectrophotometer, Thermo Fisher Scientific) scanning between 250 nm and 700 nm at a 1nm resolution. Detailed characterization of the size, distribution and morphology of the nanoparticles was performed using a particle analyzer and scanning electron microscopy (Prisma E-SEM, Thermo Fisher Scientific).

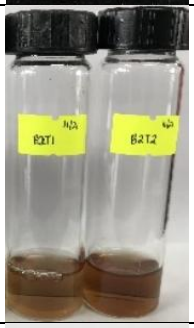
3 Results and Discussion

A total of 51 bacterial isolates and 44 fungal isolates were screened for AgNP production. Among the 95 tested isolates, only 10 isolates (3 bacteria and 7 fungi) were observed with the ability to change the color of the reaction mixture from its original pale-yellow color to dark brown (Tables 1 and 2). The change in color of the solution indicates the production of AgNPs by reduction of Ag^+ to Ag^0, mainly due to the excitation of surface plasmon vibrations in the AgNPs. The increase in color intensity of the solution was attributable to an increase in the number of nanoparticles generated, as silver ions in the aqueous solution were reduced (Elamawi and Al-Harbi 2014).

The three bacterial isolates capable of synthesizing AgNPs were B2, B3, and B5. Interestingly, all isolates belong to the class *Bacillus*. These results are in agreement with previous findings, and various previous studies confirmed that *Bacillus* strains can produce AgNPs (Saravanan et al. 2011; Deljou and Goudarzi 2016; Ahmed et al. 2020). Isolates B2, B3, and B5 (Table 3) were molecularly identified to be *Bacillus aryabhattai*, *Priestia megaterium*, and *B. megaterium*, respectively. As illustrated in Figure 1, the UV-visible absorption spectra showed absorbance peaks at approximately 420 nm. The absorption peak observed in this study is specific for AgNPs, which is similar to the results previously obtained (Priyadarshini et al. 2013; Omole et al. 2018). The scanning electron microscope micrographs (Figure 2) of the dry mass show spherical shape AgNPs with an average size of 88.10 nm, 51.08 nm, and 80.76 nm for isolates B2, B3, and B5, respectively. In addition, energy-dispersive X-ray spectra of AgNPs (Figure 3) produced by isolates B2, B3, and B5 showed peaks of silver (Ag), suggesting that the nanoparticles are indeed Ag in composition.

AgNPs in the range of 50 nm were synthesized using the bacterium *B. licheniformis*, which exhibited maximum absorbance at 440 nm in UV-vis spectroscopy (Kalimuthu et al. 2008); similarly, the synthesis of AgNPs of an approximate size of 40 nm was successful using the culture supernatant of *B. licheniformis* (Kalishwaralal et al. 2008). Equal results were obtained for silver nanoparticles with an average size of 52.5 nm using the culture

Table 1 Screening of Bacterial Isolates for Production of Silver Nanoparticles

Isolate No	Color change		Inference
	Initial	After	
Control ($AgNO_3$ only)			No change in color
B2			+
B3			+
B5			+

supernatant of *Klebsiella pneumonia* and *Escherichia coli* (Shahverdi et al. 2007). Extracellular biosynthesis of highly stable AgNPs from bacterial strain *B. megaterium* (NCIM 2326) was obtained (Saravanan et al. 2011), and the extracellular formation of AgNPs of approximate size 42 nm to 92 nm and with UV-Vis absorption at 450 nm was also found from *Bacillus* sp. (Das et al. 2014).

The seven fungal isolates capable of synthesizing AgNPs were F2, F3, F7, F24, F29, F40, and F43. These isolates belong to only two fungal classes, which are *Penicillium* and *Aspergillus*. Several studies confirmed that *Penicillium* (Hemath Naveen et al. 2010; Ma et al. 2017; Shareef et al. 2017; Taha et al. 2019) and *Aspergillus* strains (Gade et al. 2008; Jain et al. 2011; Li et al. 2011) can produce AgNPs.

Table 2 Screening of Fungal Isolates for Production of Silver Nanoparticles

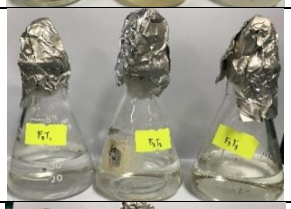
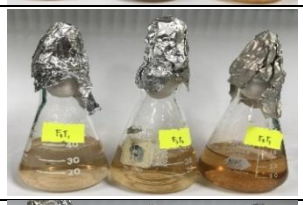
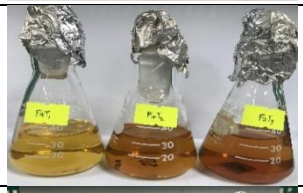
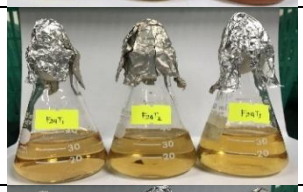
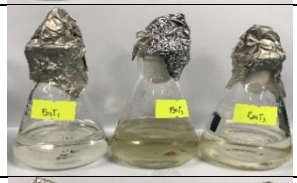
Isolate No	Color change		Inference
	Initial	After	
Control ($AgNO_3$ only)			No change in color
F2			+
F3			+
F7			+
F24			+
F29			+
F40			+
F43			+

Table 3 Molecular identification of the microorganisms capable of synthesizing AgNPs

Isolate Code	Species	Accession Number	Similarity	E Value
Bacteria				
B2	*Bacillus aryabhattai*	MT605510.1	99.34%	0
B3	*Priestia megaterium*	MW363319.1	98.95%	0
B5	*Bacillus megaterium*	JF343138.1	99.50%	0
Fungi				
F2	*Penicillium citrinum*	MT820334.1	92.15%	8e-109
F3	*Penicillium glaucoroseum*	MT530148.1	89.63%	4e-138
F7	*Aspergillus flavus*	MW805395.1	97.25%	0
F24	*Aspergillus terreus*	MT530046.1	90.62%	5e-153
F29	*Aspergillus japonicus*	EU645679.1	92.73%	4e-125

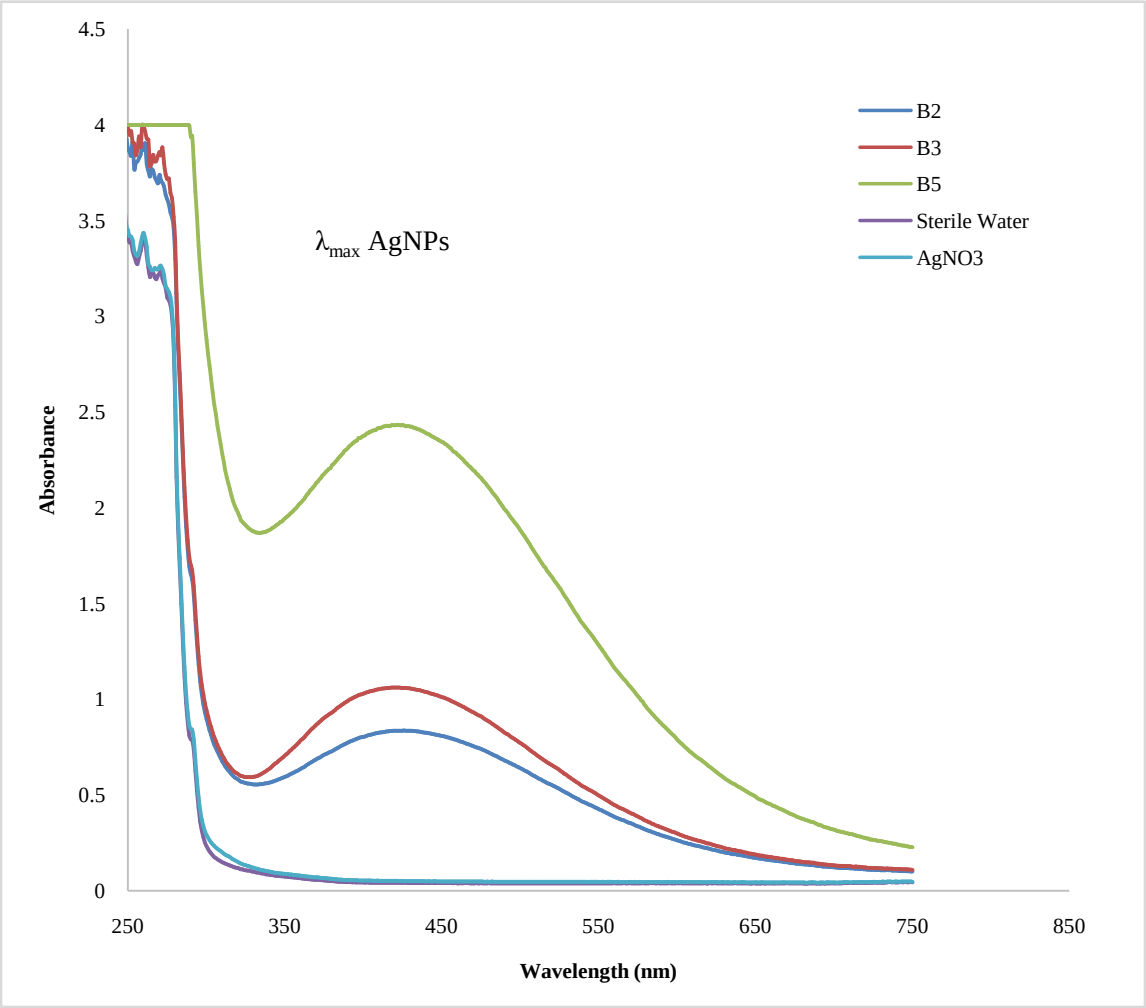


Figure 1 The UV-Vis spectra recorded for the reaction of the different bacterial cell filtrates and $AgNO_3$ solution

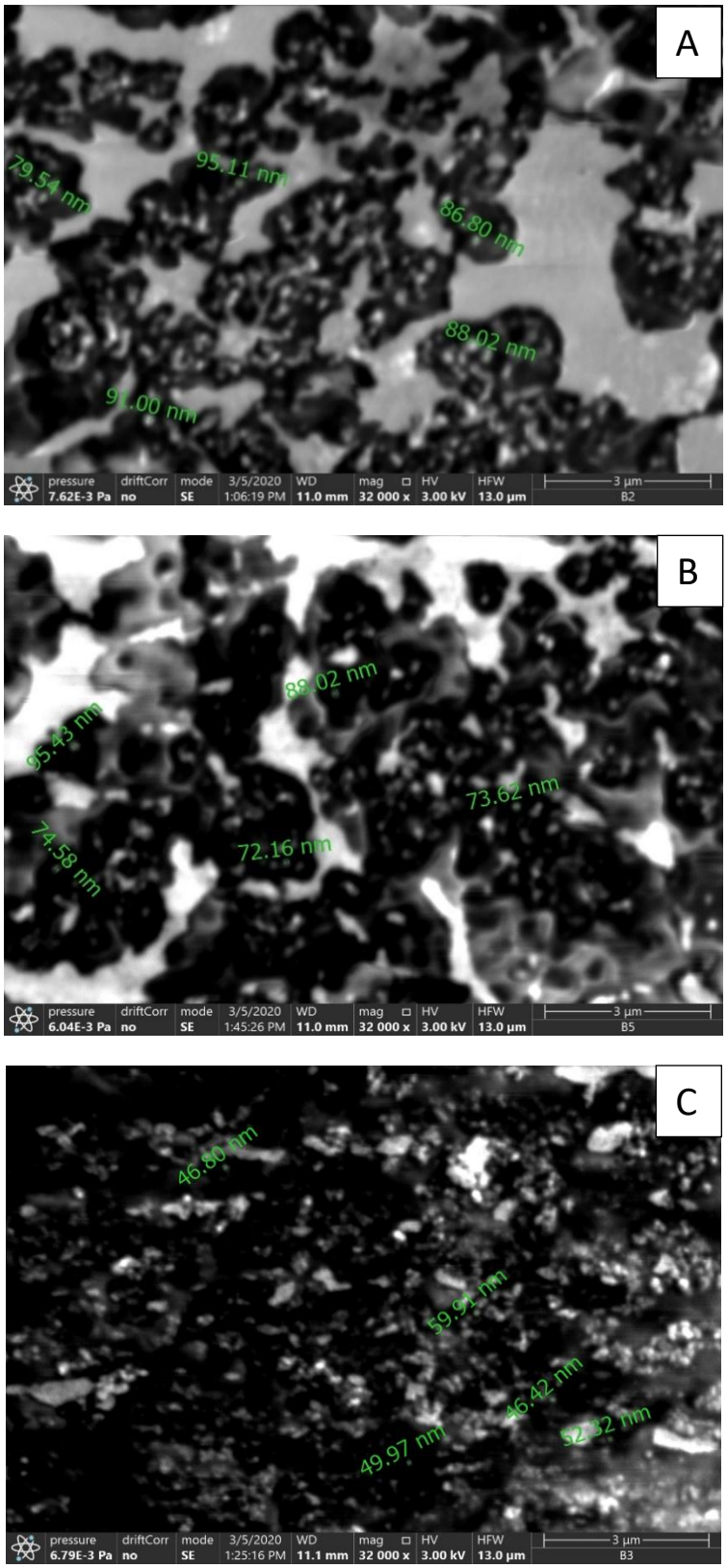
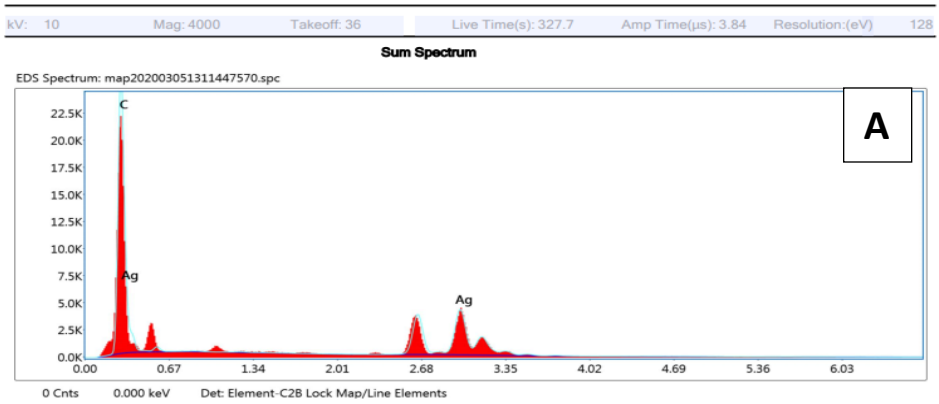
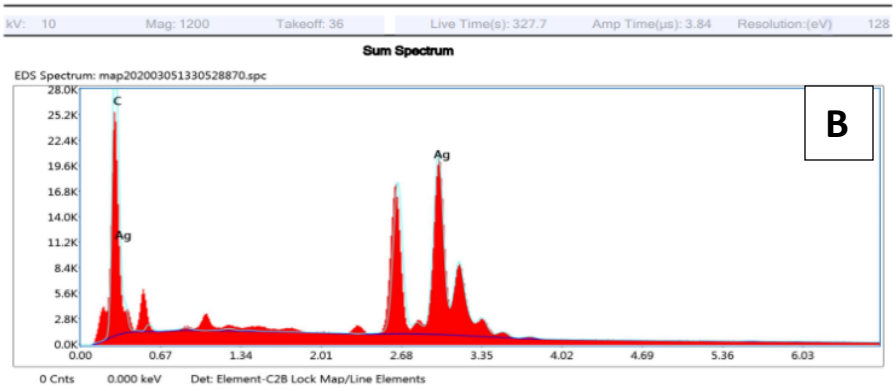


Figure 2 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) B2, b) B3 and c) B5.



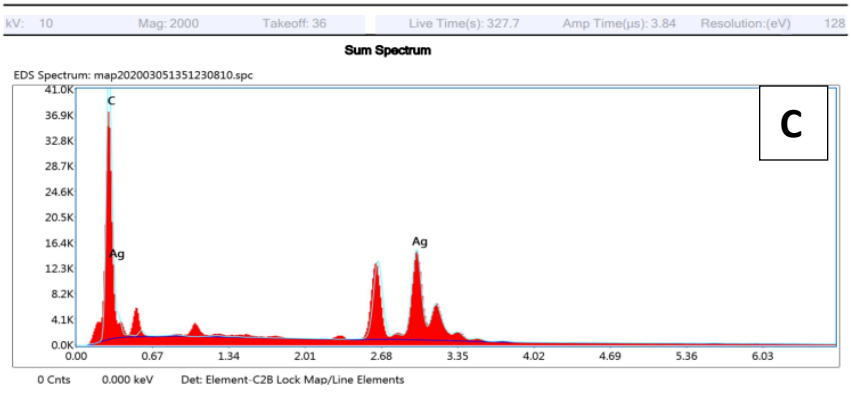
Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	34.17	82.34	3.67
AgL	65.83	17.66	3.47



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	11.53	53.93	4.28
AgL	88.47	46.07	2.88



Smart Quant Results

Element	Weight %	Atomic %	Error %
C K	20.61	69.98	3.95
AgL	79.39	30.02	3.02

Figure 3 EDX Spectra of AgNPs produced by isolates a) B2, b) B3 and c) B5

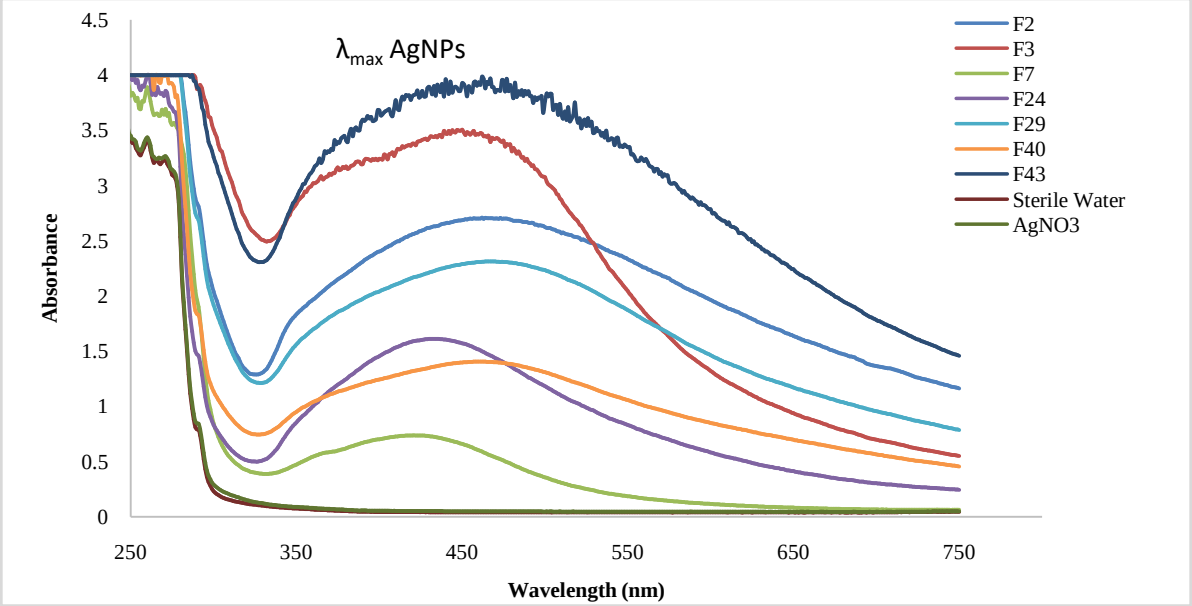


Figure 4 The UV-Vis spectra recorded for the reaction of the different fungal cell filtrates and $AgNO_3$ solution

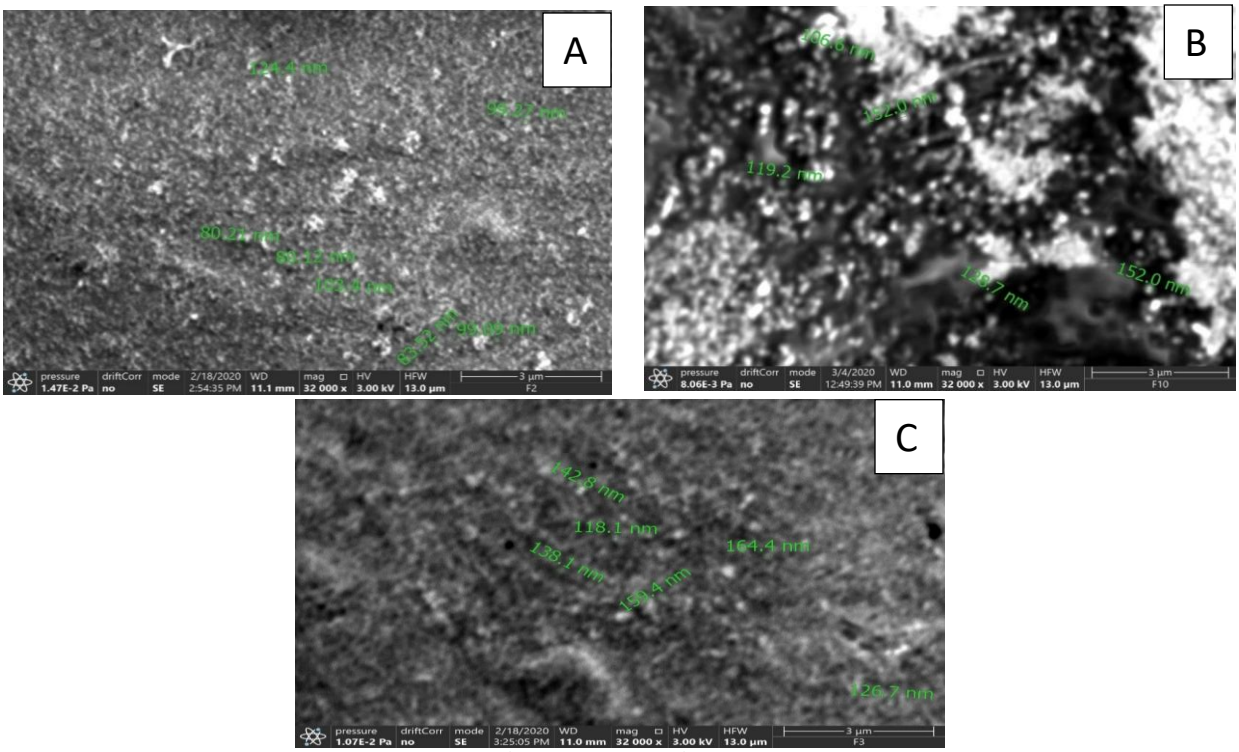


Figure 5 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) F2, b) F3 and c) F43

Isolates F2, F3 and F43 were molecularly identified as *P. citrinum*, *P. glaucoroseum*, and *P. oxalicum*, respectively. As shown in Figure 4, the UV-visible absorption spectra showed absorbance at approximately 450 nm. The scanning electron microscope micrographs (Figure 5) of the dry mass show aggregated spherical-shaped AgNPs with an average size of 88.31 nm, 141.58 nm, and 131.7 nm for isolates F2, F3, and F43, respectively. The likelihood of aggregation is high for small-sized particles because of the large surface area and attractive force between the particles (Honary et al. 2013). In addition, energy dispersive X-ray spectra of AgNPs (Figure 6) produced by isolates F2, F3, and F43 showed peaks corresponding to silver (Ag), suggesting the presence of Ag in the nanomaterial.

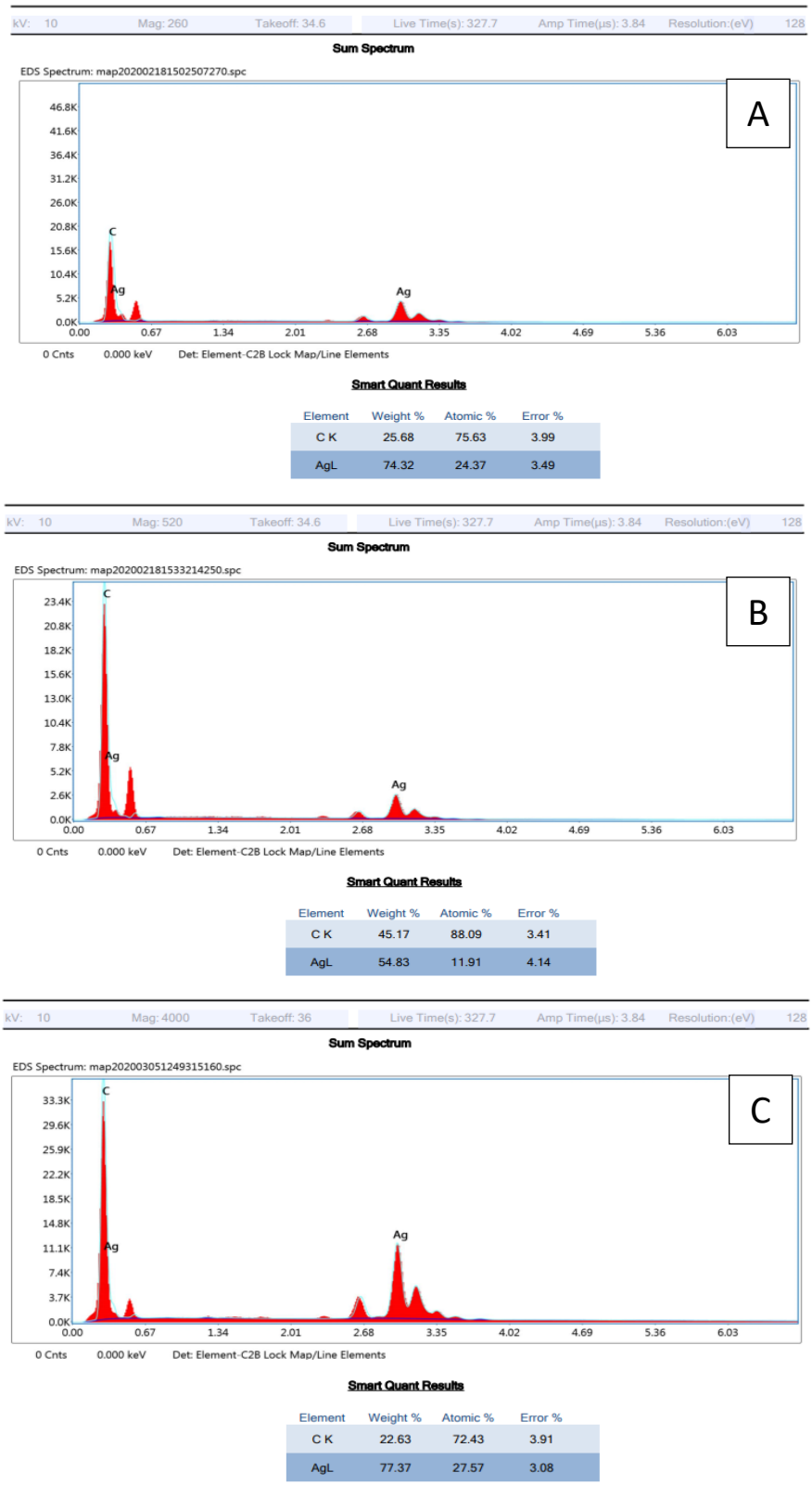


Figure 6 EDX Spectra of AgNPs Produced by isolates a) F2, b) F3 and c) F43.

The *Penicillium* family is an emerging nanofactory for the biosynthesis of green nanomaterials. There are 25 *Penicillium* species reportedly used for the biosynthesis of nanomaterials (Barabadi et al. 2019); notably, AgNPs were predominantly produced by exploiting *Penicillium* species. In another study, the crude cellular extract of *P. oxalicum* GRS-1 produced spherical AgNPs with sizes ranging from 10 to 40 nm (Rose et al. 2019). In addition, AgNPs produced from *P. oxalicum* have a characteristic strong broad peak at 456 nm and are also spherical (Bhattacharjee et al. 2017). Moreover, the production of spherical-shaped and well-dispersed AgNPs with an average particle size of 2 to 5 nm from *P. citrinum* was confirmed (Danagoudar et al. 2020), which exhibited an absorption band around 400 to 420 nm and a particle size of 90 to 120 nm (Honary et al. 2013).

Isolates F7, F24, and F29 were molecularly identified as *Aspergillus flavus, A. terreus*, and *A. japonicus,* respectively. F40 was morphologically similar to F29, hence assumed also to be *A. japonicus*. As shown in Figure 4, the UV-visible absorption spectra showed absorbance at approximately 420 nm to 450 nm. The scanning electron microscope micrographs (Figure 7) of the dry mass show aggregated spherical shaped AgNPs with an average size of 124.62, 143.54, 159.86, and 85.92 nm, respectively. Moreover, EDX spectra of AgNPs (Figure 8) produced by isolates F7, F24, and F29 showed peaks corresponding to silver (Ag), suggesting the presence of Ag in the nanomaterial.

Various previous studies confirmed the capability of some *Aspergillus* species in the production of silver nanoparticles. In a study done by Li et al. (2011), polydispersed spherical particles ranging in size from 1 to 20 nm were produced using culture supernatants of *A. terreus*. They also reported that reduced nicotinamide adenine dinucleotide (NADH) was an essential reducing agent for biosynthesis and that AgNP production may be an enzyme-mediated extracellular reaction process. In another study, the fungus *A. flavus* produced monodispersed AgNPs that showed an absorption peak at 420 nm and an average size of 8.92 nm (Vigneshwaran et al. 2007). Extracellular synthesis of cubic

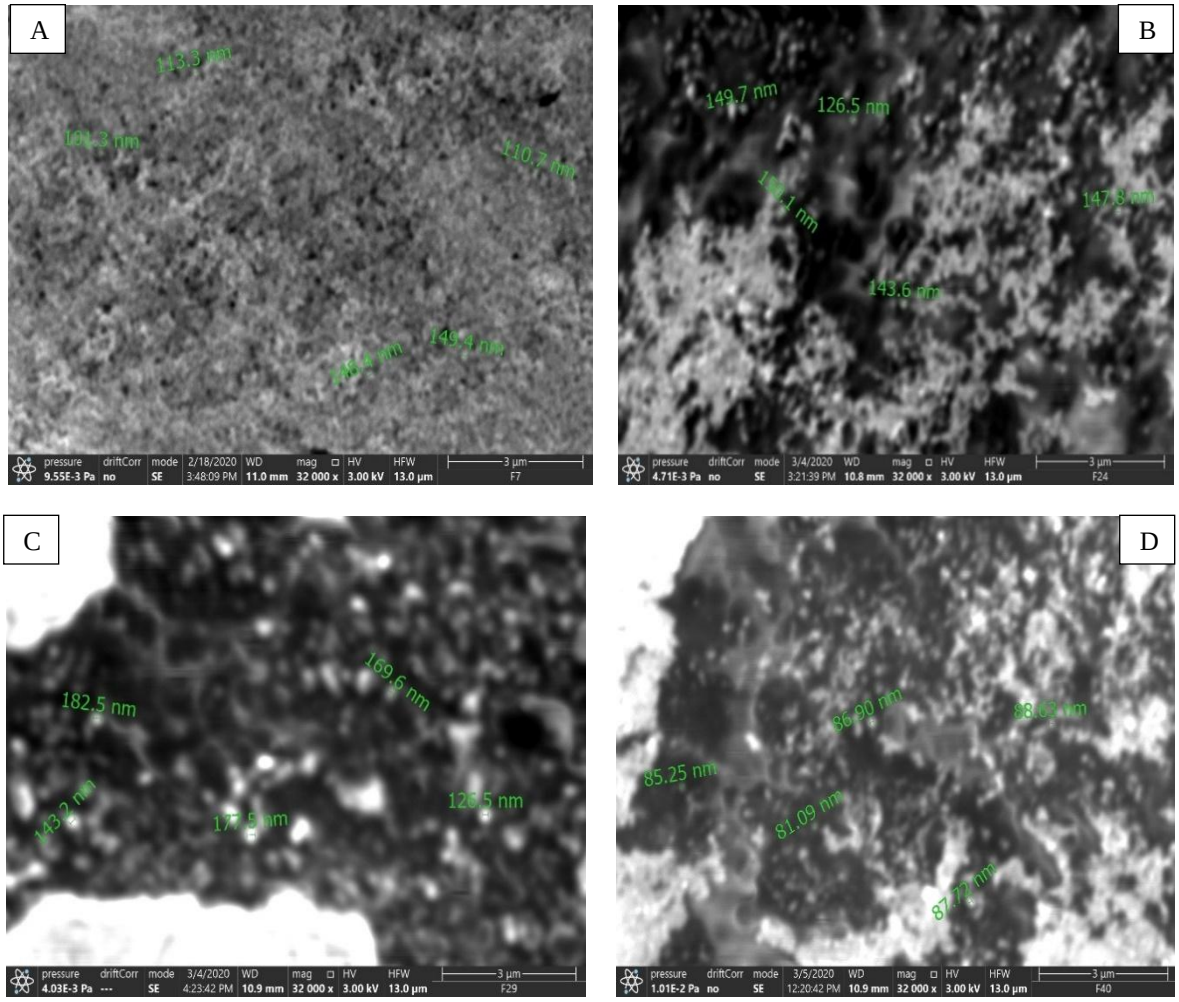


Figure 7 Scanning electron micrographs (magnification: 32,000X) of AgNPs produced by isolates a) F7, b) F24 c) F29 and d) F40

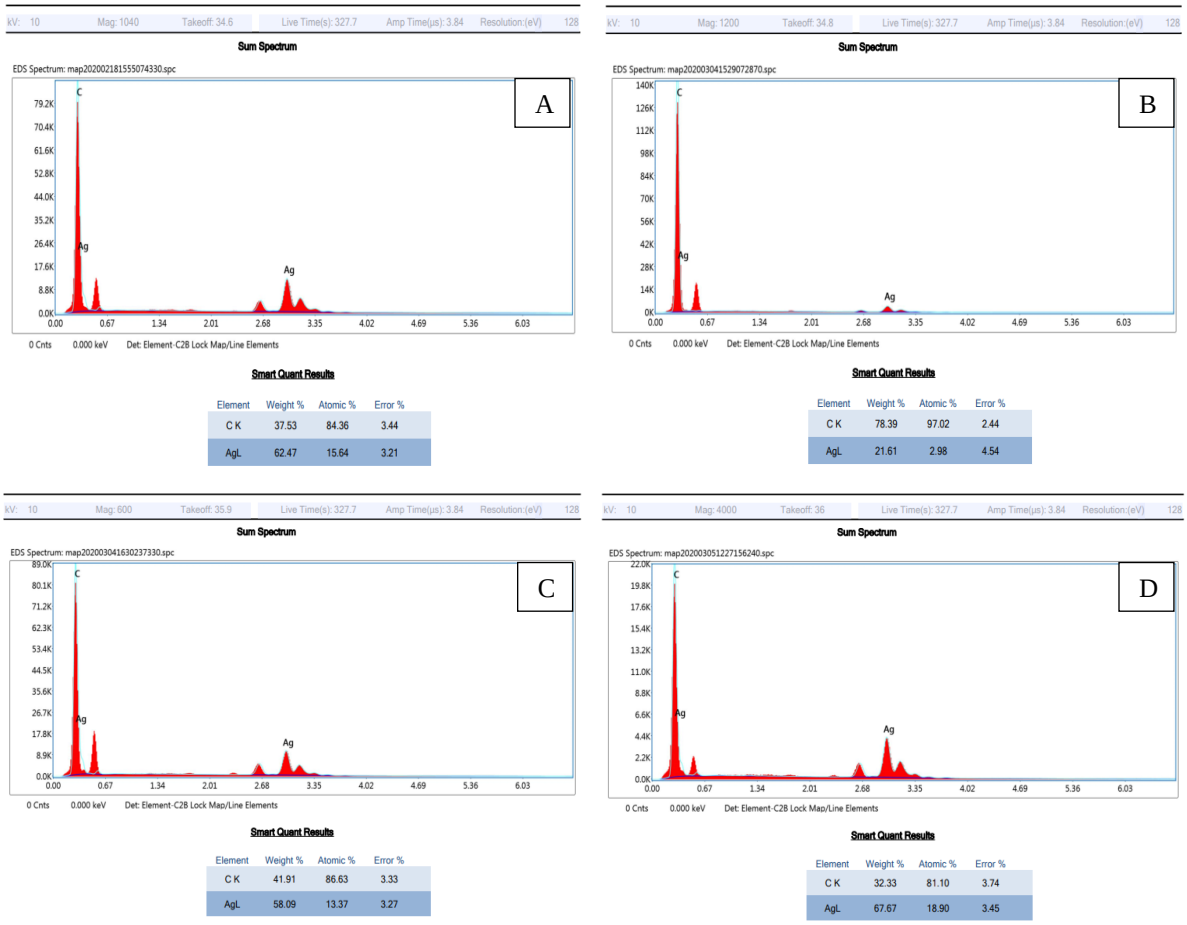


Figure 8 EDX Spectra of AgNPs produced by isolates a) F7, b) F24 c) F29 and d) F40.

structured AgNPs showed a characteristic absorbance peak of 420 nm and an average size of 33.5 nm using *A. flavus* (Sulaiman et al. 2015). Extracellular AgNPs were synthesized from cellular extracts of an *Aspergillus* consortium, which consisted of *A. niger*, *A. michelle*, and *A. japonicus* (Samuel and Guggenbichler 2004).

Conclusions

Results of the study responded to the challenge of investigating new isolates that are capable of reliable and efficient synthesis of AgNPs. A total of ten isolates belonging to the bacterial class *Bacillus* and fungal classes *Penicillium* and *Aspergillus* were found capable of producing AgNPs. The AgNPs produced have characteristic absorption peaks at 420 nm to 450 nm, mostly spherical and of varied sizes. EDX spectra showed peaks belonging to silver (Ag), suggesting the presence of Ag in the nanoparticles.

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Peruvian plant resources as potential natural controllers of adult *Aedes aegypti*

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ABSTRACT

Aedes aegypti is an important vector of tropical diseases like Dengue, Zika, Chikungunya, and Yellow Fever and affects mainly countries located in tropical and subtropical zones, including Peru. Synthetic insecticides are used to control this vector, but they also cause a residual effect on the environment, whereas the vector has developed resistance to these compounds, so there is a current need to search for new control alternatives, such as the use of abundant natural resources. Therefore, this work aimed to evaluate the biocidal activity of extracts and oils from *Cymbopogum citratus*, *Rosmarinus officinalis*, and *Minthostachys mollis* on adult *Aedes aegypti*, as well as to evaluate their quality parameters. Furthermore, the chemical profile of the three species was assessed by ultra-high-performance liquid chromatography coupled with mass spectrometry (LC-MS/MS). The results showed that the aqueous/ethanolic extracts and the essential oils from the three evaluated species presented a biocidal effect on adult *A. aegypti*. Regarding the analysis of the chemical profile, 15 compounds were identified in *R. officinalis*, while 29 compounds were identified from *C. citratus* and 30 compounds from *M. mollis*. Moreover, the extracts and oils presented quality parameters according to standards. In conclusion, the biocidal potential of the *C. citratus*, *R. officinalis*, and *M. mollis* on *A. aegypti* adults was reported so that they can be seen as a real natural alternative for the control of tropical diseases transmitted by this vector so that plant products are more eco-friendly and subject to lower resistance by target organisms.

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1 Introduction

In recent years, globalization, unplanned urbanization, and environmental problems, including climate change, are considerable factors that influence the transmission of diseases, among which vector-borne diseases are the most common (Chala and Hamde 2021). Vector-borne diseases of public health importance are those infectious diseases that are spread by organisms that carry viruses, parasites, or bacteria from one infected person (or animal) to another. These diseases account for 17% of the estimated global burden of infectious diseases and are more frequent in tropical and subtropical areas and places with problems with appropriate water access and sanitation (OPS 2019; WHO 2020).

In Peru, there is a group of diseases that share the same vector: *Aedes aegypti*, which is a hematophagous diptera distributed mainly in tropical areas, responsible for the transmission of several arboviruses that cause diseases such as dengue, Zika, yellow fever, and chikungunya (Cabezas et al. 2015; Dueñas-López 2022). The control of these diseases is linked to the eradication of the mosquito vector so that the habits of the mosquito guide its management to the elimination of breeding sites, which in turn are maintained due to poor hygiene habits of the population, which tends to discard garbage in patios, streets and vacant lots (Pereira et al. 2022).

The use of chemical insecticides is primarily adopted in Peru due to their effectiveness in reducing the populations of larvae and adults. The most widely used insecticides are organophosphates (temephos for eliminating larvae during focal treatment) and fenthion, fenitrothion and malathion for eliminating adult mosquitoes) and pyrethroids (deltamethrin, lambdacyalothrin, cypermethrin, and cyfluthrin). However, the WHO periodically monitors the emergence of resistance to the recommended pesticides and indicates the use of alternative substances that can be used, including in Peru (Lazcano et al. 2009; MINSA 2015).

Furthermore, the development of resistance in *A. aegypti* mosquitoes to synthetic insecticides is the main problem that affects control strategies caused by the intensive use of these products due to the selection of resistance genes in the populations of this species. Some authors have mentioned that the mechanism associated with resistance to organophosphate insecticides could be linked to the elevation of esterases (Lazcano et al. 2009).

Nonetheless, botanical insecticides are another accessible and low-cost control alternative for farmers and communities since several plant species have insecticidal activity. Insecticides of plant origin have the advantage of being more biodegradable than their synthetic counterparts and are related to less development of resistance, often found with immediate availability (Demirak and Canpolat 2022). The secondary metabolites produced by plants in the face of attacks by insects and predators make them natural candidates for use in controlling insect species, including agricultural pests and disease vectors (Pino et al. 2013).

In this context, plant species found mainly in Peru as *Rosmarinus officinalis*, *Cymbopogum citratus*, and *Minthostachys mollis*, demonstrated larvicidal, insecticidal, and repellent activities against different insects (Gillij et al. 2008; Duarte et al. 2015; Soonwera and Sittichok 2020; Rocha et al. 2022), so the objective of this present study was to evaluate the insecticidal potential of these species on adult *A. aegypti*.

2 Materials and Methods

2.1 Insects

Adults of *A. aegypti* were obtained from the towns of Rio Seco (Trujillo), provided by the Institute for Research in Microbiology and Tropical Parasitology (INIMYPAT) of the Program of Microbiology and Parasitology, Universidad Nacional de Trujillo (Peru).

2.2 Plant material

The collection of the plant species was carried out by the classic method of herborization. 12 kg of the *Rosmarinus officinalis* leaves were collected from Trigopampa, located at geographic coordinates with south latitude 07º53.351´, west longitude 078º34.600´, altitude 2,617 m, Otuzco province, La Libertad region, Peru, in April. On the other hand, the same amount (kg) of the *C. citratus* leaves were collected from Galindo, located at geographic coordinates with south latitude 08°4’47,08812’’, west longitude 078°5439,348´, altitude 89 m, Laredo district, Trujillo province, Peru, in February. Also, 12 kg of the *M. mollis* leaves were collected from Galindo, located at geographic coordinates with south latitude 07º53.356´, west longitude 078º34.611´, altitude 2,616 m, Otuzco province, La Libertad region, Peru, in April.

The plant material was selected from each plant species by separating the different parts to avoid mixing with each other and avoiding the inclusion of organic and inorganic residues. Then, a complete specimen of each plant was provided to the Truxillense Herbarium of the National University of Trujillo, to be deposited with the Herbarium codes N° 60304 for *R. officinalis*, N° 60305 for *C. citratus* and code N° 60306 for *M. mollis*.

2.3 Preparation of plant samples

Selected plant leaves were dried at room temperature under shade, then placed in Kraft paper bags in an oven at 40 °C for 48 hours. The dried leaves were pulverized and sieved (up to a particle size of 2.0 mm) and then properly stored in amber bottles, remaining in a place without humidity and direct light.

For the essential oil extraction, 10 kg of plant material from each plant was subjected to steam distillation for 2 hours. Then, the essential oils were purified with anhydrous sodium sulfate, received in an amber glass bottle, sealed to prevent contact with light and oxygen, and stored under refrigeration at 4°C until later use. To estimate the essential oil yield, 100 g of leaves were placed in a 1 L capacity flask and conditioned in the Clevenger equipment, proceeding to distill for 2 hours. The oil obtained was measured in the graduated tube. The essential oil yield was expressed as a volume/weight percentage (ml of essential oil per 100g of plant material) (Miranda and Cuellar 2002).

To obtain the extracts, 100 g of the dried leaves were weighed, packed in a nylon bag, and then introduced into a balloon over an electric stove. Afterward, 250 mL of different solvents of increasing polarity (hexane, ethyl acetate, ethanol, distilled water) was added to begin the extraction process. Finally, each extract was filtered through a vacuum pump and stored in an amber bottle until use (Miranda and Cuellar 2002).

2.4 Determination of total solids concentration

An aliquot of 1 mL was taken from each extract, placed on a previously weighed capsule, and then put in an oven at 40°C for 24 hours. After that time, it was introduced into the desiccator (with silica gel) to cool for 30 minutes. Calculations were made by weight difference in mg/mL. This procedure was performed in triplicate (Miranda and Cuellar 2002).

2.5 Determination of the quality parameters of the plant species

2.5.1 Determination of moisture

The loss on drying method carried out the determination of water. In a previously tared cardboard box, 100 g of the fresh vegetable drug was weighed and dried in an oven at 40 °C for 48 hours until a constant weight was obtained. After, the sample was cooled in desiccators for 30 minutes (Miranda and Cuellar 2002).

2.5.2 Determination of total ashes

The determination of complete ashes was carried out by the gravimetry method. For this, 2 g of the crushed vegetable drug was weighed in a previously tared porcelain crucible, carbonized in the kitchen, and then incinerated in a muffle oven at 700°C for 2 hours. After, the sample was cooled in a desiccator for 30 minutes (Miranda and Cuellar 2002).

2.5.3 Extraction of ethanol/water-soluble substances

The extraction was carried out by the hot extraction method. Based on the solubility of soluble substances in water and ethanol 96°GL, 5g of previously dried and crushed plant leaves were weighed in a 100 mL round-bottom flask for each system. After this, 50 mL of solvent (water and ethanol 96°GL) were added, respectively, stirred, and allowed to stand for 1 hour. A reflux condenser was attached to the flask, and the sample was subjected to a constant boil for 45 min. Then, it was quickly shaken and filtered, transferred to a flask, and refluxed again. The extracts were combined and calibrated to 100 mL. Finally, 5 mL of each extract was measured in a previously tared porcelain dish, brought to dryness in a water bath, allowed to cool in a desiccator, and weighed without delay. The procedure was performed in triplicate. In addition, the calculations were obtained by gravimetry, and the content of extractable matter was expressed in mg/g of dry vegetable matter (Miranda and Cuellar 2002).

2.6 Phytochemical screening

For the phytochemical analysis, the Draggendorff test was carried out for the determination of alkaloids, as well as the foam test, for the identification of saponins, in addition to the Ballet test (for lactones), ferric chloride test (for phenolics and tannins), Borntrager test (for quinones), Nihidrine test (for free amino acids and amines), Fehling test (for reducer sugars), Gelatin test (for tannins) and Liebermann-Burchard test, for identification of terpenes, steroids, and terpenoids (Wagner and Bladt 2004).

2.7 Chemical identification of plant extracts

The chemical identification of plant extracts was carried out by ultra-high performance liquid chromatography coupled with double mass spectrometry (UHPLC-MS/MS) analysis. In a clean and dry vial, 10 mg of the samples were placed. 10 mL of ACN-H_2O solution (8:2) was added and stirred in ultrasound equipment for 20 minutes at room temperature. The obtained solutions were diluted 10 times with the same solvent to reach 0.1 mg/mL final concentration. Chromatographic separation was achieved on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) equipped with aLuna© Omega C18 100 Å, Phenomenex (150 x 2.1 mm, 1.6µm) column. Formic acid 1% (v/v) in H_2O (A) and MeCN (B) mobile phases were used. The gradient conditions were as follows: 0-1 min 90-10% B; 1-18 min 90-10% B; 18-20 min 5-95% B; 20-25 min 5-95% B. The flow of the mobile phase was 300 µL/min, and the injection volume is of 3 µL. The column temperature was kept at 40 °C. The UV chromatograms were obtained in a range between 200 and 750 nm.

The mass spectrometer Q Exactive Plus (Thermo Scientific) was equipped with an electrospray ion (ESI) source operated in positive and negative ionization modes. The "spray voltage" was kept at 3.5-2.5KV. The drying temperature was 250-300 °C, and the sheath gas flow rate was 50 AU. Nitrogen was used as the dry, fog, and collision gas, with a heat temperature of 400 °C. The collision

energy was set at 30 eV. HRESIMS and MS/MS spectra were acquired in the m/z range of 120 to 1200 amu.

2.8 Evaluation of biocidal activity

2.8.1 Stabilization of *A. aegypti* strains in the insectary

After INIMYPAT provided the papers containing *A. aegypti* from Rio Seco (Trujillo), they were transferred to plastic sources (48.5cm x 35cm x 5cm) with dechlorinated water and placed in the larval rearing boxes (polystyrene). The feeding was made with fish food (finely crushed to powder), and placed in the dechlorinated water from the first presence of larvae. When they reached the pupal stage, they were transported to the insectary, where they were placed in the rearing cages for adults and waited until the adult emerged. Once the adults emerged, they were fed with a 5% sugar solution; the females were provided with the help of a guinea pig (with blood so that it could carry out the oviposition process). For this, the guinea pig was anesthetized with Ketamine (0.1 ml) and placed in the adult rearing cage for 45 minutes so that the females could feed. Three days after the copulation between male and female, the eggs were placed in the strips of craft paper (ovitrap), and after five days (for the embryogenesis stage), the strips were removed and stored in a box at room temperature. This process was carried out until the obtaining of F2 to carry out the bioguided tests (Brogdon and Chan 1998).

2.8.2 Bioassay in *Aedes aegypti* adults

The CDC bottle biological assay method was used. Previously washed, four 250 ml wide-mouth glass bottles (CORNING) were used for each biological assay: three for the test replicates and one for the positive control (insecticide). The extracts (hexane, ethyl acetate, ethanol, and aqueous) and essential oils from the plants described in this study were evaluated. The bottles used for the experimentation were washed with hot soapy water and rinsed at least three times thoroughly. To dry the bottles, they were placed in an oven at 50°C for 15-20 minutes until completely dry. Then, each bottle was impregnated by placing 1 ml of the solution under study from each plant to be evaluated, exposing the entire internal surface of the bottle to the solution.

This procedure was carried out in a fume hood to facilitate the solvent's evaporation and guarantee the researcher's safety when working with organic solvents. The bottles were allowed to dry before starting the tests, covering them with paper or aluminum foil to protect the formulated solution from degradation due to the effect of light. Following the steps above, the positive control bottle was impregnated with 2% malathion. Soon after, using a vacuum cleaner, 15 female mosquitoes are introduced into each bottle. The timer was activated, and the bottles were examined at Time 0; the number of dead and/or alive mosquitoes was counted every 15 minutes until the total number of mosquitoes was dead or until 2 hours had passed since the beginning (Brogdon and Chan 1998).

The criteria proposed by the World Health Organization (WHO) to assess the significance of the resistance values detected are: a) 98%–100% mortality during the recommended diagnosis period, which will indicate susceptibility in the population; b) 90%–97% mortality during the recommended diagnosis time will suggest the possibility of resistance and must be confirmed; c) <90% mortality during the recommended diagnosis time suggests resistance (WHO 2016).

2.8.3 Statistical Analysis

The data were submitted to the analysis of variance (ANOVA) and with Tukey's multiple comparison tests to determine the significant differences between the experimental and control groups with a significance level of 0.05. The InfoStat version 2018 program was used.

3 Results

3.1 Yield of essential and determination of total solids concentration

The yields of essential oils from *R. officinalis*, *C. citratus*, and *M. mollis* were 0.8, 1.0, and 0.3 % RAE (v/w), respectively. Also, the concentration of total solids in the extracts and oils was determined for each species. For *R. officinalis*, the solid concentrations in the extracts were the following: 190.03 mg/mL for the aqueous extract, 75.30 mg/mL for the ethanolic extract, and 207 µg/mL for the essential oil, while for *C. citratus*, values were: 261.23 mg/mL for the aqueous extract, 57.90 mg/mL for the ethanolic extract, and 20 µg/mL for the essential oil. For *M. mollis*, the solid concentrations were: 165.30 mg/mL for the aqueous extract, 90.30 mg/mL for the ethanolic extract, and 20 µg/mL for the essential oil (Table 1).

Table 1 Determination of the total solids concentration in the oil and extracts

Plant Species	Essential Oils	Ethanolic Extract 70° GL	Aqueous Extract
R. officinalis	20µg/mL	75.30 mg/mL	190.03 mg/mL
C. citratus	20µg/mL	57.90 mg/mL	261.23 mg/mL
M. mollis	20µg/mL	90.30 mg/mL	165.30 mg/mL

Table 2 Quality parameters of the studied plant species

Quality Parameters	Plant Species		
	R. officinalis	*C. citratus*	*M. mollis*
Water Weight Loss	52.3%	71.4%	65.3%
Total Ashes	5.32%	4.17%	7.66%
Extracted Substances	Metabolites Concentration		
H_2O	12.94 mg/ mL	21.74 mg/mL	16.86mg/mL
Etanol	11.34 mg/ mL	16.86 mg/mL	12.54mg/mL

Table 3 Phytochemical screening of the studied plant species

Assays	Metabolites	Plant Species					
		R. officinalis		*C. citratus*		*M. mollis*	
		Etanol	Water	Etanol	Water	Etanol	Water
Ferric chloride	Polyphenols	++	++	+++	+++	+++	+++
Gelatin test	Tannins	+	-	+	-	++	-
Liebermann-Burchard	Triterpenes/Steroids	-	NE	-	NE	+	NE
Borntrager	Quinones	-	NE	-	NE	NE	NE
Dragendorff	Alkaloids	-	-	-	-	++	-
Baljet	Lactones	-	NE	-	NE	-	NE
Ninhidrina	Free amines	++	++	+++	+++	+++	+++
Felhing	Reducing sugars	++	++	+++	+++	+++	+++

Here Identification: Presence (+), Absence (-); Intensity: Low (+), Moderate (++), High (+++); Not executed: (NE)

3.2 Determination of the quality parameters of the plant species

After evaluating the quality parameters of the species under study, the following results were obtained for *Rosmarinus officinalis*: water weight loss of 52.3% and total ashes equal to 5.32%. The water/ethanol soluble substances concentrations were 12.94 mg/mL (aqueous extract) and 11.34 mg/mL (ethanolic extract). For *C. citratus*, it presented a water weight loss of 71.4% and total ashes equal to 4.17 %. The concentration of water/ethanol soluble substances was 21.74 mg/mL (aqueous extract) and 16.86 mg/ml (ethanolic extract). For *M. mollis*, the water weight loss was 65.3%, and total ashes were equal to 7.66%. The concentration of water/ethanol soluble substances was 16.86 mg/mL (aqueous extract) and 12.54 mg/mL (ethanolic extract), so it can be concluded that the best extraction of secondary metabolites in both species was obtained by the aqueous extract (Table 2).

3.3 Phytochemical screening

Phytochemical screening was carried out for the three plant species. All species reported the presence of polyphenols, tannins, free amines and reducing sugars. Also, triterpenes/steroids and alkaloids were found in *M. mollis* (Table 3).

3.4 Chemical Identification by UHPLC-MS/MS

Altogether, 15 substances were identified in the ethanolic extract of *R. officinalis* (Table 4), which was shown to be composed of organic acids, flavonoids, lipid acids, and terpenoids. Among them, major reported compounds were sucrose [M-H = 341], pyroglutamic acid [M-H = 128], diethylallarate [M-H = 265], two glycosilated quercetin: quercetin-3-O-rutinoside [M-H = 609], quercetin-3-O-glucoside [M-H = 463], and three lipids: hexadecasphinganine [M+H = 274], and two isomers of heptadecasphinganine [M+H = 288] were the most common one. On the other hand, 29 substances can be identified in the extract from *C. citratus* (Table 5), in which the major compounds were sucrose, 1,3-O-di-trans-p-coumaroylglycerol [M-H = 383], 1-coumaroyl-3-feruloylglycerol [M-H = 413], as well as two lipids acids:7-Hydroxy-13,15,17-octadecatrienoic acid [M-H = 293], and 7-hydroxy-9,13-octadecadienoic acid[M-H = 295]. Moreover, 30 substances can be identified in the extract from *M. mollis* (Table 6),

Table 4 Substances identified in *R. officinalis* ethanolic extract by UHPLC-MS/MS

Substances	Rt	M-H	M+H	Main fragments (m/z)
Glutamic acid	2.12	146		128, 102, 85
Sucrose	2.16	341		179, 161, 143, 113, 101, 89, 71
Quinic acid	2.25	191		173, 127, 111
Malic acid	2.43	133		115, 72, 71
Pyroglutamic acid	2.63	128		98, 84, 82
Diethylallarate	2.73	265		179, 145
Quercetin-3-O-rutinoside	11.54	609		463, 343, 300, 271, 178
Quercetin-3-O-glucoside	11.90	463		300, 271, 178
Kaempferol-rutinoside	12.06	593		285, 255
Quercitrin	12.54	447		300, 271, 178
Hexadecasphinganine	16.15		274	256, 230, 106
Heptadecasphinganine isomer 1	16.68		288	270, 106
Heptadecasphinganine isomer 2	16.85		288	270, 106
9-Octadecenamide	17.62	282		265, 247, 149, 135, 121
Gingerol	17.70	293		236, 221

Rt: Retention time

Table 5 Substances identified in *C. citratus* ethanolic extract by UHPLC-MS/MS

Substances	Rt	M-H	Main fragments (m/z)
Sucrose	2.16	341	179, 161, 143, 113, 101, 89, 71
Shikimicacid	2.46	173	155, 137, 129, 111
Pyroglutamic acid	2.63	128	98, 84, 82
Aesculetin	11.41	177	135, 105, 89
Caffeic acid	11.53	179	135, 107
2”-O-Rhamnosyl- 6-C-Glucosyl luteolin	11.90	593	473, 447, 429, 327, 285
Apigenin-pentosyl-glucose	11.91	563	503, 425, 399, 298
6-C-Glucosyl luteolin	12.08	447	327, 285
Apigenin-rhamnosyl-glucose	12.52	577	415, 323, 293, 269
Luteolin 7-neohesperidoside	12.81	593	447, 285
Apigenin-8-C-glucoside-2'-rhamnoside	12.86	577	415, 311, 298, 269
6-C-Pentosyl-8-C-hexosyl apigenin	12.90	563	473, 417, 399, 298
7-C-Glucosyl luteolin	13.06	447	327, 285
Trans-coumaric acid	13.20	163	119, 93
Ferulic acid	13.61	193	178, 149, 134
3',6'-diferuloylsucrose	14.29	693	517, 337, 193
Acetyl-3',6'-diferuloylsucrose	14.97	735	559, 337, 193
Acetyl-3',6'-diferuloylsucrose isomer	15.18	735	559, 337, 193
8-C-rhamnoside apigenin	15.24	415	311, 253
6-C-rhamnoside apigenin	15.73	415	397, 353, 311. 253
Luteolin	15.87	285	175, 151
9,12,13-Trihydroxy-10-octadecenoic acid	16.72	329	311, 139
1,3-O-di-trans-p-Coumaroylglycerol	16,99	383	219, 163, 119
1-Coumaroyl-3-feruloylglycerol	17.15	413	398, 249, 219, 163, 119
Diferuloylglycerol	17.27	443	428, 249, 193, 175, 149, 134
16-Hydroxy-9-oxo-10,12,14-octadecatrienoic acid	18.46	307	289, 235, 211, 199, 185
12,13-Dihydroxy-9-octadecenoic acid	20.18	313	295, 277, 201, 171, 155, 127
7-Hydroxy-13,15,17-octadecatrienoic acid	21.53	293	275, 256, 223, 195, 177, 111
7-hydroxy-9,13-octadecadienoic acid	22.28	295	277, 259, 193, 183, 171, 113

Rt: Retention time

Table 6 Substances identified in *M. mollis* ethanolic extract by UHPLC-MS/MS

Substances	Rt	M-H	M+H	Main fragments (m/z)
Mannitol	2.18	181		163, 149, 131, 119, 101, 89, 71, 59
Quinic acid	2.30	191		173, 127, 111
Shikimic acid	2.46	173		155, 137, 129, 111
Malic acid	2.54	133		115, 72, 71
Caffeoylquinic acid	6.75	353		191, 179, 173, 161, 135, 111, 93
Caffeic acid	11.79	179		135, 107
Quercetin-3-O-rutinoside	12.27	609		300, 271, 178
Kaempferol-3-O-rutinoside	12.83	593		447, 285, 255
Naringenin-7-O-rutinoside	12.92	579		271, 227, 175, 151
Isoquercitrin	13.22	463		300, 271, 178
Naringenin-4´-O-rutinoside	13.44	579		271, 227, 175, 151
Kaempferol-3-O-glucoside	13.57	447		284, 255
Naringenin-7-O-glucoside	13.91	433		271, 227, 175, 151
Rosmarinic acid	14.33	359		197, 179
Isosakuranetin-7-O-rutinoside	14.90	639 [M-H+FA]		285, 226
Isosakuranin	15.75	447		285, 270, 241, 196
Chalconosakuranetin	15.83	493 [M-H+FA]		285, 179, 161, 135
Tricoumaroylspermidine	16.09	582		436, 342, 316, 145
9,12,13-Trihydroxy-10,15-octadecadienoic acid	16.25	327		309, 291, 137
9,12,13-Trihydroxy-10-octadecenoic acid	16.78	329		311, 139
Naringenin	16.94	271		227, 151, 107
Hesperetin	17.24	301		286, 257, 242
Irigenin	17.83	359		344, 329, 314, 193
2-Amino-1,3-heptadecanediol	18.50		288	270, 106, 88, 70
Eupatilin	19.14	343		328, 313, 298, 270
Isosakuranetin	19.26	285		270, 243, 151
Citronellicacid	20.01		153	135, 109, 107, 93, 81
Gardenin B	21.66		359	344, 329, 298, 135
3-Hydroxy-11-ursen-28,13-olide	22.44		455	437, 409, 391, 219
Enoxolone	22.73	471		425, 407, 271, 217

Rt: Retention time

in which the major compounds were isosakuranetin-7-O-rutinoside [M-H+FA = 639], naringenin [M-H = 271], isosakuranetin [M-H = 285], and citronellic acid [M+H = 153].

3.5 Evaluation of biocidal activity

The analysis of variance (ANOVA) reported that the analyzed data are statistically significant $p<0.05$, which establishes that there are differences between the means of the percent mortality (biocidal effect) using each plant extract, essential oil, or positive control against adults of *A. aegypti*. The biocidal activity evaluation on *A. aegypti* showed that all the essential oils caused 100% mortality of *A. aegypti* adults within 105 minutes and above 90% mortality in 30 minutes, being more efficient than all the extracts evaluated.

The statistically significant difference was determined between groups; the Tukey statistical test was used, where it was reported a $p=0.9999$ between the essential oils and the positive control (2% malathion). On the other hand, the comparison between aqueous and

ethanolic extracts reported a *p*-value above 0.9732. In all cases, the significance level was more significant than p=0.05 of standard significance, which in turn means that these extracts have comparable efficacy since there is no statistically significant difference in their biocidal effect against adults of *A. aegypti*. All ethanolic extracts caused 100% mortality in 105 min. However, the aqueous extracts of *R. officinalis* and *C. citratus* caused observed mortality in shorter periods than ethanolic extracts (30 min versus 45 min for *R. officinalis* and 15 min versus 45 min for *C. citratus*, respectively) (Figures 1, 2, and 3).

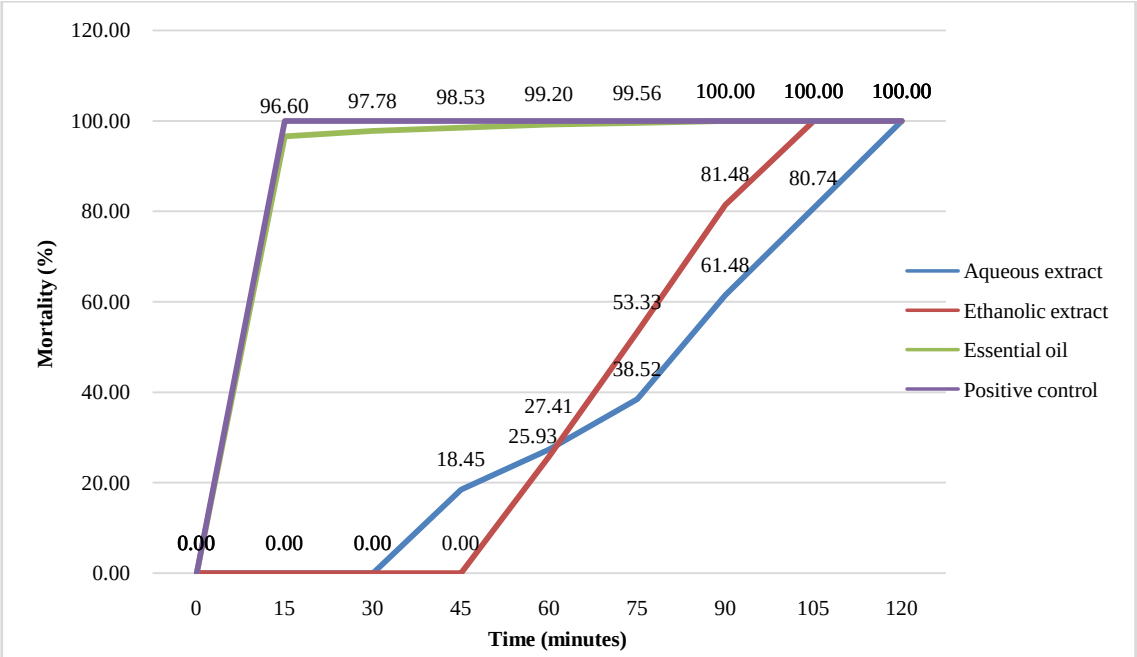

Figure 1 Percentage of mortality of adults of *A. aegypti* exposed to different extracts and oils from *R. officinalis* and 2% malathion (positive control)

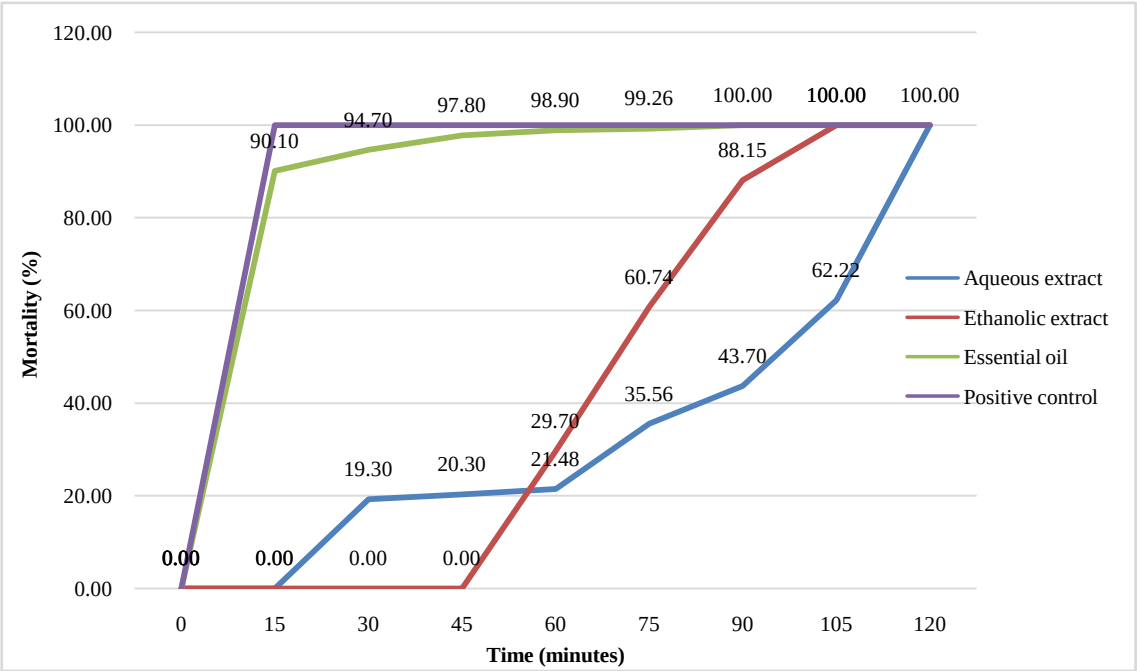

Figure 2 Percentage of mortality of adults of *A. aegypti* exposed to different extracts and oils from *C. citratus* and 2% malathion (positive control)

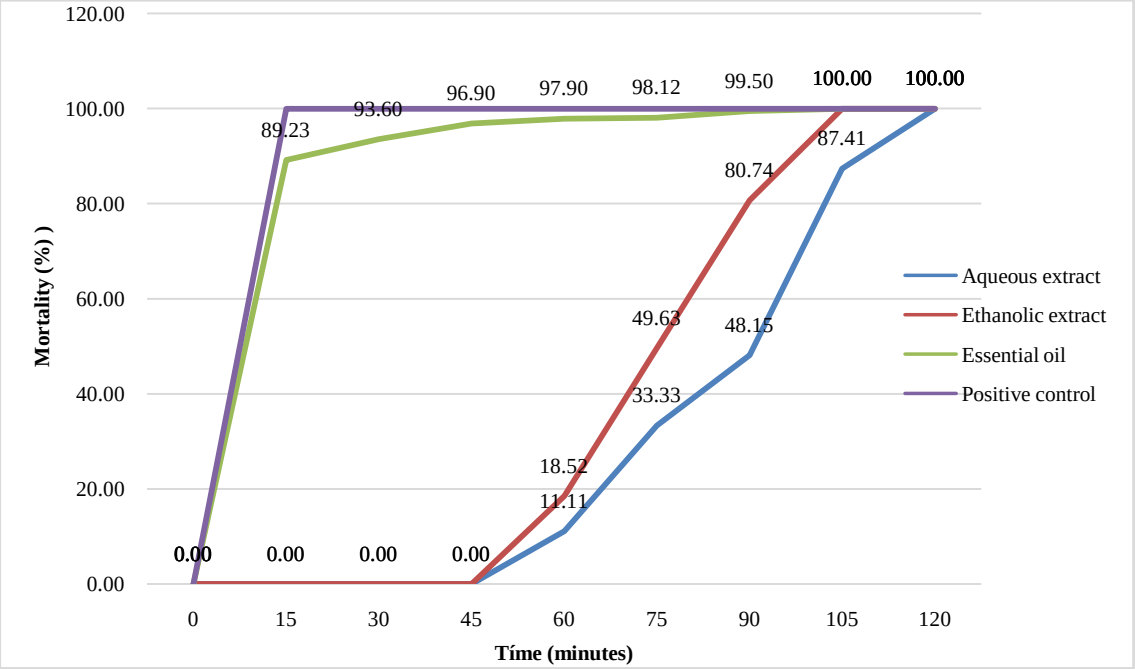

Figure 3 Percentage of mortality of adults of *A. aegypti* exposed to different extracts and oils from *M. mollis* and 2% malathion (positive control)

4 Discussion

The plant species *R. officinalis* yielded 0.8% of the fresh leaves' essential oil. The literature indicates that the yield of essential leaf oil is 0.61% for the tissue in the fresh state and 0.98% for the dry tissue, so compared to the result obtained, the value found in this work is within the expected range. It has been shown that the main components of the essential oil of *R. officinalis* are camphor (5.0-21%), 1,8-cineole (15-55%), α-pinene (9.0-26%), borneol (1.5-5.0%), camphene (2.5–12%), β-pinene (2.0–9.0%) and limonene (1.5–5.0%) in proportions that vary according to the vegetative stage and bioclimatic conditions (López 2008; Andrade et al. 2018). In turn, *C. citratus* presented a yield of 1.0% for the essential oil from its fresh leaves. The literature reports that the fresh plant of *C. citratus* provides essential oil between 0.5 – 0.7%, and the obtained oil is of a transparent yellow liquid with a citrus scent. These results agree with the findings of Rodriguez et al. (2006), those who received 0.7% of essential oil from this plant species collected from the town of Trujillo, included in the same region mentioned in this study (Rodriguez et al. 2006). Compared with the yield obtained, the plant species provides a high percentage of essential oil. This could be due to different agroclimatic factors such as the salinity of the soil, altitude, and water content, which in turn could also influence the quality and quantity of essential oils (Ekpenyong et al. 2014). According to different studies with *C. citratus*, essential oils, geranial, neral, and myrcene were the main components of the species (Soto-Ortiz et al. 2002; Rodriguez et al. 2006; Gbenou et al. 2013). On the other hand, the yield of the essential oil obtained from the fresh leaves of *M. mollis* was 0.3 %, which is in agreement with the essential oil ranges (0.25 - 4.93%) reported in the literature. The predominant components of essential oil are pulgeone and menthone. This includes a study by Orbegozo and Rodriguez (2018), which obtained 0.6% v/w in a species collected in the same region (Santiago de Chuco, La Libertad). All the essential oil values obtained in this study are within the acceptable ranges, and the place of origin was found to have a slight effect on this parameter (Van Baren et al. 2014; Orbegozo and Rodriguez 2018). In addition, *M. mollis* is very heterogeneous in its genetics and morphology, and other abiotic factors previously mentioned can influence the composition of the essential oil (Linares 2020).

Furthermore, the values found for the quality parameters of three species studied in this work proved to be within the range recommended by the reference documents in Peru (Villar 1999; Miranda 2002; British Pharmacopoeia 2022;). In addition, the study of Orbegozo and Rodriguez (2018) demonstrated the value of 7.58 % of total ashes in a sample of *M. mollis* from the La Libertad region, corroborating the result described in this work.

The study determining the concentration of solids in extracts and oils demonstrated greater water efficiency in concentrating the metabolites in all the species evaluated. The quantitative and qualitative yield of the extraction depends on the nature of the extracted compounds and the solvent used, as well as on factors such as the nature of the sample, concentration of the solvent,

temperature, contact time, particle size, and mass-solvent ratio among others (Soto and Rosales 2016).

In the chemical identification by UHPLC-MS/MS, 15 substances were identified for *R. officinalis*, whereas 29 compounds were for *C. citratus* and 30 for *M. mollis*. Flavonoids, mainly querecetin compounds, besides organic acids, sugars, lipids, and gingerol, a phenolic constituent, were the main substances identified in *R. officinalis*; these results agree with the previous literature of this species (Achour et al. 2017; Mena et al. 2020). In the *C. citratus* analysis, phenolic acids like caffeic, ferulic, and coumaric acid conjugates, as well as the flavonoids apigenin and luteolin and lipid acids were the principal bioactive constituents and are also found in other works as species markers (Sousa et al. 2021; Bhaskar et al. 2021). Moreover, the chemical analysis of *M. mollis* extracts showed the presence of flavonoids, including isosakuranetin and naringenin, as major compounds, besides phenolic acids and terpenoids, which in turn are characteristics of the genus *Minthostachys* (Faraone et al. 2021).

The lethality of essential oils can vary greatly, depending on factors such as chemical composition, plant species, part of the plant extracted, maturity, and extraction method (Isman 2020). Essential oils are a mixture of various chemical components that produce different toxic effects on the insect's organism, so it is likely that *A. aegypti* cannot easily develop an adaptation that leads to resistance (Wahyuni 2012; Dias and Moraes 2014). Many studies have shown the neurotoxic actions of essential oils, causing paralysis followed by death, in insects. This characteristic allows considering the components of essential oils as potential bioinsecticides. This mechanism might occur through the inhibition of acetylcholinesterase (AChE), or triggering effects similar to those produced by conventional insecticides such as temephos or organophosphate cholinesterase inhibitors used in vector-borne disease control programs (Kostyukowsky et al. 2002; WHO 2009). Another mechanism is through the octopaminergic system. Available data showed that essential oils could increase the level of cAMP and calcium in nerve cells. Therefore, essential oils are interesting bioinsecticide candidates (Jankowska et al. 2018). Furthermore, the literature revealed that essential oils rich in phenylpropanoids, oxygenated sesquiterpenes, and monoterpene hydrocarbons have significant larvicidal activity against *A. aegypti* (Dias and Moraes 2014). According to Rodriguez et al. (2006), the main compounds responsible for insecticidal activity are of low polarity (Rodriguez et al. 2006).

Regarding this point, Isman (2000) indicates that monoterpenes and phenylpropanoids were the most identified compounds in essential oils, extracts, and purified fractions, which stand out in larvicidal activity (Isman 2000). In addition, to postulate that the toxicity of these phytometabolites may be associated with their non-polar property, which increases the ability of the compound to penetrate the hydrophobic cuticle of the larvae and enhances its harmful effect on the insect compared to polar compounds (Vincent and Wegst 2004). Scientific evidence showed that the essential oils of *R. officinalis*, *C. citratus*, and *M. mollis* have insect repellent, insecticide, larvicide, adulticide, ovicide, and oviposition dissuasive activities, being applied in the control of pathogens and insects (Prajapati et al. 2005; Gillij et al. 2008; Waliwitya et al. 2009; Vera et al. 2014; Soonwera and Phasomkusolsil 2016; Alegre 2016; Azeem et al. 2019; Oladeji et al. 2019; Soonwera and Sittichok, 2020;). Moreover, the components found in these essential oils, such as pulegone, menthone, thymol, eugenol, trans-anetol, and citronellal have shown high larvicidal activity (Waliwitya et al. 2009).

The insecticidal activity of the extracts from *R. officinalis* is probably related to the presence of quercetin, kaempferol conjugates, and gingerol. Quercetin possesses an antifeedant effect against *A. aegypti*. The proposed mechanism suggests that quercetin oxidation by larvae generates reactive oxygen species, which in turn can degrade the nutritional quality of food present in the gut lumen of the insects. In addition, quercetin also inhibits transhydrogenase activity, negatively impacting their development and therefore leading them to death (Pessoa et al. 2018). Also, kaempferol inhibits the ecdysone 20-monooxygenase enzyme in females, suppressing their oviposition (Mitchell et al. 1993), and gingerol is a growth insect regulator and has antifeedant activity (Agarwal et al. 2001). In turn, the activity of *C. citratus* extracts can be explained by the presence of apigenin conjugates that share the exact mechanism of action with kaempferol and by the high content of phenolic acids that can inhibit insect acetylcholinesterase (Maazoun et al. 2017). Also, the presence of caffeic acid and rosmarinic acid in *M. mollis* extracts acts on the reduction of insect herbivory in several ways, such as reducing fertility, discouraging feeding, oviposition, and shortening the insect life span (Isman 2006; Dawkar et al. 2013). This effect is added to the mechanisms related to flavonoids and kaempferol conjugates and acid triterpenes (enoxolone and 3-Hydroxy-11-ursen-28,13-olide), which in turn provide antifeedant action in insects (González-Coloma et al. 2011).

In sum, the essential oils and extracts from the three species, found mainly in Peru, provide a promising source for insecticidal applications due to their significant biocidal activity and possible toxicological safety for mammals and the environment since they are more easily degraded by natural ecosystem mechanisms (Bhatt et al. 2013).

Conclusions

The research demonstrated the insecticidal activity of essential oils and extracts of the plant Peruvian resources *R. officinalis*, *C. citratus*, and *M. mollis* against *A. aegypti* adults. The study also

determined the chemical profile of ethanolic extracts from three species, including 15 substances for R. officinalis, 29 for C. citratus, and 30 for *M. mollis*. Furthermore, the study of quality parameters for the essential oil and extracts indicated that they agree with recommended quality standards so that these phytoproducts can be seen as more eco-friendly biological controllers of *A. aegypti* when compared with synthetic insecticides.

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Conflict of Interest

The authors have no conflict of interests.

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In silico targeting enterotoxin from *Staphylococcus aureus* with selected flavonoids: Hope for the discovery of natural anti-mastitis agents

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ABSTRACT

Staphylococcus aureus is a facultative anaerobe and catalase-positive bacterium responsible for various skin infections and life-threatening problems, including bacteremia and pneumonia. This bacterium produces a bunch of superantigens in the blood called enterotoxin. This toxin is responsible for food poisoning and toxic shock syndrome. Moreover, Bovine mastitis is also associated with *S. aureus*. Further, *S. aureus* related to drug resistance makes the infection more dreadful. Now a day, various natural compounds such as phytochemicals are gaining importance as they are effective against many diseases, including *S. aureus* infections. The present study used molecular docking of three ligands, i.e., Kaempferol, Apigenin, and Quercetin, with enterotoxin A from *S. aureus*. The docking study revealed that the binding energy of ligands with receptors was -6.6 to -6.9 Kcal/mol. Kaempferol had the highest binding affinity of -6.9 Kcal/mol, suggesting it has a potential against *S. aureus*. Therefore, in the current research, we have tried to identify occurring compounds that might be used to develop an effective anti-*S.aureus* agent. The findings are encouraging and will aid researchers in creating new mastitis-fighting medications based on natural phytochemicals.

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1 Introduction

Gram-positive *Staphylococcus aureus* (*S. aureus*) bacteria can cause a superficial skin infection to severe illnesses like necrotizing pneumonia and bacteremia (Cheung et al. 2021). This bacterium can be detected in the normal skin microbiota of humans and animals, and healthy individuals carry it at 20-30% (Guo et al. 2020). *S. aureus*-caused bovine mastitis has cost the cattle breeding industry money due to decreased milk production and quality, higher culling and mortality rates, and other factors (Deb et al. 2013; Chakraborty et al. 2019). 30% of bovine mastitis is subclinical mastitis driven by *S. aureus* (Halasa et al. 2007; Sharun et al. 2021). According to studies, *S. aureus* infections resulted in the loss of 380 tonnes of milk annually worldwide (Loiselle et al. 2009; Zhou et al. 2018). Because of the high morbidity and antibiotic resistance prevalence, *S. aureus* infection is one of the most concerning infections for researchers and physicians. Antibiotic-resistant infections have been projected to have topped 10 million annual deaths, and by 2050, they will outnumber cancer deaths (Ahmad-Mansour et al. 2021). They showed high resistance to the majority of protein-degrading enzymes and hence continue to operate in the digestive tract after consumption. Developing an impressive list of protein toxins is crucial to *S. aureus* pathogenicity (Cassat and Thomsen 2021). These can function alone or in combination to induce various human disorders. Short-secreted proteins soluble in saline solutions and water are known as enterotoxins (Hennekinne et al. 2012). These enterotoxins share structural and biochemical characteristics and are heat resistant. Pneumonia, toxic shock syndrome, sepsis-related infections, and food poisoning are only a few of the most common disorders linked to enterotoxins (Lin and Peterson 2010). Many recent studies have suggested that staphylococcal enterotoxins (SEs) showed an important role in the presentation of other human diseases, such as respiratory tract diseases (Huvenne et al. 2013) and autoimmune disorders (Li et al. 2015). The enterotoxins of S. *aureus* are potent non-specific T-cell stimulators (superantigens) that lead to an uncontrolled immunological response (Lin et al. 2010). A large cytokine load produces toxic shock syndrome, characterized by fever, organ dysfunction, and significant mortality (Goda et al. 2021). Compared to other *S. aureus* toxins, enterotoxins require only a small amount to be dangerous to humans. As a result, this enterotoxin could be used to identify new therapeutic candidates to treat *S. aureus* infections (Goda et al. 2021). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which have become epidemic in several countries, are representative of these challenges (Harkins et al. 2017). *S. aureus* is the most common cause of nosocomial and community-acquired bacterial infection of the blood, skin, soft tissue, and other locations in the United States, with MRSA strains accounting for the vast majority in many areas (Rose et al., 2021). Treatment includes antibiotics such as daptomycin, linezolid, nafcillin, and oxacillin. In previous literature, researchers have done insilico studies and showed that enterotoxin A, TSST-1, exfoliative toxin A, and γ-hemolysin can interact with many drugs and proved that these toxins can be drug target (Mohana and Venugopal 2017). Selvaraj (2020) showed that nafcillin analogues interact with enterotoxin I inhibits *S. aureus* growth by using docking analysis (Selvaraj 2020). As a result, molecular docking research is being used to investigate the molecular interactions of Apigenin, quercetin and kaempferolwith enterotoxin I. The study's objective is to conduct molecular docking to assess the effectiveness of natural flavonoids against the enterotoxin I receptor on *S. aureus* in mastitis.

2 Materials and Methods

2.1 Preparation of Enterotoxin 3-D structure for docking

An online resource, RCSB-PDB, was used to acquire the three-dimensional structure of the receptor protein enterotoxin A from *S. aureus* with PDB ID IESF and a resolution of 1.9 Å. Water molecules and heteroatoms from receptor proteins were disallowed during docking.

2.2 Ligand library preparation and analysis of physiochemical properties

Apigenin, quercetin, and kaempferol, three phytochemicals chosen for docking experiments, had their three-dimensional structures retrieved in sdf format from PubChem (Figure 1). An online tool examined ligand absorption, distribution, metabolism, and elimination at a pH of 7 (Jayaram et al., 2012). Lipinski's rule of five was used to determine whether a chemical compound possesses the chemical and physical properties that would make it likely to be an orally active medication in humans (Lipinski, 2004). Physiochemical properties, which include LogP (<5), molecular weight (m.w.) (<500 Da), H-bond donor (5), molar refractivity, H-bond acceptor (<10), and drug likeliness were listed in Table 1.

Table 1 Physiochemical Properties of ligands

ADME Properties	Apigenin	Quercetin	Kaempferol
Molecular weight (<500 Da)	270	302	286
LogP (<5)	2.4	2.01	2.3
H-bond donor (5)	3		4
H-bond acceptor (<10)	5	7	6
Molar Refractivity	70.8	74.0	72.3

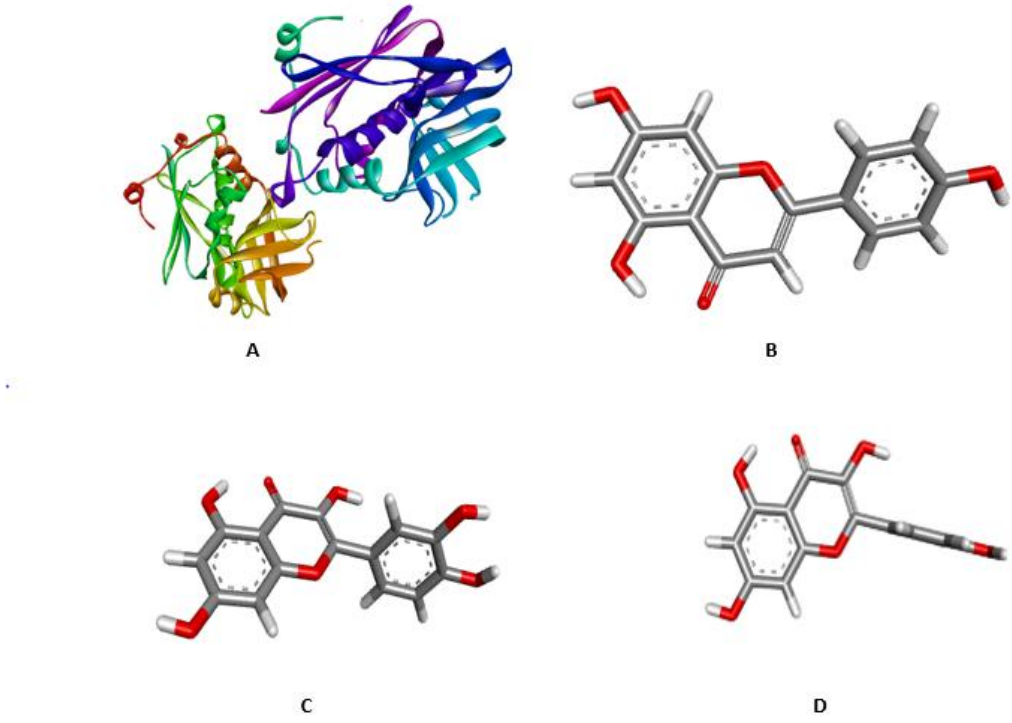


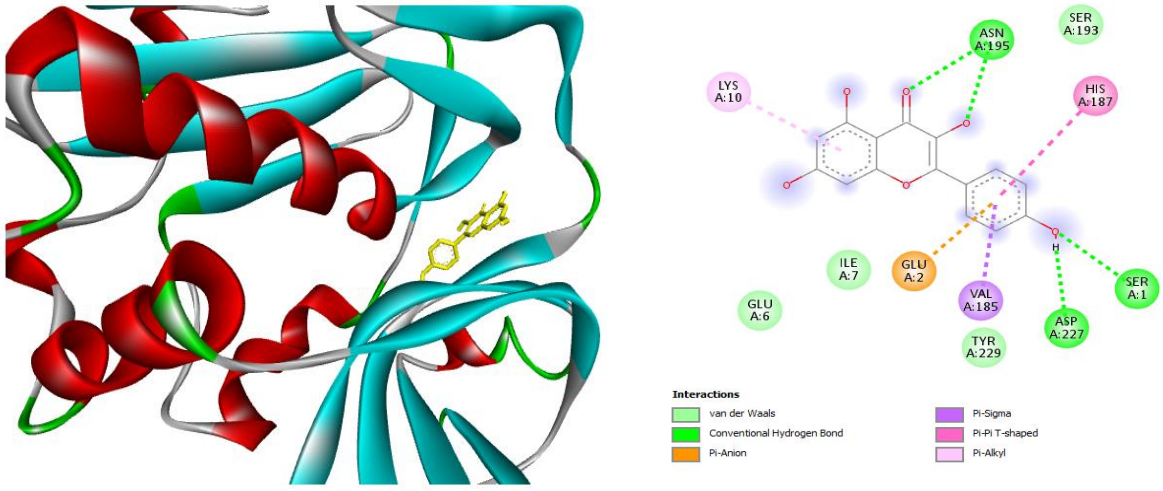
Figure 1 3-D structure of receptor and ligands; Enterotoxin A (A), Kaempferol (B), Apigenin (C) and Quercetin (D)

2.3 Computational Docking of ligands with Enterotoxin A from *Staphylococcus aureus*

PyRx v0.8 (utilizes Auto Dock vina) was used for molecular docking studies. Energy minimization of selected ligands was done by applying Universal Force Field (UFF), and ligand structure format was converted to pdbqt format via OpenBabel (O'Boyle et al., 2011). Docking studies were done as blind docking with an exhaustiveness value of 10. Discovery Studio Visualizer studied molecules that have a high binding affinity.

3 Results and Discussion

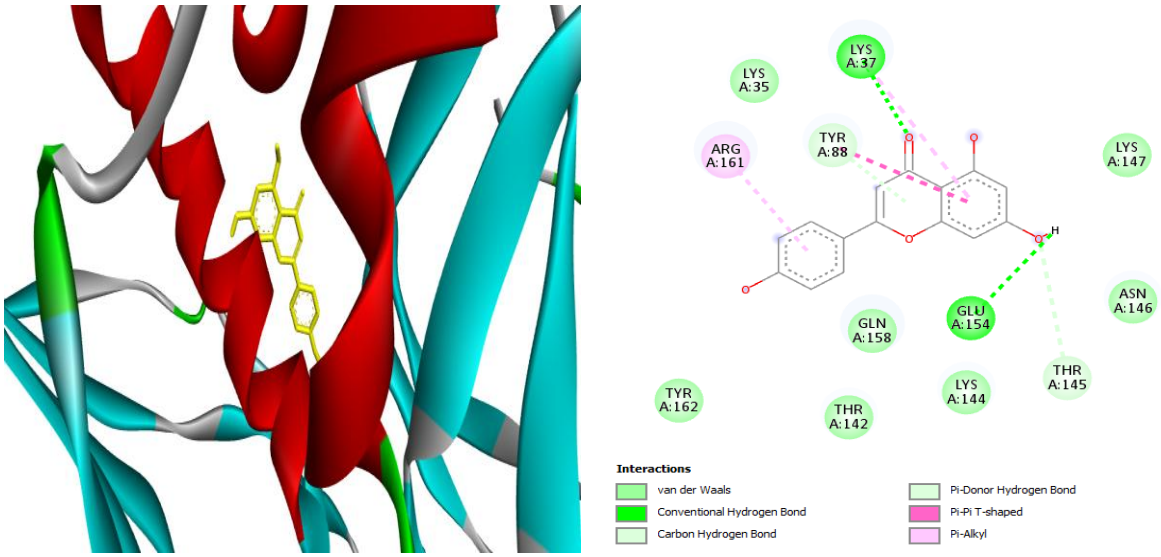
Recently, computational docking has gained popularity as a tool for drug creation (Tuli et al., 2021a; Tuli et al., 2021b; Tuli et al. 2022). *In silico* docking is cost-effective and relatively takes less



(A) **(B)**

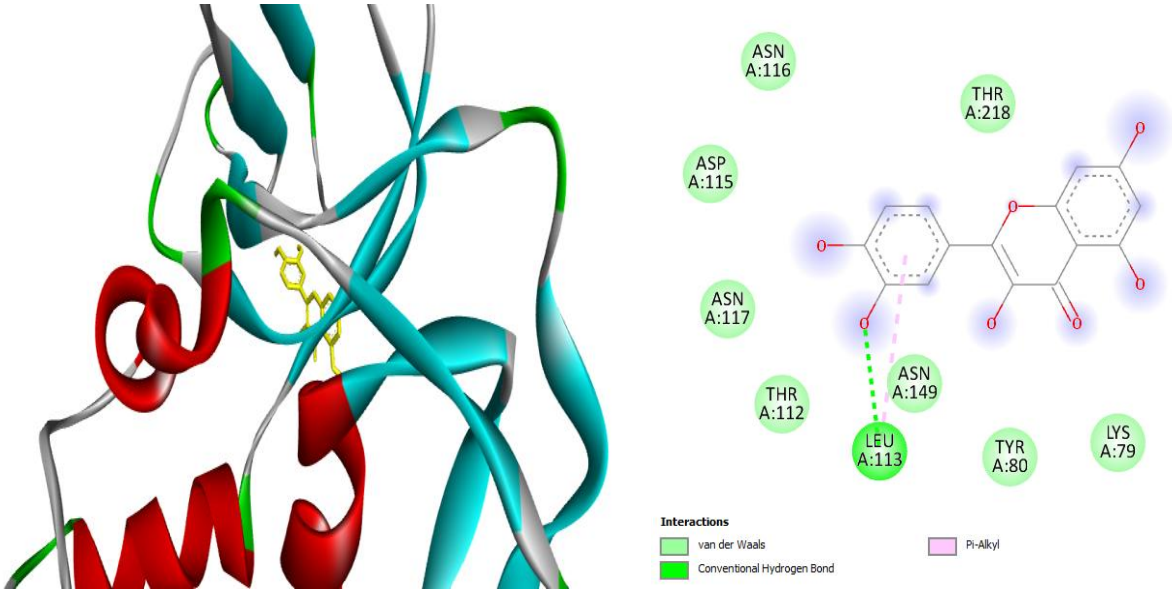
Figure 2 Binding of enterotoxin A from *S. aureus* and Kaempferol. Best interaction position in the receptor protein's pocket (A); 2-D image of interacting residues of receptor protein with ligand (B)

time for drug designing. In virtual screening by PyRx, the binding affinity score of the receptor-ligand complex was calculated, and it was reported that the higher the binding affinity of a molecule, have higher the stability of the molecule. In the present study, docking of enterotoxin and ligands (Kaempferol, Apigenin, and Quercetin) was done. According to docking research, the binding affinities of kaempferol, apigenin, and quercetin to the receptor molecule were between -6.9 and -6.6 Kcal/mol. With enterotoxin, kaempferol had the most binding affinity, measuring -6.9 Kcal/mol. Kaempferol forms hydrogen bonds via Asn195 (two H-bonds), ser1, and Asp227 residues with receptor molecules (Figure 2).Further, enterotoxin interacts with apigenin with a binding affinity of -6.7 Kcal/mol via two hydrogen bonds with Lys37 and Tyr88 residues (Figure 3). In silico studies revealed that Quercetin forms one hydrogen bond via Leu113 with receptor molecule having a binding affinity of -6.6 Kcal/mol. Binding affinity with different RMSD value are mentioned in Table 2 and figure 4.



(A) **(B)**

Figure 3 Binding of enterotoxin A from *S. aureus* and Apigenin. Best interaction position in the receptor protein's pocket (A); 2-D image of interacting residues of receptor protein with ligand (B).



(A) **(B)**

Figure 4 Binding of enterotoxin A from *S. aureus* and Quercetin. Best interaction position in the receptor protein's pocket (A); 2-D image of interacting residues of receptor protein with ligand (B).

Table 2 Binding affinity of Kaempferol, Apigenin, Quercetin, and RMSD value in 10 different modes

Ligand	Binding Affinity (ΔG Kcal/mol)	rmsd/ub	rmsd/lb
Kaempferol			
Kaempferol_5280863_uff_E=362.50	-6.9	0	0
Kaempferol_5280863_uff_E=362.51	-6.6	23.183	21.137
Kaempferol_5280863_uff_E=362.52	-6.5	27.186	24.641
Kaempferol_5280863_uff_E=362.53	-6.3	32.493	31.336
Kaempferol_5280863_uff_E=362.54	-6.3	36.575	34.532
Kaempferol_5280863_uff_E=362.55	-6.3	33.563	31.742
Kaempferol_5280863_uff_E=362.56	-6.3	26.532	24.765
Kaempferol_5280863_uff_E=362.57	-6.2	32.737	30.996
Kaempferol_5280863_uff_E=362.58	-6.2	23.749	21.029
Apigenin			
Apigenin_5280443_uff_E=233.26	-6.7	0	0
Apigenin_5280443_uff_E=233.27	-6.6	20.623	18.599
Apigenin_5280443_uff_E=233.28	-6.5	21.293	17.805
Apigenin_5280443_uff_E=233.29	-6.4	27.244	24.783
Apigenin_5280443_uff_E=233.30	-6.2	15.229	13.34
Apigenin_5280443_uff_E=233.31	-6.2	19.845	18.173
Apigenin_5280443_uff_E=233.32	-6.2	25.921	23.913
Apigenin_5280443_uff_E=233.33	-6.1	20.391	17.962
Apigenin_5280443_uff_E=233.34	-6	6.63	1.642
Quercetin			
Quercetin_5280343_uff_E=380.43	-6.6	0	0
Quercetin_5280343_uff_E=380.44	-6.6	21.064	18.067
Quercetin_5280343_uff_E=380.45	-6.5	7.014	2.239
Quercetin_5280343_uff_E=380.46	-6.5	29.101	27.426
Quercetin_5280343_uff_E=380.47	-6.4	28.757	28.019
Quercetin_5280343_uff_E=380.48	-6.3	7.126	2.313
Quercetin_5280343_uff_E=380.49	-6.3	20.823	18.296
Quercetin_5280343_uff_E=380.50	-6.3	28.672	27.76
Quercetin_5280343_uff_E=380.51	-6.1	26.008	23.263

Previous studies reported docking of nafcillin analogues with enterotoxin I from *S. aureus*. It was shown that Thr74 and Asn15 residues of enterotoxin interacted to form hydrogen bonding (Selvaraj 2020). Kurjog et al. (2018) reported that natural antitoxin compounds such as 28-Norolean-12-en-3-one and Betulin might give an effective treatment against *S.aureus* (Kurjogi et al. 2018). Moreover, phytochemicals extracted from medicinal plants might act as potential drugs against diseases like Covid-19 and typhoid (Bansal et al. 2022; Tuli et al. 2022). Somehow, our results agree with the previous study of Emran et al. (2015) those who reported luteolin from *Bacopa monnier* had the highest binding score for penicillin-binding protein from *S.aureus* (Emran et al. 2015). Protohypericin, Berbamine, Hypericin, Galangin, and Berberine were the best active compounds against *S. aureus* (Dorcheh et al.

2022). Shidiki and Vyas (2022) reported that taxifolin compounds and diferulic acid were potential inhibitors against *S. aureus* (Shidiki and Vyas 2022). It has been in silico proven that chrysin and luteolin have the highest efficiency for receptors of enterotoxin A of *S. aureus* (Kumar et al. 2022).

Conclusion

Staphylococcus aureus is a facultative anaerobe causing pus-foaming skin infections in humans. In the current study, natural phytochemicals were docked in-silico with enterotoxin A *S. aureus*. The binding affinity of studied ligands, i.e., Kaempferol, Apigenin, and Quercetin were found to be in the -6.6 to -6.9 Kcal/mol range. Ligands used in the current study follow Lipinsik's rule of five and may be used as *anti-staphylococcus aureus* agents. Before using these drugs as anti-*Staphylococcus aureus* agents, *in-vitro* and *in-vivo* studies are suggested to carry out against *S.aureus* infection. If it is proven that the phytochemicals mentioned above can be effective against *S. aureus*, they can be used to treat diseases caused by *S. aureus*.

Conflict of Interest

There are no conflicts of interest among the authors.

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Effect of culture medium composition on somatic embryos induction and maturation of pineapple [*Ananas comosus* (L.) var. (Smooth Cayenne)]

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ABSTRACT

The cultivation of pineapple contributes 1.6% of the gross Ivorian national product (GDP). However, this crop is facing a severe production crisis due to the aging of the orchards. Revising this sector requires the rejuvenation of orchards with healthy and improved planting material. This work was conducted to study the conditions for the efficient in vitro production of restorative pineapple planting material by somatic embryogenesis. The effects of seven culture media consisting of a different combination of nitrogen sources (casein hydrolyzate, glutamine, and glycine), cytokinins (kinetin or BAP), and auxins (2,4-D or picloram) were tested on somatic embryos induction and maturation in pineapple. Results of the study revealed that EIM_1 (EIM added with 3 $mg.L^{-1}$ picloram, 0.05 $mg.L^{-1}$BAP, 2 $mg.L^{-1}$ glycine, 1000 $mg.L^{-1}$glutamine, 100 $mg.L^{-1}$casein hydrolyzate) and EIM_5 (EIM added with 2 $mg.L^{-1}$glycine, 100 $mg.L^{-1}$casein hydrolyzate, 0.2 $mg.L^{-1}$kinetin) media induced the highest numbers of embryogenic cells, i.e., 154 and 149 cells respectively. Further, the EIM_5 medium was more embryogenic, with the most significant number of mature embryos (66 mature embryos), and allowed the observation of all embryonic maturation stages. Embryogenic cell induction in pineapple is thought to be controlled by a low NH_4^+/NO_3^- ratio in interactions with phytohormones. In the presence of 2,4-D, embryogenic cell maturation was improved by kinetin addition to the culture medium containing glycine and casein hydrolyzate.

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1 Introduction

Pineapple, originating from America, is eaten fresh or used for producing canned foods (Dick et al. 2015; Lucas 2020). It ranks third among tropical fruits, after banana and mango, with an average production of approximately 30 million tons (FAO 2020). In Côte d'Ivoire, fresh pineapple exports generate more than $223 million annually, and its cultivation contributes 1.6% to agricultural GDP (Nouza 2011; Anonymous 2022). The smooth cayenne is the main cultivated variety in Côte d'Ivoire because of its adaptation to climatic conditions (Leal and Coppens d'Eeckenbrugge 1996). Indeed, the smooth cayenne has been a pillar of the international pineapple trade. Côte d'Ivoire was the first pineapple supplier to the European Union because of the hegemony of the smooth cayenne (97% of the market). However, Ivorian production has steadily decreased since the end of the 1980s (Vagneron et al. 2009; Africa 24, 2022). For example, the production was 238,000 tons in 2000, dropping to less than 50,000 tons in 2021, revealing a drastic drop of more than 79% (Houessionon 2022). The degeneration of the plant material can explain this decrease. To overcome this difficulty, producers resort to higher than regular doses of phytosanitary products such as fibrophos (400 kg/ha), dolomite (500-750 kg/ha), kieserite (500 kg/ha), NPK (200 kg/ha), urea (1 g/plant), and potassium sulfate (2.5 g). Excess supply of these phytosanitary products causes an accumulation of chemical residues in the fruit beyond the maximum residue limit (for example: ethoprophos 0.01 mg/kg; fosetyl alumin 15 mg/kg; ethephon 2ppm) and makes the fruits more acidic. This is associated with the drop in exports from Côte d'Ivoire to the European market. Today, Côte d'Ivoire shares less than 2% in the European market (EUROSTAT 2014). In this context, the renewal of orchards with high-performance varieties is essential for improving smooth cayenne's fruit quality and yield. Thus, varietal selection programs based on interspecific hybridization have been initiated. However, they did not lead to the creation of varieties that could replace smooth cayenne because the conventional improvement of pineapple is difficult (Leal and Coppens d'Eeckenbrugge 1996; Akbar et al. 2003; Kouadio et al. 2017). However, *in vitro* culture of plant tissues by somatic embryogenesis appears to be an exciting tool for pineapple improvement. Indeed, somatic embryogenesis induces the formation of plants that conform to the mother plant, are rejuvenated, healthy, homogeneous, and have a high yield. In addition, these plants are free of contamination and pesticide residues, making them ideal materials for the renewal of orchards (Yapo et al. 2011). Many researchers have highlighted the mass multiplication of pineapples by somatic embryogenesis (Yapo 2013; Kouadio et al. 2017; Cacaï et al. 2021; Kessel-Domini et al. 2022). However, somatic embryogenesis is impacted by several culture parameters such as plant genotype, environmental conditions, cultural medium composition, and level of phytohormones (Rhimi et al. 2006; Kessel-Domini et al. 2022). Thus, the selection of culture media and phytohormones must meet the plant's nutrient requirements to allow its genetic potential to be fully expressed (Kouakou 2003; Usman et al. 2011). Therefore, growth regulators strongly influence somatic embryogenesis, and the most commonly used phytohormones include auxins and cytokinins (Kouadio et al. 2017; Cacaï et al. 2021). Also, many authors have suggested that a significant concentration of carbohydrates, amino acids, and nitrates are required for embryogenic skills acquisition during embryogenesis (Thiruvengadam et al. 2006; Yapo et al. 2011; Kouadio et al. 2017).

According to Daquinta et al. (1996), dicamba (auxin) induced embryogenic calli in smooth cayenne leaves and reported low embryogenic callus induction (approximately 55%). Sripaoraya et al. (2003) developed a methodology for somatic embryogenesis induction from the cultivar Phuket (Queens group) leaves. These authors reported an embryo induction rate of 58.3% using MS medium supplemented with sucrose (3%) and picloram (3.0 mg/L). However, the induction rate decreased with the increase in picloram concentration. Subsequently, different explants were used in smooth cayenne to develop more efficient protocols to induce somatic embryogenesis (Firoozabady and Moy 2004). After embryogenesis initiation, the maturation of embryos is obtained by culturing the embryogenic cells onto other media different from the induction media (Yapo 2013). However, the uses of the same medium for somatic embryo induction and maturation have been used by some researchers and not reported much difference. To simplify the somatic embryogenesis protocol and reduce the costs of pineapple production, this study sought a modified culture medium optimal for induction, followed by efficient somatic embryo maturation for healthy, high-performing plant material mass production. This study aimed to investigate the appropriate cultural medium and *in vitro* conditions for efficient and healthy pineapple planting material production by somatic embryogenesis.

2 Material and methods

2.1 Plant material

Pineapple (*Ananas comosus* var. Smooth cayenne, cv. CI 16) suckers used in this study were collected from the National Center of Agronomic Research (Anguédédou Station, Côte d'Ivoire). This study used the leaf base as the explant for the induction of somatic embryogenesis.

2.2 Methods

2.2.1 Disinfection of explants and shoot induction

The explants were disinfected using the method described by Yapo et al. (2011). Pineapple suckers were cleared of mature leaves and

root bases using a knife. Terminal buds were trimmed to approximately 2 cm. Under a laminar air flow hood, these buds were thoroughly washed with water. Then, they were disinfected for the 20s with alcohol (70%) and soaked in 3.6% (active chlorine) sodium hypochlorite for 30 min. With sterile distilled water, the buds were rinsed four times. The buds were removed entirely from the leaves using a blade mounted on a scalpel. These surface disinfected explants were transferred onto shoot initiation medium (MS basal medium with vitamin B_5, which was added with 0.2 $g.L^{-1}$ of glutamine, 0.01 $mg.L^{-1}$ of kinetin, and 30 $g.L^{-1}$ of sucrose). The pH of the prepared medium was adjusted to 5.8, and the medium was solidified by adding phytagel (2.5 $g.L^{-1}$).

2.2.2 Callus induction

In this study, calli were induced using Murashige and Skoog (1962) basal medium including vitamin B_5 (MSB_5 medium), supplemented with 3 $mg.L^{-1}$ picloram, 2 $mg.L^{-1}$ glycine, 1000 $mg.L^{-1}$ glutamine, 100 $mg.L^{-1}$ casein hydrolysate, 30 $g.L^{-1}$sucrose (Kouadio et al. 2017). The pH of the culture medium was adjusted to 5.5 and then solidified by adding 6 $g.L^{-1}$ of agar. The prepared medium was autoclaved under a pressure of 1 bar for 30 min at 121°C. Approximately 5 mm of leaf base from regenerated shoots was seeded onto the callus induction medium under a laminar flow hood. The jars containing the explants were incubated for four weeks. The incubation was carried out in a culture room at 25°C, with a 16 h photoperiod and a light flow of 2000 lux. The resulting friable calli were transferred to a somatic embryo induction medium.

2.2.3 Somatic embryos induction

Embryo induction medium (EIM) is composed of solid Murashige and Skoog and Gamborg B-5 (Gamborg et al. 1968) basal medium supplemented with vitamin B_5 (MSB_5) and the double concentration of KNO_3, the half concentration of NH_4NO_3 (MSB_5 - ½ [NH_4NO_3] + [KNO_3]), agar (6 $g.L^{-1}$), sucrose (30 $g.L^{-1}$), a combination of amino acids (glutamine, glycine, and casein hydrolysate), and hormones (BAP, kinetin, picloram, or 2,4-D). Seven embryo induction media selected from Kouadio et al. (2017) were tested in this study (Table 1). MIC_7 BAP medium (picloram-glycine-glutamine-casein-BAP) identified by Kouadio et al. (2017) as the best callogenesis medium served as a control in this study. Unlike the other test media, the MSB_5 basal medium was not modified.

Under a laminar flow hood, 1000 mg of friable calli were seeded into jars containing 10 mL of EIM medium with two calli explants per dish. Calli were subcultured on renewed media to maintain cell viability after four weeks of incubation.

After two subcultures, embryogenic structures (proembryos: clusters of rounded, regularly outlined, thick-walled cells with dense cytoplasm) were researched using a light microscope (Nikon TMS) (Vits et al. 1994; Nomura and Koumamine 1995). Then, the embryogenic structures observed were counted using a colony counter (COMECTA SA), and the embryogenic cell induction rate (ECIR) or embryogenic index (Cao Jing-Lin et al. 2008) was evaluated as follows:

$$\text{ECIR} = \frac{\text{NEC}}{\text{TNIC}} \text{ X } 100$$

Here NEC = number of embryogenic cells induced by medium; TNIC = Total number of induced embryogenic cells

2.2.4 Somatic embryos maturation

Approximately 500 mg of embryogenic calli were cultured on 10 mL of EIM medium with two explants per Petri dish for embryo maturation. The different cultures were incubated for three months in a dark room with monthly subcultures. At the end of each subculture, embryos were collected and observed under a laminar flow hood using a stereo microscope (Nikon TMS) at 100x or 400x magnification to look for evidence of embryo maturation according to the method of Thiruvengadam et al. (2006). Observed mature embryos were counted using a colony counter (COMECTA SA).

Table 1 MS media composition of somatic embryos induction medium (EIM)

Components /Medium	Culture media						
	MIC_7BAP (control)	EIM_1	EIM_2	EIM_3	EIM_4	EIM_5	EIM_6
Glycine ($mg.L^{-1}$)	2	2		2		2	2
Glutamine ($mg.L^{-1}$)	1000	1000	1000		1000		1000
Caseinhydrolyzate ($mg.L^{-1}$)	100	100		100		100	100
Picloram ($mg.L^{-1}$)	3	3					
2.4-D ($mg.L^{-1}$)			3	3	3	3	3
Kinetin ($mg.L^{-1}$)					0.2	0.2	0.2
BAP ($mg.L^{-1}$)	0.05	0.05					

The rate of mature embryos (RME) was calculated using the following equation:

$$\text{RME} = \frac{\text{NMEC}}{\text{NEP}} \text{ X } 100$$

Here NMEC =number of mature embryogenic cells; NEP = number of embryos present on the callus

2.2.5 Culture condition

Apart from embryo maturation, all cultures were incubated under a 16 h photoperiod, with illumination (2000 lux) provided by long light tubes in a culture room at 25°C.

2.2.6 Statistical analysis

The embryogenesis culture media effect was assessed based on the embryos' average number, induction rate, and maturation rate. Before the study, percentage rates were submitted to an angular transformation (arcsin√x). The analyses of data were performed using Statistica 7.1 software. One and two-criterion variance analyses were carried out on the mean values of parameters. Newman-Keuls test (5%) was used to classify the means when a significant difference was revealed between the two means.

3 Results

3.1 Influence of the culture medium composition on somatic embryos induction

Evaluation of the influence of different medium compositions on somatic embryo induction revealed that all culture media induced embryogenic cells (Figure 1). The composition of the selected media influenced the embryogenic cell induction rate (ECIR) and the number of induced embryogenic cells (NEC). The results also revealed that EIM_1 and EIM_5 induced the highest number of NEC (154 and 149 cells, respectively). These were followed by EIM_3 (50 cells) and EIM_2 (48 cells) media with statistically identical numbers of embryogenic cells. Furthermore, EIM_4 (10 cells) and EIM_6 (3 cells) media showed the lowest NEC (Table 2). These

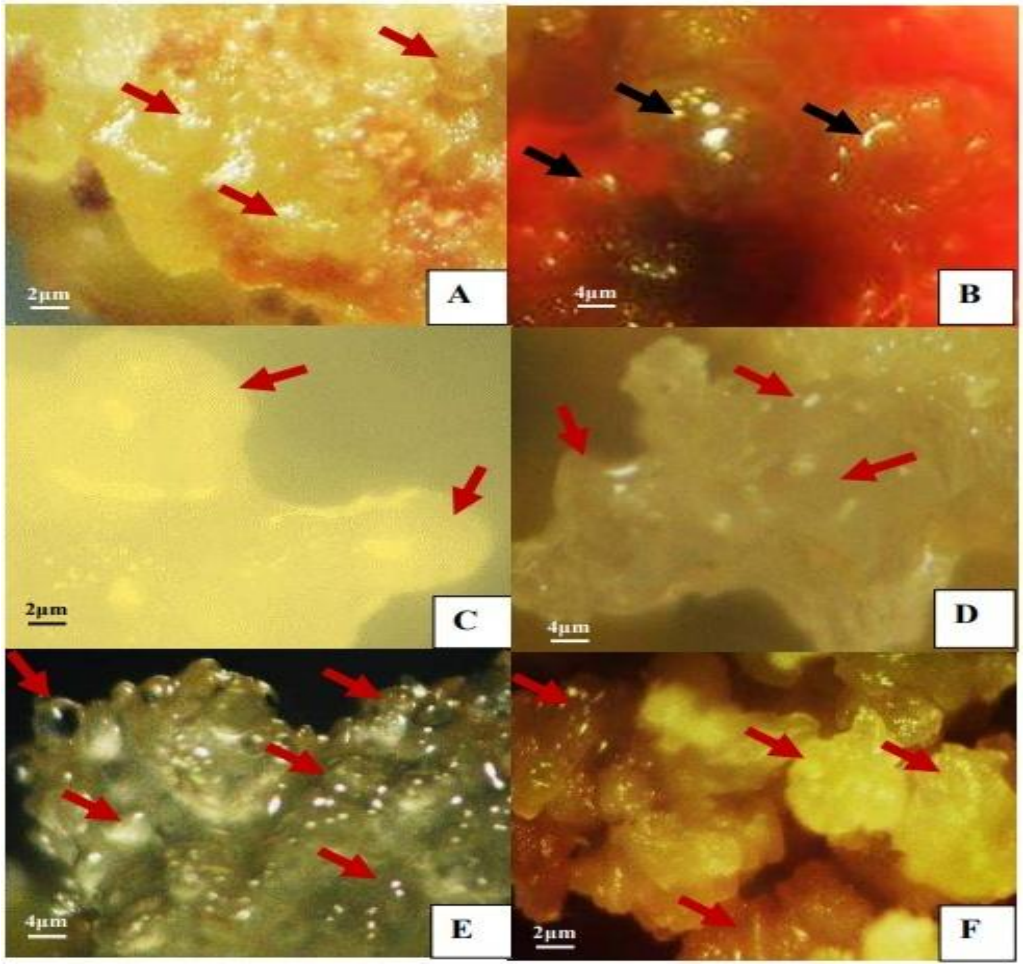

Figure 1 Embryogenic cell observed on pineapple calli from the selected cultures media; A: EIM_1; B: EIM_2; C: EIM_3; D: EIM_4; E: EIM_5; F: EIM_6; Arrows indicate the embryogenic cells.

results showed that the EIM1 medium induced a higher NEC (154 cells) and was reported lowest in EIM_6 media (3 cells). These two media were different, and the hormonal combination significantly influenced NEC.

In addition, analysis of EIM_5 media composition revealed that the addition of the kinetin (cytokinin) to the medium containing glycine and casein significantly improved embryogenic cell induction (149 in EIM_5 media) as compared to the EIM_3 (50). However, the EIM_2 and EIM_4 media composition analysis revealed that adding kinetin to the EIM2 medium containing glutamine inhibited the formation of embryogenic cells, showing only 48 and 10 cells, respectively. Similar trends were reported in the embryogenic cell induction rate (ECIR) (Table 2).

3.2 Influence of culture medium composition on the maturation of somatic embryos

Figure 2 revealed a significant evolution of the embryogenic structures on all selected culture media. Further, the culture

Table 2 Influence of culture media composition on the embryogenic cells induction

Culture media	Somatic embryogenesis parameters	
	Induction rate of embryogenic cells (%)	Number of embryogenic cells (NEC)
MIC_7BAP (control)	0 ± 0^{e}	0^{e}
EIM_1	37.20 ± 2.37^{a}	154^{a}
EIM_2	11.59 ± 2.07^{b}	48^{b}
EIM_3	12.08 ± 1.01^{b}	50^{b}
EIM_4	2.42 ± 1.53^{c}	10^{c}
EIM_5	35.99 ± 3.61^{a}	149^{a}
EIM_6	0.72 ± 0.46^{d}	3^{d}

In a column, numbers with the same letter are not significantly different (Newman-Keuls test at the 5% threshold).

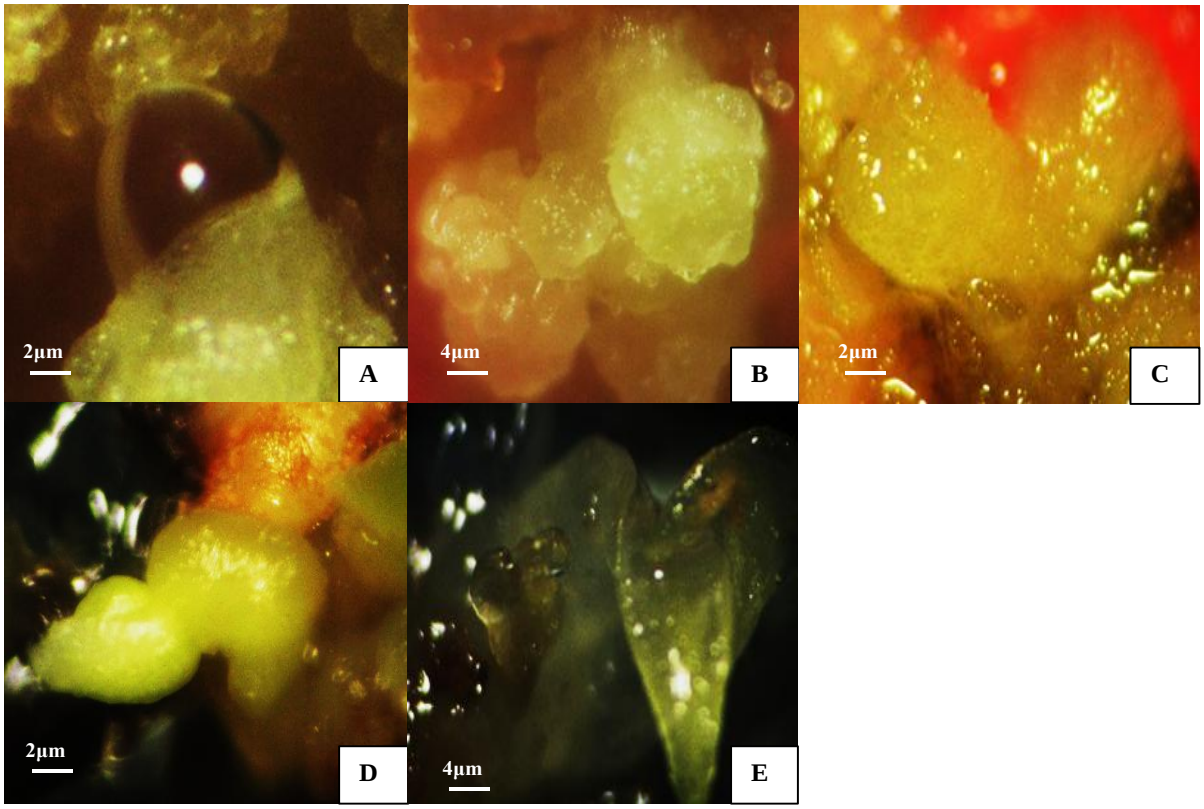

Figure 2 Different stages of evolution of pineapple somatic embryos observed on EIM_5 maturation medium; EIM_5 (EIM added with 3 $mg.L^{-1}$ of 2,4-D,0.2 $mg.L^{-1}$ of KIN,2 $mg.L^{-1}$ of glycine, 100 $mg.L^{-1}$ of casein hydrolyzate); A: globular stage embryo; B: cordiform stage embryo; C: heart stage embryo; D: torpedo stage embryo; E: cotyledonary stage embryo

Table 3 Influence of culture media composition on the maturation of the somatic embryo in pineapple

Culture media	Parameters	
	Mature embryo rate (%)	Number of mature embryogenesis cells/callus (num)
MIC_7BAP (Control)	0 ± 0^{c}	0^{d}
EIM_1	30.40 ± 1.27^{b}	19.67 ± 0.88^{b}
EIM_2	76.74 ± 1.81^{a}	23.00 ± 2.52^{b}
EIM_3	72.76 ± 3.38^{a}	22.33 ± 0.88^{b}
EIM_4	54.40 ± 2.00^{ab}	3.33 ± 0.88^{c}
EIM_5	76.33 ± 2.59^{a}	66.00 ± 4.91^{a}
EIM_6	35.58 ± 1.84^{b}	2.02 ± 0.65^{c}

In a column, numbers with the same letter are not significantly different (Newman-Keuls test at the 5% threshold)

medium composition significantly influenced the mature embryo rate (RME) (Table 3). EIM_2 and EIM_5 media induced a significantly higher rate of mature embryos (76.74 and 76.33%, respectively). These were followed by the EIM_1 (30.40%) and EIM_6 (35.58%) media. Analysis of variance also showed that medium composition strongly influenced the number of mature embryogenic cells (NMEC). The highest number of mature embryos (66 embryos) was recorded in the EIM_5 medium, followed by EIM_2 (23 embryos), EIM_3 (22.33 embryos), and EIM_1 (19.67 embryos), which had a statistically identical NMEC. Among the tested media, EIM_4 (3.33 embryos) and EIM_6 (1.33 embryos) media were found to be least effective in the induction of mature embryos. The EIM_5 medium, which gives a good evolution of embryos towards maturation with 66 mature embryos, was retained as the maturation medium. Globular, cordiform, heart, torpedo, and cotyledonary embryo stages were observed in this medium (Figure 2).

4 Discussion

4.1 Influence of the culture medium composition on somatic embryos induction

The results of this study revealed that the combination of EIM_5 media significantly affects somatic embryogenesis and development. In this manner, it can be established that MS media supplemented with glycine, 2,4-D, and kinetin is suitable for somatic embryogenesis. The results of this study agree with the findings of Cacaï et al. (2021), who suggested that somatic embryo induction in pineapple was favored by picloram combined with BAP in contrast to the kinetin and 2,4-D combination. Similarly, Zouzou et al. (2008) and Kouakou et al. (2009) also reported that the 2,4-D and kinetin combination was significantly effective for embryo induction in cotton. Kone (2010) also reported that the combination of 2,4-D and TDZ favor the embryogenesis in *Vigna subterranean*. These results suggest cytokinin-associated auxins' had a varietal effect in the induction of embryogenic structures (Dóra et al. 2020). Moreover, Yapo (2013) also reported that phytohormones also influence somatic embryo induction in pineapples. This supposes that pineapple calli produce endogenous picloram and BAP, which increases their stressful action to trigger cell differentiation into embryos. These results also suggest a robust synergistic action of picloram and BAP for embryogenic cell induction compared to the interaction of 2,4-D and kinetin. Furthermore, this difference in expression between EIM_1 and EIM_5 correlated with the types of amino acids (source of nitrogen). Similarly, Yapo et al. (2011) reported that somatic embryogenesis in pineapple is improved by adding the amino acids in the culture medium. These amino acids immediately provide a readily available nitrogen source to cells which initiates the stimulation of embryogenesis. These researchers suggested that nitrogen is selectively absorbed by cells in the form of nitrates to induce embryonic development. Thus, high nitrate levels significantly influenced the induction of embryogenic cells. A low NH_4^+/NO_3^- ratio induced the high number of embryogenic cells observed on the EIM_1 medium. Thus, nitrate appears to be essential for somatic embryo induction (Yapo 2013). The low embryogenic cell number observed in the EIM_3 medium compared to the EIM_5 medium showed that the kinetin addition to the culture medium stimulated the embryogenic cell induction. Arnold et al. (2002) have reported that phytohormones induce callus differentiation in polarized cells and initiate somatic embryogenesis. However, a decrease in embryogenic cells after adding 2-4D to the EIM_2 medium shows that somatic embryogenesis in pineapple depends on the combination of amino acids in the culture medium. Callus sensitivity to phytohormones is impacted by the presence of nitrogen in the culture medium (Fuentes-Cerda et al. 2006; Cangahuala-Inocente et al. 2007; Kouadio et al. 2017). The present study reported that the embryogenic cell induction in pineapple is controlled by a low NH_4^+/NO_3^- ratio and phytohormone interactions. EIM_1 and EIM_5 media are the most embryogenic in pineapple.

4.2 Influence of culture medium composition on somatic embryo maturation

This study reported that the composition of the culture medium influenced the induced embryo's maturation. Thus, all selected media allowed the initiation of embryogenic structures and had a similar effect on embryo maturation. These results corroborate with the authors, who have reported that somatic embryo maturation requires a proper combination of cytokinin and auxin (Fotso et al. 2008; Cacaï et al. 2021). According to Yapo (2013), weak auxin and cytokinin are essential to induce embryo maturation once embryogenesis is initiated. Indeed, the auxin and cytokinin combination stimulates cell division in the embryos, leading to their differentiation by allowing them to go through different embryonic stages (Fotso et al. 2008). In the current study, different stages (globular, cordate, heart, torpedo, and cotyledonary stages) of embryo evolution were observed, demonstrating that the embryos obtained are mature. Similar results were reported by Yapo (2013) for pineapple and Kouakou (2009) for cotton. These results suggest the influence of environment, nitrogen source, and phytohormones on maturation, and this could also be explained by the fact that embryos would accumulate starch reserves during maturation. The starch synthesized and accumulated in cells is an essential energy source for mitotic activities (Profumo et al. 1986).

Moreover, the work of Koné (2003) and Kouakou (2009) showed that cotton embryos that evolve to the cotyledonary stage (the most developed stage of maturation) have a dense cytoplasm, i.e., filled with starch reserves. According to Virgo-Brown (1987), the interaction of kinetin (0.5 $mg.L^{-1}$) and 2,4-D (2 $mg.L^{-1}$) in the N6 basal medium promoted embryo maturation in sorghum. Jayanthi et al. (2001) also showed that the association of 2,4-D and BAP or kinetin influenced embryogenesis. This combination also supports the maturation of embryos in most of the used culture media in this study.

However, this study revealed the non-maturation of some embryogenic cells during the induction phase. In addition, combinations of amino acids with cytokinins significantly affect the maturation of some embryogenic cells. In the current study, the association of glutamine with 2-4D inhibits the maturation of embryogenic cells, as observed for EIM_2 and EIM_4 media. These results suggest a toxic or competitive effect of 2-4D on glutamine. Furthermore, the abnormal cytoplasmic accumulation of starch reserves could explain the low rate of embryo maturation observed in EIM1 and EIM6. In addition, some cells without starch reserves accumulate phenolic compounds that inhibit maturation (Virgo-Brown 1987; Robert et al. 1989). The composition of these media inhibited hydrolases in particular and, consequently, low energy availability to embryogenic cells. Therefore, the embryos obtained remained blocked at the globular stage.

In contrast, adding kinetin to the EIM_3 and EIM_5 medium supplemented with glycine and casein hydrolyzate stimulated the maturation of the embryogenic cells. These results corroborate previous studies that reported that the cytokinin (BAP or kinetin) addition onto the culture medium supplemented with 2,4-D stimulates the maturation of the somatic embryos (Fotso et al. 2008; Jayanthi et al. 2001). These results could be explained by the synergistic effects of cytokinins and auxins on embryo maturation. Moreover, working on cotton plant embryos, Kouakou (2009) reported that cells have differential sensitivity to compounds in the culture medium that condition mitotic activity. He also noted that adding kinetin and 2,4-D onto the culture medium promoted the induction of embryogenic structures and embryo maturation in cotton. These results indicate the action of cytokinins and auxins on somatic embryo maturation depends on plant species.

Conclusions

The present study aimed to establish the appropriate conditions for efficient somatic embryogenesis and mass production of healthy and efficient pineapple plant material to renew the Ivorian orchard. Results of the study revealed the effects of seven culture media consisting of various amino acids (glutamine, glycine, and casein hydrolysate), cytokinins (kinetin and BAP), and auxins (2,4-D and picloram) on the induction and maturation of smooth cayenne (*Ananas comosus* L.) somatic embryos. Results of the study show that the embryogenic cell induction in pineapple was under the control of a low NH^+_4/NO^-_3 ratio and phytohormone interactions. Among the tested media combinations, EIM_5 medium (EIM added with 2 $mg.L^{-1}$ of glycine, 100 $mg.L^{-1}$ of casein hydrolyzate, and 0.2 $mg.L^{-1}$of kinetin) was the most embryogenic, and maturation of embryogenic cells media. These results suggested that adding kinetin to the culture medium containing casein hydrolysate and glycine in the presence of 2,4-D significantly affects the somatic embryo's initiation and maturation.

Author contribution

KOKS and KTH designed the experiment. KOKS, SO, and KD conducted the experiments and performed data analyses. KOKS and YSES prepared all of the figures, and all authors contributed to data interpretation. KOKS wrote the first draft of the manuscript, and KOKS and KTH edited the draft. All authors reviewed the manuscript.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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iNCOVACC COVID-19 vaccine: A Twitter based Social Media Analysis Using Natural Language Processing, Sentiment Analysis, and Topic Modelling

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ABSTRACT

Most, if not all, the vaccine candidates designed to counteract COVID-19 due to SARS-CoV-2 infection require parenteral administration. Mucosal immunity established by vaccination could significantly contribute to containing the SARS-CoV-2 pandemic, which is spread by infected respiratory secretions. The world has been impacted on many fronts by the COVID-19 pandemic since early 2020 and has yet to recover entirely from the impact of the crisis. In late 2022 and early 2023, China experienced a new surge of COVID-19 outbreaks, mainly in the country's northeastern region. With the threat of new variants like XBB 1.5 and BF.7, India might experience a similar COVID-19 surge as China and needs to be prepared to avoid destruction again. An intranasal vaccine can elicit multiple immunological responses, including IgG neutralization, mucosal IgA production, and T-cell responses. In order to prevent further infection and the spread of COVID-19, local immune responses in the nasal mucosa are required. iNCOVACC is a recombinant vaccine vectored by an adenovirus that contains a SARS-CoV-2 spike protein that has been pre-fusion stabilized. This vaccine candidate has shown promise in both early and late-stage clinical trials. iNCOVACC has been designed for intranasal administration via nasal drops. The nasal delivery system was created to reduce expenses for those living in poor and moderate-income

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countries. The newly introduced intranasal COVID vaccine will be beneficial in mass immunizing the public as it does not need any syringe and can be proven to be an effective method to boost immunity against the SARS-CoV-2 virus. This study uses natural language processing (NLP) techniques to analyze the Indian citizen's perceptions of the newly developed iNCOVACC vaccine in social media. For this study, we have used social media posts (tweets) as data. We have analyzed 125,300 tweets to study the general perception of Indian citizens regarding the iNCOVACC vaccine. Our results have indicated 43.19% of social media posts discussing the COVID-19 nasal vaccine in a neutral tone, nearly 34.29% of social media posts are positive, and 22.5% of social media posts discussions are negative. The general positive feeling that the iNCOVACC vaccine will work and the risks in the new vaccine are the two significant aspects Indian citizens voice out in social media posts about the iNCOVACC vaccine.

1 Introduction

In the last two years, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of coronavirus disease 2019 (COVID-19), has profoundly affected global health, the economy, and social stability (Dhama et al. 2020; Chen et al. 2022). SARS-CoV-2 is a member of the coronavirus family and primarily affects the respiratory system of its host (Akkız 2022). Though the COVID-19 crisis was subdued mainly in the latter part of 2021, the emergence of the Omicron variant and subsequent new Omcrion variants, including BF.7 and XBB 1.5 in late 2022, created a new wave of COVID-19 throughout the world, and it is a matter of concern (Dhama et al. 2022a; Zhou et al. 2022; Dhama et al. 2023). The introduction of the vaccine in mid-2020 has dramatically reduced the effect of COVID-19 and the number of deaths (Coccia 2022). However, the emergence of new variants and their ability to evade the immunity provided by the vaccine created a new issue for scientists and governments worldwide (Praveen et al. 2022). Global health is negatively impacted by the SARS-CoV-2-caused COVID-19 pandemic due to the virus' rapid dissemination and rapid mutation rate, stressing the significance of effective vaccines to prevent future illness and mortality. There are now over 500 vaccinations being researched and developed, with over 150 vaccine candidates undergoing clinical review and 24 vaccines approved for use in humans in times of emergency.

Vaccines capitalize on the unique ability of the human immune system to recognize and recall previously encountered pathogens. An ideal vaccination would stop the disease before it causes serious illness, hospitalization, or death quickly and in various ways. In the aftermath of vaccination, T cells and antibody-making B cells mediate the adaptive immune response (Chakraborty et al. 2022; Sah et al. 2022). Presently, the only way to receive a COVID-19 vaccination is via an intrusive intramuscular (IM) injection, but scientists are hard at work developing a vaccine that may be given via the less invasive nasal or oral routes. Vaccines against COVID-19 that have been approved by the World Health Organization and are given intramuscularly elicit antibody-mediated and cell-mediated immunity to prevent viral replication and offer resistance to the emergence of COVID-19. However, the current IM vaccines are designed to generate a systemic immune response rather than mucosal protection. The protections provided by IM vaccinations may, therefore, not be adequate to deal with virus multiplication and shedding in the upper respiratory tract and may not prevent SARS-CoV-2 infection through the nasal route. Unvaccinated individuals may still be susceptible to infection with SARS-CoV-2 if they do not mount a local secretory IgA antibody immunological response (Alu et al. 2022; Nakahashi-Ouchida et al. 2023).

Inducing sterilizing immunity in the upper airway is not a goal of most vaccinations. Therefore, they primarily defend against lower respiratory tract illnesses. The introduction of vaccines through the nasal route has the potential to not only protect against the clinical manifestations of disease but also to stop the spread of the virus among susceptible people. If anybody wants to stop the spread of viruses, they should get vaccinated in a method that makes their upper airway utterly immune to them (Dhama et al. 2022a; He et al. 2023). An intranasal vaccination has promise because it mimics the natural route of infection, may be administered by the patient, and has the potential to capture a sizeable market share in the long run. Regarding the upper and lower respiratory tracts, intranasal immunization induced strong neutralizing antibody responses and mucosal IgA and T cell responses, essentially eliminating the SARS-CoV-2 infections (Dhama et al. 2022b).

Intranasal vaccination can provide a safe and effective means of eliciting long-lasting systemic and humoral immune responses and mucosal immunity in the upper and lower respiratory tracts because the nasal compartment is the first-line barrier to SARS-CoV-2 entry that must be breached before the virus can spread and disseminate to the lungs. The immunological response prompted by an intranasal vaccine is extensive, including the production of neutralizing IgG, mucosal IgA, and T cells. Prevention of both infection and transmission of COVID-19 requires immune responses at the site of infection (in the nasal mucosa). SARS-CoV-2 vaccinations intranasally protect against acquiring the virus, its replication, it's shedding, and the development and spread of disease (Slomski 2022; He et al. 2023; Nakahashi-Ouchida et al. 2023). Studies have indicated that intranasal delivery of vaccines is

favored over needle injection since it is perceived as less uncomfortable and intrusive to the body, is equally effective, and is associated with fewer side effects (Alu et al. 2022; Slomski 2022; He et al. 2023; Nakahashi-Ouchida et al. 2023).

Previous studies have mentioned the fear of vaccines, and the general mistrust of vaccines resulted in the low intake of vaccines in many parts of the world (Praveen et al. 2021b; Praveen et al. 2021; Chakraborty et al. 2022). In late January 2023, Bharat Biotech developed a new vaccine named iNCOVACC, an intranasal COVID-19 vaccine (Times 2023). Intranasal vaccines are superior to conventional vaccines since the nasal mucosa is often the initial site of infection (Chavda et al. 2021). Furthermore, nasal mucosal vaccination lessens the need for syringes and medical waste, making it a resource-saving and environmentally friendly approach appropriate for a sustainable healthcare paradigm. The mucosal vaccines are also practical for self-help vaccination, which guarantees individual comfort and improves individual compliance, making them suitable for mass immunizations in the general public. China is experiencing a crisis with a surge in COVID-19 cases, and India may soon have a similar situation (Kelleni 2023). To get through this challenging times, the Indian government and health officials must be prepared, and iNCOVACC intranasal COVID vaccine can be promoted among the public to increase immunity among the Indian population.

iNCOVACC contains a SARS-CoV-2 spike protein pre-fusion stabilized and delivered as a recombinant vaccine vectored by an adenovirus that lacks replication. Clinical trials of this vaccine candidate in phases I, II, and III met with positive conclusions. iNCOVACC has been designed for intranasal administration via nasal drops. The nasal delivery device was created to be affordable in low- and middle-income countries (Shahnoor et al. 2023).

Previous research has shown that some people doubt vaccinations and do not accept new methods easily (Praveen et al. 2021). Our study analyzes the Indian citizens' perspective on the new intranasal COVID-19 vaccine. It is crucial to comprehend how the general public perceives the vaccine. Before beginning the process of mass vaccination, this study will assist government officials and policymakers in understanding the difficulties that must be resolved.

2 Materials and Methods

We have used social media posts of Indian citizens to understand ordinary citizens' mental attitudes toward the COVID-19 intranasal vaccine. Governments and policymakers should be aware of the public's views on any health policies they consider implementing since implementing a policy that most citizens do not support will result in not attaining the desired outcomes. In this research, sentiment analysis and topic modeling are two natural language processing (NLP) approaches we employed to analyze the general public's perceptions of India's iNCOVACC COVID-19 nasal vaccine.

For this study, we collected all the tweets concerning Indians talking about the iNCOVACC nasal COVID vaccine. We have scrapped all tweets containing the word 'iNCOVACC' using the Python library Twint. Using the python scrapper we have built, we have scrapped down all the tweets posted by Indians between the 4th week of January 2023 to the 3rd week of February 2023 that contains the word 'incovacc.' For this study, we selected Twitter as our data source. We have gathered tweets from India using the geographical filtering function in the Python library Twint. We have chosen only tweets in the English language for this study. One hundred twenty-five thousand three hundred distinct English tweets were used for the analysis after excluding the duplicated tweets and the tweets from other languages. We select the same amount of tweets for four weeks in 2023 (4th week of January to 3rd week of February 2023) in our corpus to balance out any potential disadvantage resulting from the uneven sample.

Before the analysis, we processed the data through various data-cleaning methods. Data cleaning is essential in the study as it removes unwanted entities from the corpus (Praveen et al. 2021a). Stop words, numbers, punctuation, and hyperlinks that weren't necessary for our data analysis were removed through this method. Stop words in the corpus lack inherent meaning and are hence unnecessary for analysis. Stop words typically refer to articles like 'a' and 'an' and prepositions like 'is,' 'that,' and 'of,' which have no meaning or purpose. Following removing the stop words from the corpus, we also removed other unwanted entities like numbers, punctuations, and hyperlinks. Further, we have performed the stemming and lemmatization of the data for our study. Stemming is the process of reducing the words into their root type by removing the end letters (Praveen and Ittamalla 2020a), such as "pens- > pen" and "likes-> like," and lemmatization is the act of organizing various word kinds into groups to reduce the dimensionality (Praveen et al. 2021c).

2.1 Sentimental analysis

Sentiment analysis is a machine learning technique used for gathering and examining subjective evaluations of various characteristics of a thing or entity in textual data (Praveen and Ittamalla 2020b). The sentimental analysis technique aims to calculate the sentimental score by analyzing the data, which might be a phrase, sentence, or full text (Praveen et al. 2020b). We performed sentiment analysis in our study to comprehend how Indian social media users felt about the iNCOVACC COVID-19 nasal vaccinations. Realizing the general public's opinions regarding a specific concern, such as a particular health policy, can

help governments and policymakers determine whether the general public will support the policy they implement. Using the sentimental analysis technique, the author's tone appears in their text as either positive, negative, or neutral. The Python library TextBlob was utilized for the sentiment analysis process. The TextBlob library analyzes every word in the documents in the corpus using powerful machine-learning algorithms, categorizing the overall sentiments as positive, negative, or neutral (Praveen and Ittamalla 2021a). Each word in the document is scored individually using the Text Blob library. The total score of the document is calculated by a pooling operation (averaging all sentiments), and the final sentiment of each document is determined (Praveen and Ittamalla 2022).

2.2 Topic Modelling

Sentiment analysis lets us comprehend how the general public feels about a health policy. Yet, topic modeling is necessary to understand the variables influencing emotions fully. We performed topic modeling on the data to understand the major aspects of Indian citizens' voices about the newly developed iNCOVACC vaccine. In this study model, we used Latent Dirichlet Allocation (LDA) topic modeling to analyze the principal issues raised by Indian citizens regarding the COVID-19 nasal vaccination. Topic modeling is an information retrieval technique that helps understand the premises based on which the big data corpus is built (Praveen et al. 2020a; Praveen and Ittamalla 2021b). Previous to LDA, latent semantic indexing was used to derive the topics based on which the corpus is built (Praveen et al. 2021). However, the latent semantic indexing method cannot do document-level understanding. Latent Dirichlet Allocation topic modeling works under the assumption that all the documents presented in the corpus are a mixture of the number of topics, where each topic is a multinomial distribution of words (Praveen et al. 2020a). LDA employs machine learning algorithms to understand the latent variables from the unstructured data. To facilitate a better understanding of the topics identified, we used LDAvis.

3 Results and Discussion

3.1 Sentiment Analysis

This research was divided into two phases. In the first phase, sentiment analysis was done to ascertain how individuals feel about iNCOVACC COVID-19 nasal vaccines. The general sentiment of each text in the corpus is evaluated by TextBlob algorithms, which look at each word in the tweet to assess if it is positive, negative, or neutral. For this study, we scrapped 125,300 tweets about Indians talking about iNCOVACC COVID-19 nasal vaccine. For an accurate comparison, we chose an equal number of tweets from each week of the month in the corpus. The sentimental analysis study showed that out of 125,300 tweets, 54129 tweets (43.19%) were neutral about the iNCOVACC COVID-19 nasal vaccine, 42977 tweets (34.29%) revealed positive sentiments and 28194 tweets (22.5%) had negative sentiments. Our study by sentiment analysis showed that about 77.48% of the Indian population's social media posts were either positive or neutral. The results of our research are mentioned in Table 1. Figure 1 and Figure 2 represent the graphical representation of table 1.

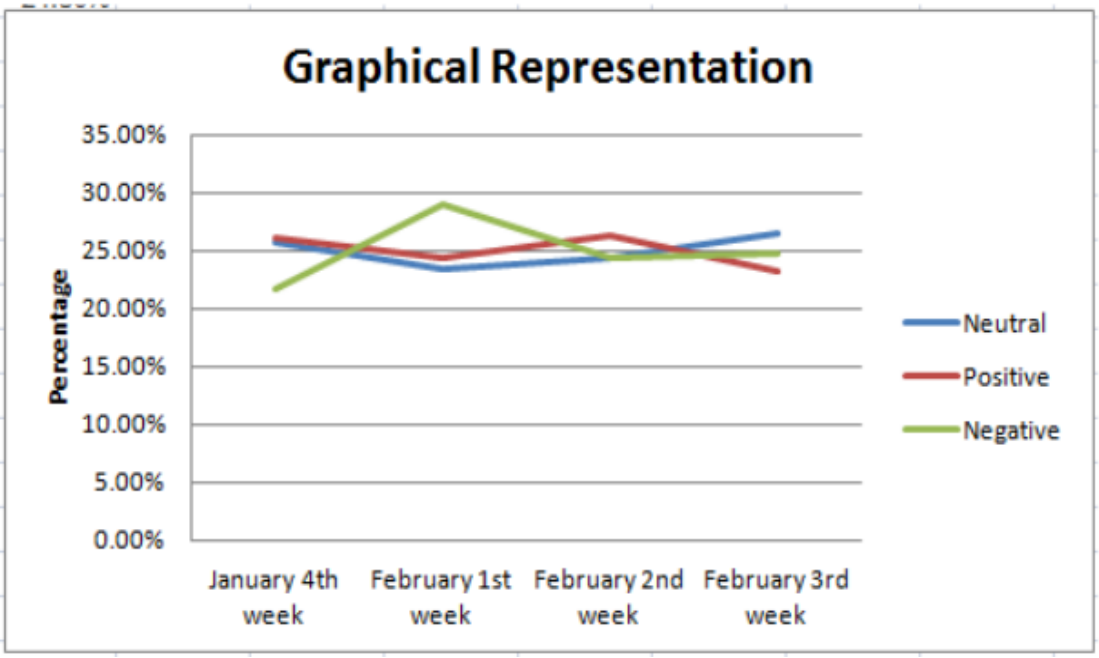

Figure 1 Graphical representation of Table 1 (By percentage)

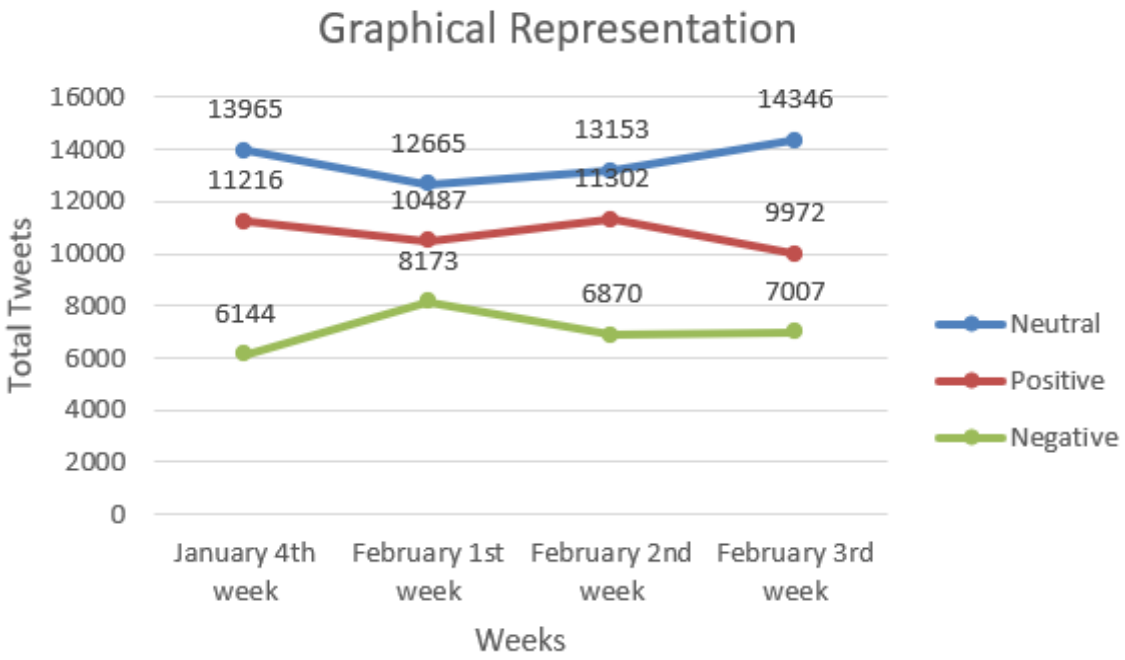

Figure 2 Graphical representation of Table 1 (By Number of Tweets)

Table 1 Sentimental Analysis Research

Week	Total Tweets	Neutral	%	Positive	%	Negative	%
January 4th week	31,325	13965	25.8	11216	26.09	6144	21.7
February 1st week	31,325	12665	23.4	10487	24.4	8173	28.9
February 2nd week	31,325	13153	24.3	11302	26.2	6870	24.3
February 3rd week	31,325	14346	26.5	9972	23.2	7007	24.8
Total	125,300	54,129		42,977		28,194	

3.2 Topic Modeling

Though sentimental analysis provided insight into how the general Indian population felt about the iNCOVACC COVID-19 nasal vaccine and its impacts, it did not aid in our understanding of the critical factors that influence that attitude. We further performed Latent Dirichlet Allocation topic modeling for the tweets about the nasal vaccine to understand the significant aspects the Indian population voices in their social media post about the newly introduced iNCOVACC COVID-19 vaccine. According to the findings of our topic modeling, Indian citizens, while discussing about the iNCOVACC COVID-19 nasal vaccines they discuss various aspects such as the functioning of nasal vaccine, risks of taking a vaccine, availability of the vaccine, fear of infection, safeness for children, registering for this vaccine, whether worth the risk despite of the risks it has, about the need for the vaccine, and general positive feelings about the vaccine.

Many previous studies have used machine learning and deep learning techniques to understand common people's perceptions of vaccines and vaccine hesitancy. Hussain et al. (2021) analyzed over 300,000 Facebook and Twitter posts belonging to the United Kingdom (UK) and the United States (US) related to COVID-19 vaccines. They concluded that nearly 58% of UK social media posts were positive sentiments, and 22% and 17% of UK citizens' posts about vaccines were negative and neutral sentiments, respectively. On the other hand, 56%, 24%, and 18% of the social media posts belonging to the US were made of positive, negative, and neutral sentiments. Lanyi et al. (2022) analyzed over 90,000 social media posts of citizens of the UK. They identified that mistrust towards vaccines, safety aspects towards vaccines, feeling that vaccines are ineffective, and accessibility of the vaccine were some of the key issues that contributed to vaccine hesitancy. Gautam et al. (2022) analyzed over 6000 Indian tweets about the COVID-19 vaccine and concluded that nearly 44.1% of the tweets

Table 2 Topic Modeling

Topic	Top words
Feeling that nasal vaccine work	vaccine, nasal, work, immunity, go, mucosal
Risks in vaccine	vaccine, rinse, nasal, die, people, risk
Availability of the vaccine	reception, appointment, book around, hospital
How it works	nasal, gene, long, enter, production, body
Fear of infection	infection, respiratory, get, covid, much, outrage
Children	child, nasal, vaccine, safe, book, immunity
Registering for the vaccine	intranasal, vaccine, visit, hospital, website, private
Whether worth the risk	approve, get, risk, worth, life, choice
Wondering about the need for the vaccine	need, wonder, covaxin, potus, incovacc, strategic
General positive feeling about the vaccine	great, bharatbiotech, video, proud, world, efficacy

were on a positive tone, 17.6% and 38.2% of tweets were of negative and neutral sentiments, respectively. Villavicenio et al. (2021) have used Naïve Bayes to understand the perception of Filipinos regarding the COVID-19 vaccines. They analyzed 11,974 tweets and concluded that 83.38% were positive. Phrases like "trust science," "vaccine works, and "a dose of hope" were observed several times in the analysis. Ljajic et al. (2022) analyzed 8817 tweets relating to COVID-19, and they concluded that issues such as vaccine effectiveness, the belief that natural immunity is better, a general mistrust over science, vaccines are just an experiment, and conspiracy theories are some factors for vaccine hesitancy. Prabagar et al. (2022) analyzed 400,000 tweets about COVID-19 and concluded that conspiracy theories and the fear of side effects were the most important factors contributing to vaccine hesitancy. From our analysis results, it can be understood that the perception towards the iNCOVACC vaccine is almost as same as that of any previous vaccines.

Conclusion

The study shows that only 34.29% of the Indian population has a positive sentiment toward the COVID-19 nasal vaccines. A previous study analyzing the sentiment of Indians towards the COVID-19 vaccine when the first vaccine for COVID-19 was introduced revealed that 35% of the sentiments of the Indian population were positive. It can be observed that almost the same percentage of Indians who felt optimistic about the COVID-19 vaccine when the first vaccine was introduced also feel positive about the newly introduced iNCOVACC vaccine. Our study also pointed out that 43% of the sentiments about the newly introduced vaccine were neutral. The government, health officials, and policymakers need to introduce advertising and promotion-based initiatives to target 43% of the netural sentiments to convert into positive sentiments. Our topic modeling results show that along with the positive aspects of the vaccines, a large sector of the Indian population also shared their concerns and doubts regarding the vaccine. The risks of taking the newly introduced vaccine, fear of infection on taking the vaccine, whether the vaccine is safe for children, whether it is worth to avail the vaccine despite the risks it has and the need for the new vaccine, despite the availability of the previous vaccines are the concerns shared by Indian population regarding the newly introduced iNCOVACC vaccine. To accomplish the desired outcomes of protective immunity among the citizens of India and protect their health during the COVID-19 pandemic, the Indian governments and policymakers should implement and promote efficient awareness programs and policies through social media and all forms of necessary communications. Strategic planning should be appropriately done to motivate more individuals to come up and take the vaccine.

This worldwide epidemic shows us that the healthcare industry's current regulatory systems cannot expedite the approval of products like vaccinations unless they are under extreme pressure. As vaccine development has not been a priority, it has required a worldwide pandemic to unite worldwide scientists and encourage them to work together on creating a vaccine and other therapies for COVID-19. Healthcare crises of this magnitude necessitate a dedicated regulatory and funding structure to reduce casualties as much as possible. The IM vaccine delivery elucidates a durable systemic IgG response and generates memory B and T cells; a subsequent booster dose administered via the intranasal route recruits memory B and T cells in the upper respiratory tract to provide mucosal protection and prevent the spread of the virus. Most companies developing new COVID-19 vaccines also conduct clinical trials of nasal-based vaccination platforms as part of a booster dosage strategy. We believe the many present initiatives will soon lead to new-generation vaccines and regulatory mechanisms, which will help us overcome the current problem.

The public should be educated about the benefits of intranasal vaccines and the immunological mechanisms that make them superior to those of IM vaccines, and the public should be given

the option of receiving immunizations via either route. In other words, intranasal immunization can prevent virus infection and transmission by inducing sterilizing mucosal and systemic immunity. Intranasal vaccines are expected to aid in the fight against the ongoing COVID-19 pandemic and other possible viral infectious diseases. Pharmaceutical firms may choose to adopt nasal vaccinations and provide comprehensive information on these vaccines due to their benefits and increased adherence. Those who are usually reluctant to get immunizations could be more likely to do so if they were offered these. Vaccine reluctance is a multifaceted problem, but our research can help us better design effective messages to reduce vaccine skepticism and increase vaccination rates. Implicit measures, such as the Implicit Association Test (IAT), could be used to probe people's perceptions of nasal vaccines to tease out their true motivations and biases.

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Heavy Metal Tolerance profile among Bacterial species Isolated from Hydrocarbon polluted sites and their mobile genetic elements

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ABSTRACT

This present study evaluated the plasmid incidence in bacteria and their genetic elements in heavy metals tolerant-antibiotics resistant microbes isolated from petroleum hydrocarbon polluted sites. The plasmid isolation was carried out using the fermentas Genejet plasmid miniprep kit (Thermofisher Scientific Inc, USA). Screening for class 1, 2, and 3 integrons, incompatibility group P testing, plasmid replicon typing, plasmid restriction analysis, and other analysis was performed using standard laboratory procedures. Plasmid incidences were higher among multiple heavy metal-tolerant bacterial species from hydrocarbon-polluted sites than those from the pristine site. Further, Class 1 integron incidence was significantly higher among the integrons in heavy metal tolerant bacterial isolates isolated from the polluted ecosystems than those from pristine ecosystems. Plasmid replicon type of bacteria with multiple heavy metal tolerance and antibiotics resistance indexes revealed that IncN plasmid replicon type carrying class 1 integron. This encodes resistance to sulphamethazole/trimethoprim, ampicillin, and tolerance to Cd, Ni, and Cu in *Klebsiella pneumoniae* isolate from petroleum-polluted soil. This is the first report of IncN plasmid in environmental bacteria in Nigeria, particularly from petroleum polluted environment. The conjugation experiment confirmed the possible transferability of antibiotic resistance determinants among isolates in polluted ecosystems. From the results of this study, it can be concluded that petroleum hydrocarbon pollution vis-a-vis heavy metal selective pressure with the abundance of mobile genetic elements amongst isolates from polluted ecosystems could contribute to the dispersing of antibiotic resistance genes, thus posing a serious public health concern.

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1 Introduction

Over the years, petroleum hydrocarbons have improved several nations' socioeconomic standards. But the mismanagement of this essential commodity due to pipeline vandalization, accidental discharge, and refining processes has resulted in severe environmental threats. Many polluted soil and aquatic ecosystems have been abandoned because of ineffective reclamation methods (Ekpo et al. 2012). However, various reports have shown that excess heavy metals, especially in soil environments, reduce microorganisms' growth rate and replication. Heavy metals are found naturally in the ecosystem, but their abundance and prevalence are associated with various anthropogenic activities such as industries (Petrochemical, Mining, and gas flaring) and Agricultural sectors (Fertilizer plan, Fermentation industries, milling companies). The bioavailability of heavy metals at low concentrations may not cause a serious environmental threat, but higher accumulation in aquatic and soil ecosystems may cause a significant challenge to public health (Azarbad et al. 2015; Berg et al. 2005). They have some heavy metals that might act as essential micronutrients and are involved in cell metabolism, but bioaccumulation of these heavy metals in high proportion, such as Zn and Pb uptakes, could be toxic and cause danger to its accumulator. The biodiversity of microbial communities could be altered when the excess accumulation of heavy metals occurs in the terrestrial and aquatic ecosystems (Epelde et al. 2015).

Plasmids are autonomous self-replicating extra-chromosomal DNA elements (Chen et al. 2015), which are not beneficial for bacterial growth and proliferation but play essential roles in various other metabolic activities like drug and heavy metal resistance. Plasmids are highly mobile and spread widely between the same or different bacteria genera and even eukaryotes (Hu et al. 2017). The family *Enterobacteriaceae* is one of the typical bacterial family that has been used in the transfer of plasmids from one specie to another (Knapp et al. 2017), between enteric and other gram-negative bacteria (Chen et al. 2015) and between or within non-enteric gram-negative organisms (Sawut et al. 2018). However, plasmids are smaller but have a vast carrying capacity of not only transferring and replicating genes but also coding for antibiotic resistance, metabolic enzymes, and bacteriocin production (Gati et al. 2016). Making a copy of plasmid in a cell is determined by replication of origin, also known as the replicon. The inability of a single cell to maintain different plasmids with the same replication mechanism has given rise to incompatibility (Inc) grouping of plasmids. Incompatibility classifies plasmids by their ability to coexist stably with other plasmids in the same bacterial strain. The number of incompatibility plasmid groups increases from the 26 known Inc groups occurring among the *Enterobacteriaceae* (Frost et al. 2005). This study aimed to evaluate plasmid incidence in bacterial and its genetic elements in heavy metals tolerant-antibiotics resistant microbes from petroleum hydrocarbon polluted sites.

2 Materials and Methods

2.1 Heavy metal resistance screening in bacteria isolates

As described by Lee et al. (2009), the agar dilution method for bacterial isolation was adopted with slight modifications. A loopful of 12-16 hr bacteria culture in TSB was streaked on Mueller Hinton agar (Hardy, Diagnostic, USA) supplemented with heavy metal salts to achieve 100µg/ml each of cadmium, Chromium, cobalt, nickel, vanadium, and 600 µg/ml each of lead and copper. The rest procedure is as described by Lee et al. (2009)

2.2 Detection of Bacterial plasmid

Plasmid DNA was obtained using the Fermentas Genejet plasmid miniprep kit (Thermo-Fisher Scientific Inc, USA). New bacterial colonies were inoculated into sterile 10 ml Lauria Bertani medium (Merck, Germany) in a 50 ml capacity tube and incubated for 12-16 hours at 37°C at a shaker with 200 rpm rotations. It was followed by harvesting the bacterial cells by centrifugation at 8000 rpm using microcentrifuge model 5415 R (Eppendorf, Germany). Pelleted bacterial cells were resuspended and subjected to SDS/alkaline lysis. Two hundred and fifty microlitres (250µl) of resuspension solution with RNase A was added to the pelleted cells and vortex by using a Labinco Model L46 vortex mixer (Labinco BV, Netherlands). 250µl of lysis solution was mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly transparent. Vortexing can be avoided in other not to shear chromosomal DNA. The lysis process did not exceed five minutes to avoid supercoiled plasmid DNA denaturing. It was followed by adding a neutralization solution (350µl) and inverting the tube 4-6 times. The tube was centrifuged for 5 minutes at 13000 rpm to remove pellet cell debris and chromosomal DNA. After this, pipetting was done to extract the supernatant to the Gene-Jet spin column, the transferred supernatant was centrifuged for one minute, and the flow-through was discarded. Further, five hundred microliters (500µl) of the wash solution was added to the Gene-Jet spin column, centrifuged for 30-60 seconds, and the flow-through was discarded. Again the washing step was performed twice. After the last washing, the Gene-Jet column was centrifuged for 60 seconds to remove the residual wash solution. Following centrifugation, the Gene-Jet spin column was transferred into a fresh 1.5ml microcentrifuge tube, and 50µl of the prewarmed (at 70°C) elution buffer was added to the center of the Gene-Jet column membrane to elude the plasmid DNA. The column was incubated at room temperature for two minutes and centrifuged for two minutes. The isolated plasmid DNA was stored at -20°C. Using the standard method, plasmid DNA was detected using 1% agarose gel in lx TAE buffer.

2.3 Screening for class 1, 2, and 3 integrons

Moura et al. (2007) developed PCR to screen bacterial isolates with genes *int* 11, *int* 12, and *int* 13. One microliter (1µl) of bacterial genomic DNA was used as a template for PCR while 4µl of boiled cells of positive control strains, i.e., *Salmonella enterica serovar typhimurium* (*int*11), *Escherichia coli* (*int*11) and *Klebsiella pneumoniae* (*int*13) were used as templates. These were added to the reaction mixture containing 14.25µl of sterile milli-Q water, 3µl of 25mM MgCb (Fermentas, USA), 2.5µl of Taq buffer with $(NH_4)_2S0_4$ (Fermentas, USA), 1.25µl of DMSO (Eurobio, France), 1µl of dNTPs (BIORON, Germany), 0.75µl each of primer pairs and 0.5p.l of U Taq Polymerase (Fermentas, USA) to achieve a total volume of 25µl. Amplification was performed in a Thermal Cycler (BIORAD, USA) with the following PCR program 94°C for 9 min, 30 cycles of 94°C for 30 sec, 55°C or 50°C for 30 sec (as appropriate), and 72°C for 45 sec, with a final extension at 72°C for 10 min. PCR reaction products were analyzed on 1.5% agarose gel in lx TAE buffer, run at 80V for 60min. Amplicons were visualized after staining in ethidium bromide using a molecular imager.

2.4 Detection of Class 1 integron variable region

Class 1 integron variable region was also detected by PCR amplification (Levesques et al. 2005) using primer pairs targeting class 1 integron variable region (5'-CS: GGC ATC CAA GCA GCA AG and 3'-CS: AAG CAG ACT TGA CCT GA). The PCR mixture contained 10µl of Extensor long PCR master mix (ABgene-Thermo Scientific, UK), 1µl each of primer pairs, 7µl of milli-Q water, and lµl DNA. The amplification protocol used was denaturation at 94°C for 5min followed by 30 cycles of 94°C for 30 sec; 58.5°C for 30 sec and 68°C for 3 min with a final extension at 68°C for 10 min (Table 1). Reaction products were analyzed as described above.

2.5 Testing of incompatibility group P (IncP-1)

All positive plasmid DNAs were screened for broad host range (3HR), plasmid IncP-1 subgroups a, p, s, *y*, and 5 by PCR technique. Three primer pairs developed by Bahl et al. (2009) were used to amplify the 281 bp homologous fragments of the *trfA* gene from plasmids belonging to the different IncP-1 subgroups. The primer pairs and positive controls are presented in Table 2. Each PCR mixture contained 12.55µl of sterile milli-Q water, *2.5*µl of Buffer (Promega, USA), 2.5µl of $MgCl_2$ (Promega, USA), 2.5µl of 200µM dNTPs (BIORON, Germany); 1.25µl of each primer, *1.25*µl of DMSO (Eurobio, France), 0.2µl of GoTaq Flexi DNA Polymerase-5µl (Promega, USA) and 1µl of DNA template. The total volume of each PCR mixture was 25 µl. The PCR program was initially denatured at 98°C for 30 sec. followed by 35 cycles of 98°C for 20 sec., 67°C for 20 sec, and 72°C for 30 sec. with a final extension at 72°C for 5min. Reaction products were analyzed in 1.5% Agarose Gel in lx TAE buffer, run at 80V for 70min, and viewed using a molecular imager after staining in ethidium bromide.

2.6 Plasmid replicon typing

Three panels multiplex PCR was carried out to detect 18 plasmid replicons, as Johnson et al. (2007) described, with some required modifications in the PCR. The PCR mixture contained the primer pairs for each plasmid replicon (Table 2). The PCR condition used in this study is as follows: 5 minutes at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 90 sec at 72°C and a final extension of 5 mins at 72°C. Amplicons were visualized on 1.5% Agarose Gel in l X TAE buffer alongside a 1-kb DNA ladder.

Table 1 Oligonucleotides used for integrase gene detection

Primer pair	Target	Sequence (5' -3')	Annealing Temp. (°C)	Amplicon size (bp)
int1F int11R	*intI1*	CCT CCC GCA CGA TGA TC CCT CCC GCA CGA TGA TC	55	280
intI2F intI2R	*intI 2*	TTA TTG CTG GGA TTA GGC ACG GCT ACC CTC TGT TATC	50	233
intBF intI3R	*intI 3*	AGT GGG TGG CGA ATG AGT G TGT TCT TGT ATC CGC AGG TG	50	600

Source: Liu et al. (2018)

Table 2 Primer pairs for IncP-1 subgroups

Subgroup	Name	Forward primer (5'-3')	Reverse primer (5'-3')
α, β, ε	TrfA	TTCACSTTCTACGAGMTKTGCCAGGAC	GWCAGC1TGCGGTACTTCTCCCA
γ	trfA-y	TTCACTTTTTACGAGCTTTGCAGCGAC	GTCAGCTCGCGGTACTTCTCCCA
&	trfA5-&	TTCACGTTCTACGAGCTTTGCACAGAC	G AC AGCTCGCGGT ACTTTTCCC A

PCR was selected as per Bahl et al. (2009).

2.7 Conjugation experiment

The filter paper mating assay described by Moura et al. (2007) was adopted for the conjugation experiment. Four class 1 integron-positive bacterial isolates (Gl, G2 containing InC-N plasmid and ITS5, 'TS1 containing InC-P plasmid) were included as donors, while *Escherichia coli* CV601 resistant to kanamycin and rifampicin but sensitive to ampicillin was used as a recipient cell. Donor strains were resistant to ampicillin and trimethoprim/sulphamethazole. Donors and recipient strains were grown separately in Luaria Bertani broth Merck, Germany) and incubated with agitation (200rpm) at 37°C for 24 hours. The optical density of cells was adjusted at 600nm to 1 OD, equivalent to 8 x 10 cells/ml. The concentration of cells needed for mating was determined. Donor and recipient strains were mixed in 0.9% NaCl solution and filtered through 0.45 µm pore size nitrocellulose filters. Filters were placed on TSA plates and incubated at 37°C for 24 hours. Vortexing in 10 mL of 0.9% NaCl was carried out to wash off cells on the filter. Serial dilutions were prepared, and aliquots of 100µL were spread-plated on TSA plates supplemented with rifampicin (100 mg/L), kanamycin (30 mg/L), and ampicillin (50 mg/L). Aliquots of 5µl were also inoculated into 1ml of TSB containing rifampicin (100 mg/L), kanamycin (30 mg/L), and a disc of trimethoprim/sulphamethazole(25mg). Donor and recipient were also inoculated on the selective plates and broths for mutant detection. Assays were carried out in duplicate. Transconjugants were confirmed by repetitive extragenic palindromic sequence polymerase chain reaction (Rep-PCR), InC-N and P plasmid typing, and class 1 integron detection.

2.8 Plasmid restriction analysis

Plasmid DNA from donor cells (G1 and G2) and transconjugants were extracted using the Gene-jet plasmid miniprep kit. Plasmid DNA was double-digested using two 6-cutter enzymes: *Pst*1(CTGCA↓G) and *Bst* 17701 (GTA↓TAC), according to manufacturer's instructions (Fermentas, Lithuania). The restriction profile was analyzed in 0.8% Agarose Gel and electrophoresis was run at 40V for 3hr. The gel was stained in ethidium bromide and viewed using a molecular imager.

2.9 Rep-PCR protocol

For this, the following primers: Rep 2I-(NCG ICT TAT CIG GCC TAC) and Rep 2I-(III ICG ICG ICA TCI GGC) were used (Versalovic et al. 1991). Cell suspension of each donor, recipient, and supposed transconjugants was prepared in 100µl of sterile distilled water without boiling and used as a DNA template. The PCR mixture was made up of 11.15µl of sterile MilliQ-water, 3µl of MgCl (Promega, USA), 5µl of NH_4^+ buffer (Promega, USA), 1.5 µ1 of dNTPs (Bioron, Germany), 1.25 µl (of DMSO(Eurobio, France), 1 µl each of primer pair, 0.1µl of GoTaq Flexi DNA Polymerase (Promega, USA) and 1 µl of DNA template.

2.10 16S rDNA PCR

The protocol of Manni et al. (2008) was adopted using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and 1492R (5'GGTTACCTTGTTACGACTT-3'). PCR mixture consists of 14.25µl of sterile MilliQ-water, 2.5µl of lx buffer (Fermentas, USA), 3µl of MgCb fermentas, USA), 1.5µl of dNTPs (Bioron, Germany), 0.75µl each of primers, 15µl of DMSO (Eurobio, France), 0.5µl of Taq DNA polymerase (Fermentas, USA) and 0.5µl of DNA template. The PCR condition was initially denatured at 94°C for 3 mins and followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 mins at 72°C. The final extension was at 72°C for 10 mins. PCR products were run on 1% Agarose Gel in lx 7AE buffer at 80V for 80 min. Amplicons were visualized after staining in ethidium bromide. Products with an expected amplicon size of approximately 1400bp were purified and sent for sequencing.

2.11 PCR product purification

The PCR product was purified using JETQUICK spin column techniques (Genomed, USA) as per Manufacturer's protocol with slight conditional modification. Four hundred microlitres (400µl) of solution HI (binding solution containing guanidine hydrochloride and isopropanol) was added to 20µl of PCR product and mixed thoroughly. A JETQUICK spin column was placed into a 2ml Eppendorf tube, loaded with the mixture, and centrifuged at 13,000xg for 1 minute. After centrifugation, the flow through was discarded. The spin column was inserted into an empty receiver tube, and 500µl of reconstituted solution H_2 (ethanol, NaCl, EDTA, and Tris-HCl) was added. The column was centrifuged at 13,000xg for 1 minute. The flow-through was discarded, and the spin column was placed back in the same receiver tube and centrifuge at maximum speed for 1 minute. DNA was eluted from the spin column with 20µl of sterile dH_20 pre-warmed to 65^0C and centrifuged at 13,000xg for 2 minutes. Eluted DNA was stored in the freezer at -20°C and further used for sequencing.

2.12 DNA sequencing

All purified PCR products were sent for commercial sequencing. Sequences were compared to the NCBI nucleotide sequence database using The Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

2.13 Statistical analysis

Data collected were subjected to Analytical software (SPSS version 16, Quick Calcs online GraphPad, and Microsoft Excel). The chi-square test was used to compare the differences in the number of heavy metal-tolerant bacterial isolates from pristine and hydrocarbon-polluted ecosystems. The rate of occurrence of

plasmids and class 1 integron in bacterial isolates from both ecosystems were compared using Fisher exact test. Correlation analysis was performed to establish a relationship between heavy metal tolerant profiles of bacterial isolates, the incidence of plasmids, and class 1 integron. All statistical testing was performed at a 95% confidence level.

3 Results

3.1 Heavy metal tolerance testing

A total of 345 bacterial isolates were isolated and screened for their ability to tolerate l00µg/ml of Ni, Cr, Cd, Co, V, and 600µg/ml of Pb and Cu, respectively. The result of the study are presented in Table 3; among the 152 isolates from pristine ecosystems, the percentage tolerance to the different heavy metals was 69.7% for Cu; 66.4% for Pb; 41.4% for Cd; 36.2% for Co; 34.9% for Cr; 6.6% for Ni and 5.9% for V. Further, among the 193 isolates from polluted ecosystems (Itu in Akwa Ibom, Odukpani and Calabar in Cross River), the percentage tolerance to all heavy metals tested was above 60% and were as follows: 90.4% for Cu; 89.5% for Pb; 82.4% for Cr; 81.4% for Co; 77.2% for Cd; 74.6% for Ni and 63.4% for V. Comparatively, tolerance to heavy metals among bacterial isolates from polluted ecosystems was significantly greater than ($p<0.01$) those from pristine ecosystems (Table 3).

3.2 Plasmid and integron detection in selected isolates

Table 4 presents the rate of plasmids and Class 1 integrons occurrence among the selected bacteria isolates. However, of the 37 selected heavy metal-resistant isolates from polluted ecosystems, plasmids were detected in 19 (51.4%), while class 1 integrons were detected in 32 (86.5%). As for isolates from pristine ecosystems, plasmids were detected in 12(30.8%) out of 39, while class 1 integrons were detected in 18(42.6%). About 10.3% of isolates from pristine ecosystems harbored plasmids and class 1 integrons whereas 43.2% from polluted ecosystems harbored plasmids and class 1 integrons. The percentage occurrence of plasmids positive-class 1 integrons negative was 20.5% and 43.2% for isolates from pristine and polluted ecosystems, respectively, while plasmid negative-class 1 integrons positive occurrence was 35.9%, and 43.2% for isolates from pristine and polluted ecosystems respectively. The overall pattern/level of plasmids and class 1 integron occurrence was greater amongst isolates from polluted than from pristine ecosystems. The difference in plasmid occurrence among isolates from pristine and polluted ecosystems was not statistically significant. The difference in the number of isolates that harbored class 1 integrons with those that harbored both plasmids and class 1 integrons was statistically significant *($p=0.0003$* and *$p=0$*.0002-Fisher exact test). In addition, plasmid positive-class 1 integrons negative isolates were significantly higher among isolates from polluted ecosystems than those from

Table 3 Heavy metal tolerance among bacteria isolates from pristine and polluted ecosystems
(Number and Percentage of tolerant bacterial isolates)

	Pristine (N=152)	Polluted (N=193)	p-value (X^2)
Pb(600ug/ml)	101(66.4)	173(89.5)	<0.0001
Ni(100ug/ml)	10(6.6)	144(74.6)	<0.0001
Cr(100ug/ml)	53(34.9)	159(82.4)	<0.0001
Cd(100ug/ml)	63(41.4)	149(77.2)	<0.0001
Co(100ug/ml)	55(36.2)	157(81.4)	<0.0001
Cu(600ug/ml)	106(69.7)	175(90.4)	<0.0001
V(100ug/ml)	9(5.9)	122(63.4)	<0.0001

Table 4 Rate of occurrence of plasmids and class 1 integron in bacterial isolates from pristine and polluted ecosystems
(Number and Percentage of occurrence)

Types	Pristine (N=39)	Polluted (N=37)	p-value (Fisher exact test)
Plasmid positive	12(30.8)	19(51.4)	0.1018
Intl 1 Positive	18(46.2)	32(86.5)	0.0003*
Plasmids positive-Intl 1 Positive	4(10.3)	16(43.2)	0.0002*
Plasmids positive- Intl 1 negative	8(20.5)	16(43.2)	0.0481*
Plasmids negative- Intl 1 Positive	14(35.9)	16(43.2)	0.6395

pristine ecosystems. Class 2 or 3 integrons were detected among the selected isolates.

Figure 1 presents the correlation between the incidence of plasmids and heavy metal tolerance. A positive correlation (r=0.786) was observed between the number of bacteria isolates from the polluted ecosystem that harbored plasmids and their ability to tolerate multiple heavy metals, but this correlation was not significant (*P>0.05*). Bacteria isolates from pristine ecosystems that harbored plasmids were also found to tolerate multiple heavy metals, but this correlation was poorly significant (r=-0.414, *P>0.05).* A significant positive correlation (P<0.05, r=0.926) between the number of isolates from the polluted ecosystem that harbored class 1 integrons and their ability to tolerate multiple heavy metals was found (Figure 2). Conversely, a negative non-significant correlation (r=-0.184, *P>0.05*) was the case for class 1 integrons negative isolates from pristine ecosystems and their multiple heavy metal tolerability (Figure 2).

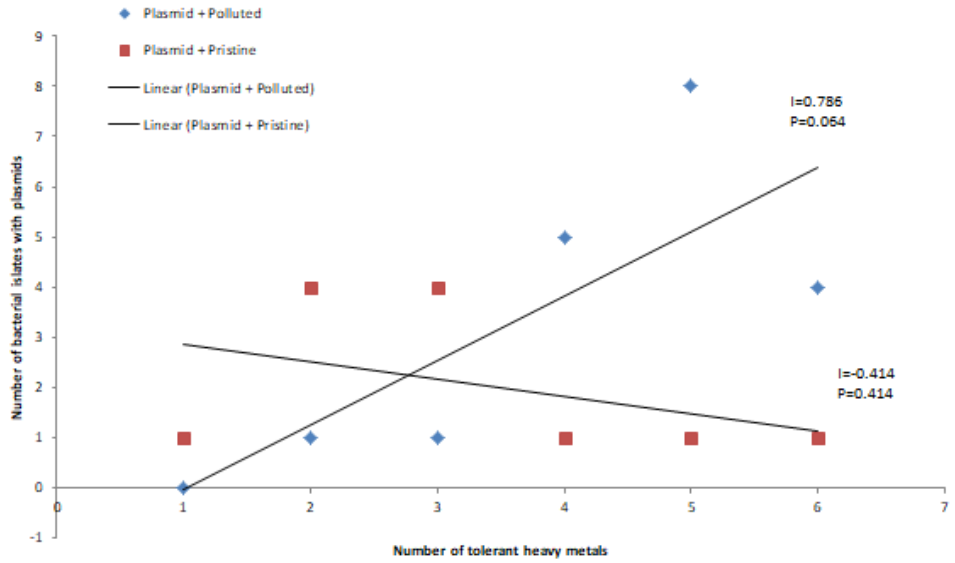

Figure 1 Correlation between heavy metal tolerant profiles with plasmids incidence among bacterial isolates isolated from pristine and polluted ecosystems

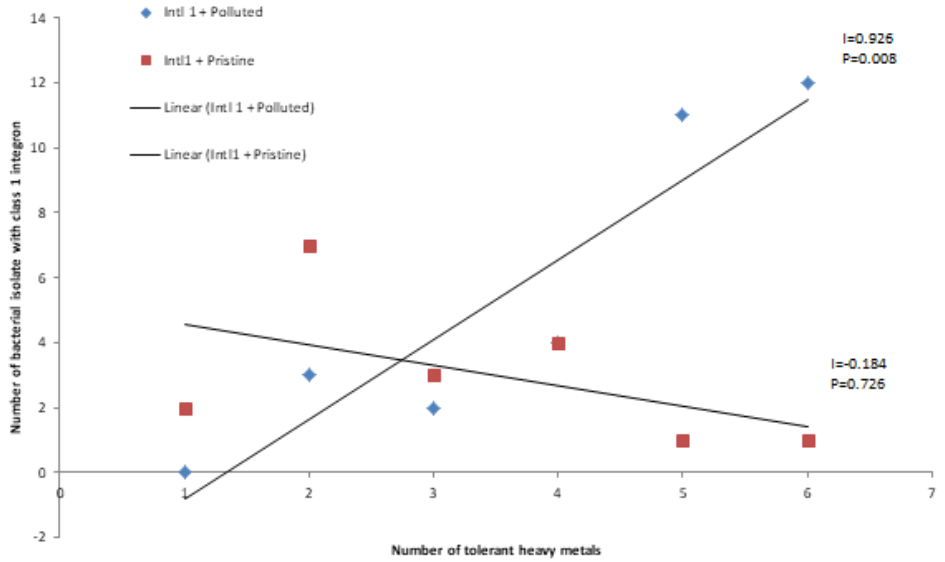

Figure 2 Correlation between heavy metal tolerant profile with the occurrence of class 1 integrons among bacterial isolates from pristine and polluted ecosystems

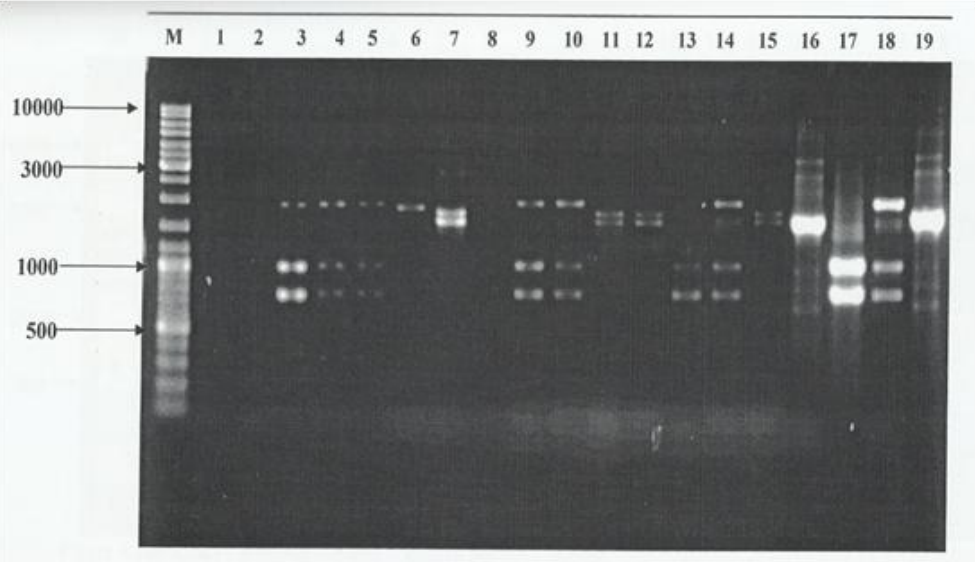

Figure 3 Class 1 integron variable region of bacterial isolates from the pristine ecosystem (M= Molecular maker (bp), 1= *Serratia sp* 4R, 2= *Pseudomonas* sp NSW6, 3= *Serratia* sp CRWI, 4= *Serratia* sp CRSI, 5= *Serratia sp* CRSI4, 6=*Pseudomonas* sp EEW8, 7=*Klebsiell*a sp CRSA13, 8= *Pseudomonas* OKW4, 9 = *Klebsiella sp* OKW2, 10=*Serratia* sp NSS11, 11=*Enterobacter* sp OKW1, 12=*Klebsiella* sp NSW5, 13=*Yersinia* sp NSS1, 14= *Bacillus* sp NSS14, 15= *Enterobacter* spp CRS9, 16= *Enterobacter* sp ITS2, 17= *Aeromonas* sp ITS1, 18 = *Enterobacter* sp ITS5)

Table 5 Distribution of class 1 integron variable region among bacterial isolate from prinstine and polluted ecosystems (Number and Percentage of occurrence)

Variable size (kbp)	Pristine n=18	Polluted n=32	Total (n=50)
6.0	-	1(3.1%)	1(12%)
4.0	-	9(28%)	9(18%)
2.0	7(39%)	7(22%)	14(28%)
1.5	5(28%)	12(38%)	17(34%)
1.2	-	7(22%)	7(14%)
1.0	9(50%)	6(19%)	15(30%)
0.7	9(50%)	3(9.4%)	12(24%)

3.3 Class 1 integron variable region detection

Class 1 integron variable region of bacterial isolates from pristine and polluted ecosystems are presented in Figure 3. Variable sizes between 0.7 and 2.0kb were detected among isolates from pristine ecosystems, while 0.7 to 6.0kb sizes were detected among isolates from petroleum hydrocarbon-polluted ecosystems. The 0.7 and l.0kb regions were highly prevalent among isolates from pristine ecosystems, followed by the 2.0kb region. Among the isolates from polluted ecosystems, the 1.5kb region was the most prevalent and was detected in 12 (38%) out of 32 isolates. Other variable regions detected were 4.0kb (28%), 2.0kb (22%), 1.2kb (22%), 1.0kb (19%), 0.7kb (9.4%), and 6.0kb (3.1%). The 1.5kb region was the most prevalent among isolates from both ecosystems (Table 5).

3.4 Plasmid analysis and typing

Plasmid profiles of some bacterial isolates from pristine and petroleum hydrocarbon-polluted ecosystems are presented in Figure 4. Each plasmid-containing isolate from both pristine and polluted ecosystems harbored at least one plasmid with a size ranging from approximately less than 2.1 kbp to 55 kbp. Most of these plasmids were of low molecular weights, with a few isolates (ITS5, ITS 1, Gl, G2, and G13) harboring high molecular weight plasmids. Plasmid positives isolates which were gram negatives and belonged to the *Enterobactericeae* group examined for the presence of plasmids of IncP-1 subgroups *trfA-α, β,ε, trfy, trfβ,* and 18 plasmid replicon types. The expected amplicon size, specific for IncP-1 subgroups, was not detected. However, plasmid replicon typing PCR revealed the presence of IncN plasmid replicon in

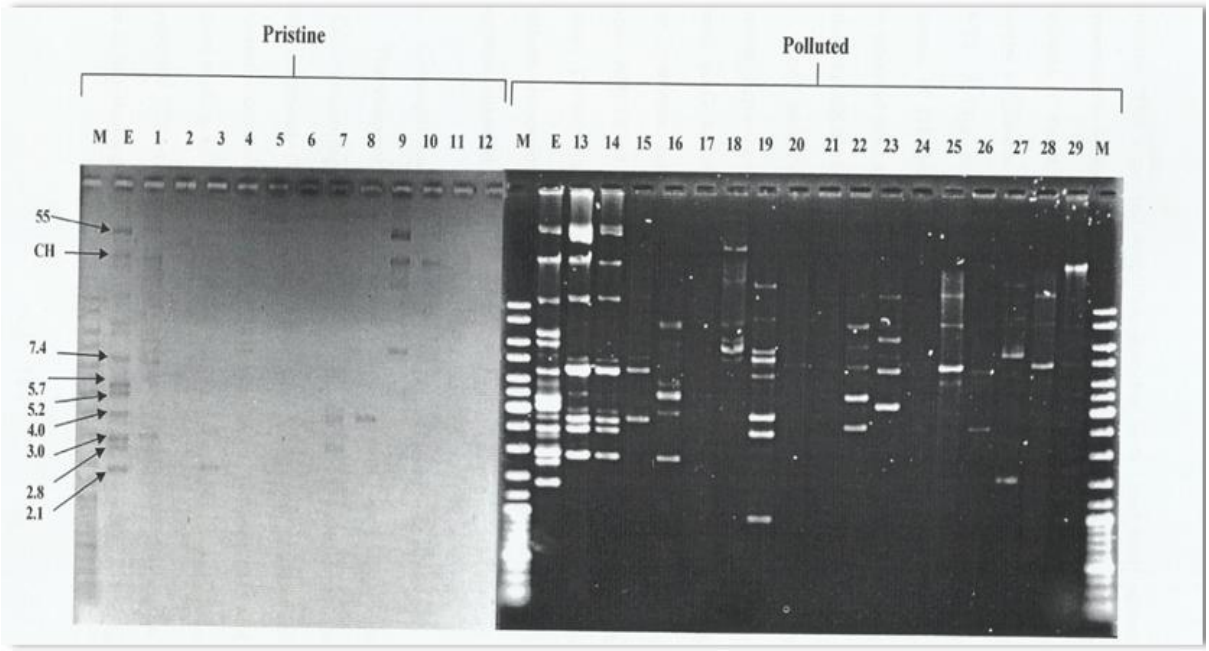

Figure 4 Plasmid profile of some bacteria isolates from pristine and petroleum hydrocarbon polluted ecosystems (M=100bp DNA ladder, E= *E.coli* CV517, 1=ITSS, 2=CRS12, 3= CRS3, 4=CRS8, 5=CRW1, 6=CRW2, 7=EEWI, 8=ITS3, 9=ITSI, 10=GENOMIC DNA, 12=NSS2, 13=GI, 14=G2, 15=G6, 16=F3, 17=E14, 18=G13, 19=G5, 20=HO6W5, 21= HO6W6, 22=G7, 23=E4, 24=F12, 25=FIS5, 26=A10, 28=F11, 29= genomic DNA)

isolates Gl and G2 from petroleum hydrocarbon polluted soil and IncP plasmid replicon in isolates ITS5 and ITS1 from pristine sediments. The expected amplicon size for IncN and IncP plasmid replicons was approximately 559bp and 534bp, respectively. These isolates' IncN and IncP plasmid amplicon sequences (Gl, G2, ITS5, and ITS 1) were analyzed by performing Clustalw alignment and comparing the aligned sequence to the NCBI database using BLAST. A high degree of similarity (100%) was observed between the IncN plasmid sequence of Gl (pCHNGl) and G2 (pCHNG2) and that of completely sequenced IncN plasmid-pNL 194 of *Klebsiella pneumoniae* strain NL194 (GenBank accession number: GU585907.1). IncP plasmid sequence of isolates-ITSl and ITS5 showed 99% similarity to complete sequences of *Salmonella enterica subsp. enteric serovar* Dublin strain 853 plasmid pSD_88 (GenBank accession number: JF267652.1), *Salmonella enterica subsp. enterica serovar Typhimurium* plasmid pYTl DNA (GenBank accession number AB576781.1) uncultured bacterium plasmids PSP21 (GenBank accession number: CP002153.1), PB11 (GenBank accession number: CP002152.1), PB5 (GenBank accession number: CP002151.1) and partial sequence of *Pseudomonas aeruginosa* plasmid R1033 (GenBank accession number: HM804085.1).

3.5 Conjugal transfer of antibiotic resistance and class 1 integron

The fingerprints of suspected transconjugants are presented in Figure 5; Gl and G2 transconjugant fingerprints (Lane 4 and 5) were similar to that of *E. coli* CV601 recipient (Lane 2), indicating that there was a transfer of ampicillin and sulphamethoxazole/trimethoprim resistance from Isolates Gl and G2 to *E. coli* recipient leading to its survival in the presence of selective antibiotics. The fingerprint of suspected ITS 1-transconjugant (Lane 3) differed from that of the *E. coli* recipient (Lane 2) but showed semblance to isolate ITS1 (Lane 2) fingerprint, indicating no conjugal transfer of ampicillin and trimethoprim/sulphamethazole resistance between ITS1 and *E. coli* recipient. Conjugal transfer of class 1 integron from isolates G1 and G2 to *E. coli* recipient was also observed. The expected amplicon size of approximately 280bp was detected in the transconjugants but not in the *E coli* recipient. This signifies the conjugal transfer of class 1 integron, which could be plasmid-borne. As analyzed, the transconjugants' class 1 integron variable region showed the presence of a 1.5 kbp region. It shows the presence of transferred high molecular weight plasmid of approximately 55kbp in G1 and G2-transconjugants (Lane 3 and 5) as compared to plasmid profiles of donors (Gl= Lane 2 and G2= Lane 4) with seven plasmids a piece. The transconjugants' plasmids were further screened to detect the plasmid of the incompatibility group N (IncN). The expected amplicon size of 559bp was detected in transconjugants indicating that the IncN plasmids of donor strains (G1 and G2) were transferred via conjugation. Restriction analysis of the transconjugants plasmids showed identical restriction profiles.

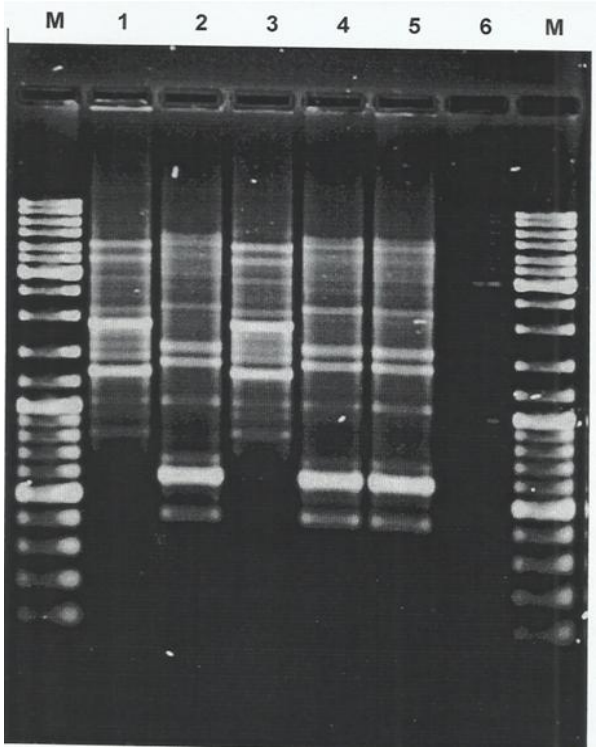

Figure 5 Rep-PCR fingerprint showing conjugal transfer of antibiotics resistance (M=DNA ladder, 1=ITSI, 2=*E.coli* recipient, 3=suspected ITSI= transconjugant, 4= G1- transconjugant, 5=G2- transconjugant, 6=negative control).

4 Discussion

Plasmid incidence in the multiple heavy metal tolerant bacterial isolates obtained from petroleum hydrocarbon polluted ecosystems was higher than their counterpart from the pristine (without pollution) ecosystem, but this difference was not statistically significant. A highly positive but non-significant correlation was observed between the incidence of plasmid and heavy metal tolerance among the bacterial isolates isolated from polluted ecosystems. This insignificant increase and correlation could be due to a mixed population of diverse bacteria genera being studied. Plasmid incidence and the deposal of environmental pollutants have been evaluated and correlated by several authors at various experimental sites. Ndeddy Aka and Babalola (2017) found no significant difference in bacterial plasmid incidence between polluted and unpolluted sites but reported an increase in the frequency of catabolic plasmids in *Pseudomonas*-like isolates in polluted marine and freshwater ecosystems than in unpolluted ecosystems. Similar observations were reported by Epelda et al. (2015) that *Vibrio* spp. from oil-polluted water had a higher incidence of plasmid-bearing strains than isolates from unpolluted water. A similar difference between bacteria isolated from toxic waste-contaminated water and bacteria isolated from either uncontaminated or domestic sewage-affected waters was reported by Tan et al. (2018). The increased incidence of plasmid among the selected bacteria from polluted ecosystems obtained in this study is not significant but suggests plasmid-mediated adaptation in the polluted ecosystems.

On the contrary, Class 1 integron incidence was significantly higher in heavy metal tolerant bacterial isolates from polluted ecosystems than those from the pristine ecosystem. This incidence correlated with the ability of these isolates to tolerate multiple heavy metals. Interestingly, bacterial isolates that harbored both plasmids and class 1 integron were significantly higher in polluted than in pristine samples, suggesting the possibility of the class 1 integron being carried on the plasmid. The diversity of integrons and integron-transferred genes in heavy-metal-contaminated mine tailings has been studied by Nemergut et al. (2004). In their study, they sequenced a gene that codes for a step in a pathway for nitro-aromatic catabolism, a group of compounds associated with mining activity. This implies that integrons may serve a significant purpose during gene transfer in response to selective environmental pressures other than the availability of antibiotics. Significantly high incidence of class 1 integron and its correlation with multiple heavy metal tolerance in bacteria from polluted ecosystems suggest a possible role of gene transfer in response to petroleum hydrocarbon pollution with concomitant heavy metal contamination. The abundance of class 1 integrons and plasmids in this study, confirms the influence of industrial pollution on the mobile genetic elements.

The low incidence of plasmids and integrons, as seen amongst bacteria from the pristine ecosystem, could not be associated with these isolates' heavy metal and multidrug drug resistance profiles. This suggests that other resistance determinants, such as the efflux pump, could have been responsible for the resistance recorded. Bratu et al. (2008) and Nemec et al. (2007) reported the role of efflux pumps in conferring multidrug resistance to gram-negative bacteria. Other works also confirm that efflux pumps confer resistance against many antibacterial agents, including beta-lactams, aminoglycosides, tetracyclines, trimethoprim, fluoroquinolones, and chloramphenicol (Su et al. 2005). Amplified class 1 integron variable region showed great diversity between isolates from pristine and petroleum hydrocarbon-polluted ecosystems. Variable region size diversity for isolates from pristine ecosystems was 0.7kbp to 2.0kbp, while for isolates from polluted ecosystems, the size varied between 0.7kbp and 6.0kbp. This suggests differences and diversity in types of heavy metal tolerance and antibiotic resistance coding gene cassettes in the studied isolates. Li et al. (2017) proposed that class 1 Integrons comprise conserved and stable variable regions, with resistance genes transferred more often as part of the entire integron structure than as individual cassettes. The high prevalence of large class 1 variable region sizes among isolates from petroleum hydrocarbon polluted ecosystems suggests the presence of complex gene cassettes possibly attributable to the complex nature of pollutants in the ecosystems.

Plasmid analysis showed the presence of multiple plasmids in both isolates from pristine and petroleum-polluted ecosystems, but the prevalence was higher among bacterial isolates from petroleum hydrocarbon-polluted ecosystems. Multiplasmid bacterial strains were first described for clinical isolates (Chen et al. 2018). Subsequently, various environmental strains with a high incidence of different plasmids were isolated (Gati et al. 2016). A significant proportion of plasmids ranges from different sizes, copy numbers, and genetic equipment in a bacterium might increase its fitness (Kado, 1998) by accepting adaption to special ecological zones such as petroleum hydrocarbon pollution with concomitant-heavy metal contamination. It is well known that clinical isolates of *E. Coli* usually possess multiple plasmids with different sizes due to exposure to various antibiotics in the treatment process (Jan et al., 2009). Chen et al. (2015) correlate the presence of multiple plasmids in a bacterial strain to the prolonged use of antibiotics. In this study, multiple plasmids in bacterial isolates from polluted ecosystems could be attributed to the long-term exposure of these bacterial isolates to petroleum hydrocarbon and heavy metal pollutants in the environment. Plasmid replicons typing PCR revealed the presence of IncN plasmid replicons in isolates G1 and G2 from petroleum hydrocarbon-polluted soil sample and IncP plasmid replicons in isolates ITS1 and ITS5 from pristine sediment sample. Analysis of the IncN plasmid sequence of G1 (pCHNGl) and G2 (pCHNG2) showed maximum similarity between them and also showed similarities with the IncN plasmid- pNL194 of *Klebsiella pneumonia* strain NL194 (GenBank accession number: GU585907.1). Incompatibility group N plasmid (IncN) is one of the most frequently encountered resistance plasmid types in *Enterobacteriaceae* of human and animal origin (Carattoli 2009). IncN plasmids have been associated with genes conferring resistance to a variety of antibiotics among pathogenic *Klebsiella pneumoniae*, *K. oxytoca*, and *E. coli* strains worldwide (Novais et al. 2007; Shen et al. 2008; Carattoli 2009; Diestra et al. 2009; Gootz et al. 2009; Bortolaia et al. 2010; Cullik et al. 2010; Poirel et al. 2011). This study is the first report on the presence of IncN plasmid in bacterial isolates in Nigeria, particularly from petroleum hydrocarbon polluted ecosystem. The 16S rRNA sequence analysis of isolates G1 and G2 identified preliminary as *Klebsiella* spp using phenotypic and biochemical characteristics showed 99% similarity to the complete sequence of *K. pneumoniae* strain DSM 30104 (GenBank accession number. NR_036794.1). This also further confirms the prevalence of IncN plasmid type in *Enterobacteriaceae.* Though isolates G1 and G2 were multiplasmidic (with seven plasmids a piece), we observed that G1 and G2 transconjugants showed the presence of high molecular weight plasmid of approximately 55kbp which encoded resistance to ampicillin and trimethoprim/sulphamethazole and tolerance to heavy metals (Cd, Ni, and Cu). Analysis of the G1 and G2 transconjugants for IncN plasmid and class 1 integron showed their presence and further confirmed earlier suggestion of plasmid-borne class 1 integron among isolates from petroleum hydrocarbon polluted ecosystems.

Conclusion

Results of the study can be concluded that petroleum hydrocarbon pollution vis-a-vis heavy metals have selective pressure with the abundance of mobile genetic elements amongst isolates from polluted ecosystems could contribute to the dissemination of antibiotic resistance genes, thus posing a severe public health concern.

Declaration

The article's preprint is available on Research square with DOI: https://doi.org/10.21203/rs.3.rs-1771685/v1. The authors declare that this is their original article, and they have taken consent from the Research square team to publish this article in the journal entitled "Journal of Experimental Biology and Agricultural Sciences eissn 2320-8694". If any conflict of interest or plagiarism arises by anybody, journal management can retract this article.

Acknowledgment

May the Lord be glorified for the divine grace given to us to complete this research work.

Ethical Approval

No ethical approval was obtained for the research.

Consent to Participate

This research was a Ph.D. dissertation in which the supervision was carried out among the supervisors (Prof. S.P Antai and Dr. G.D Iwatt).

Authors Contributions

Prof. S.P Antai and Dr. G.D Iwatt designed the Topic and Methodology, while Dr. Agbor R.B, and Dr. Ubi S. E. did the lab work and wrote the manuscript.

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Competing Interests

No competing interests.

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Journal of Experimental Biology and Agricultural Sciences, February - 2023; Volume – 11(1) page 171 – 178

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Influence of Population Growth on Supply, Demand, and Quality Issues of Water Resources in the Yarmouk River Basin in Jordan

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Water supply-demand and quality issues

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Decision-makers

ABSTRACT

This study was carried out to investigate the influence of population growth on supply, demand, and quality issues of water resources in the Yarmouk River Basin in Jordan for twenty years. The population growth data for the years 1997 and 2017 was derived from four Jordan governorates, i.e., Mafraq, Irbid, Jerash, and Ajloun, as well as for the population of the Yarmouk Basin was calculated, where a part of the population of these governorates resides within Basin. The water supply and the number of wells were also determined during this study. Various physicochemical parameters of water, like pH, EC, TDS, DO, NO_2, and NO_3, were also evaluated. Water supply, demand, and quality issues were also identified in collaboration with relevant stakeholders. The study showed an increase in the Kingdom's population in four governorates from about 1.27 to 2.88 million inhabitants, while the population of four governorates in the Yarmouk Basin increased from about 639,992 to 1.53 million inhabitants, and it is more than doubling. Comparing the population with the water supply, the numbers of wells and their uses showed significant changes, as evidenced by the substantial increase in the water supply. The studied physiochemical parameters were within the permissible limits of the National Standards. The critical water issues reported in the study area are difficulties in law enforcement and rapid population growth, which interactively affect the water supply. The study's findings will assist decision-makers in managing future water supplies as they face challenges in securing additional water, and there is an urgent need for research and future scenarios to meet water needs.

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1 Introduction

Rapid population growth has put unprecedented pressure on water resources to meet the population's demand, affecting the quantity and quality of water supplies (Mongelli et al. 2019). By 2050, more than half of the world's population (about 57%) will live in areas of water scarcity for at least one month each year (Boretti and Rosa 2019). Domestic water demand is expected to increase significantly from 2010 to 2050 in all the world regions except Western Europe. The highest water demand will increase (over 300%) in Africa and Asia (Wada et al. 2016). In developing countries, the rapid increase in population pressure has affected agricultural production (Maitima et al. 2009; Shammout et al. 2018), which also causes alterations in the earth's surface and significantly impacts groundwater recharge (Costa et al. 2003; Shammout et al. 2013).

Most studies agree that population growth via urbanization significantly increases the potential for surface runoff in a given basin and reduces the groundwater quantities and qualities (Chow et al. 1988; Shammout et al. 2018). The influence of population on water resource responses by assessing the relationship between uses and water quantities provides a basis for groundwater management practices in basins. Without proper water supply management, changes in the land basin will continue, and the surface storage and capacity of the soil to store water will also be reduced (Chow et al. 1988; Shatanawi and Shammout 2011). Hence, water management techniques may alleviate water scarcity (Boretti and Rosa 2019). There are some crucial points for countries that suffer from water scarcity as Jordan, in terms of water management scenarios, such as raising efficiency in the water distribution system and allocating alternatives to water resources that positively affect the quantities and quality of water supplies and can prevent water deterioration (MWI 2017a).

In Jordan, precipitation is restricted mainly to the winter season, ranging from over 500 mm in the highlands to less than 50 mm in the east. Moreover, about 8% of annual rainfall flows as a flood and recharges groundwater. The supply of available water resources is less than the water demand. According to Jordan's Water Strategy 2009, the country's annual per capita available water is less than 150 m^3 yearly (MWI 2009). By 2025, the per capita share of water is expected to decrease by approximately 90 m^3 per person per year, which might place the Kingdom in a state of water scarcity (El-Naser 2009). This means that the gap between water availability and demand for water resources will increase, the population will suffer from severe water poverty, and water basin biology will likely be threatened (Shammout 2020). Jordan has fifteen surface water basins (MWI 2015; MWI 2016) and two rivers, i.e., Yarmouk and Jordan Rivers, with a permanent flow. Yarmouk River is the crucial water source for the King Abdullah Canal, which supports agricultural expansion in the Jordan Valley (FAO 2009). Figure 1 shows the surface water basins in Jordan and the location of the Yarmouk River Basin among the basins.

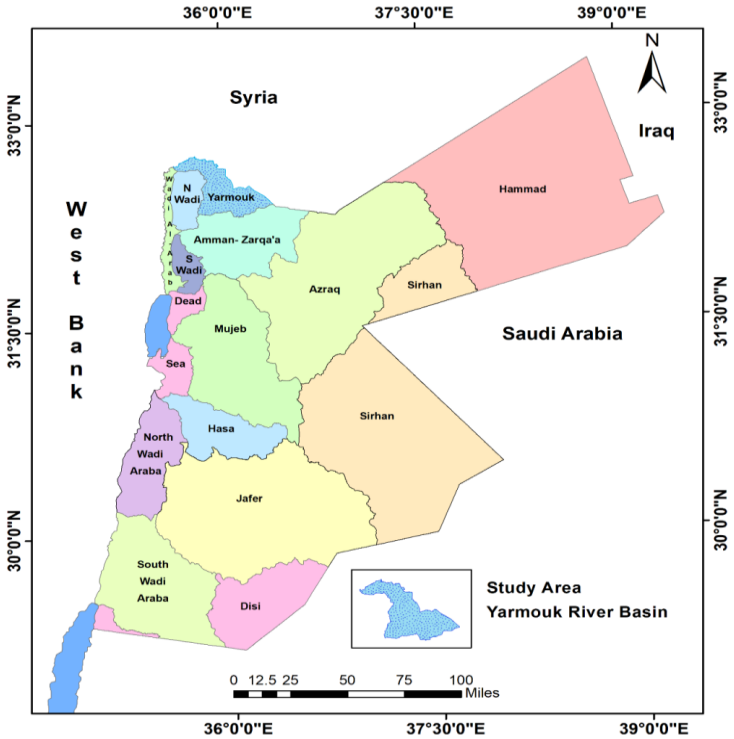

Figure 1 The surface water basins in Jordan and the location of the Yarmouk River Basin among the basins

This study was applied to the Yarmouk River Basin in Jordan, a transboundary water basin. This River Basin is under the pressure of various agricultural, domestic, and industrial activities. The problem of this Basin is exacerbated because water resources systems cannot absorb the shocks caused by the natural contradiction with the sudden increase in population and water uses, increasing in water demand for domestic and irrigation uses. The groundwater and surface water are insufficient to meet domestic and agricultural demand. These problems are attributed to the scarcity of water as well as wide fluctuations in annual rainfall, climate change, and prolonged drought over the past decades. These factors have reduced the water resources and led to the deterioration of the Yarmouk Basin. For these problems, the Yarmouk River Basin was selected as a case study for the project WE/2/08/2017, funded by the Scientific Research and Innovation Support Fund-Ministry of Higher Education and Scientific Research, Jordan. Studying the influence of population growth on supply-demand and quality issues of water resources for the Yarmouk River Basin is essential for understanding and managing the Basin. The specific objectives of this study were to determine the population growth in Jordan for four governorates and the Yarmouk Basin for the years 1979 and 2017. This study was also carried out to assess the influence of the population's needs on responses of groundwater supply wells and quality and identify water supply-demand issues that may help decision-makers manage water resources.

2 Materials and Methods

2.1 Study Site

The Yarmouk River Basin drains an area of about 1393 km^2 inside Jordan, of which more than 50% is covered by vegetation irrigated by the available water resources, mainly groundwater. A warm, semiarid climate characterizes the Yarmouk River Basin, where precipitation especially falls between October and March, and short grasses and drought-resistant shrubs are the dominant vegetation. The average annual rainfall for 1983-2015 was 239 mm/year. In January, an average temperature was recorded above 5°C, while in summer, temperatures may exceed 30°C.

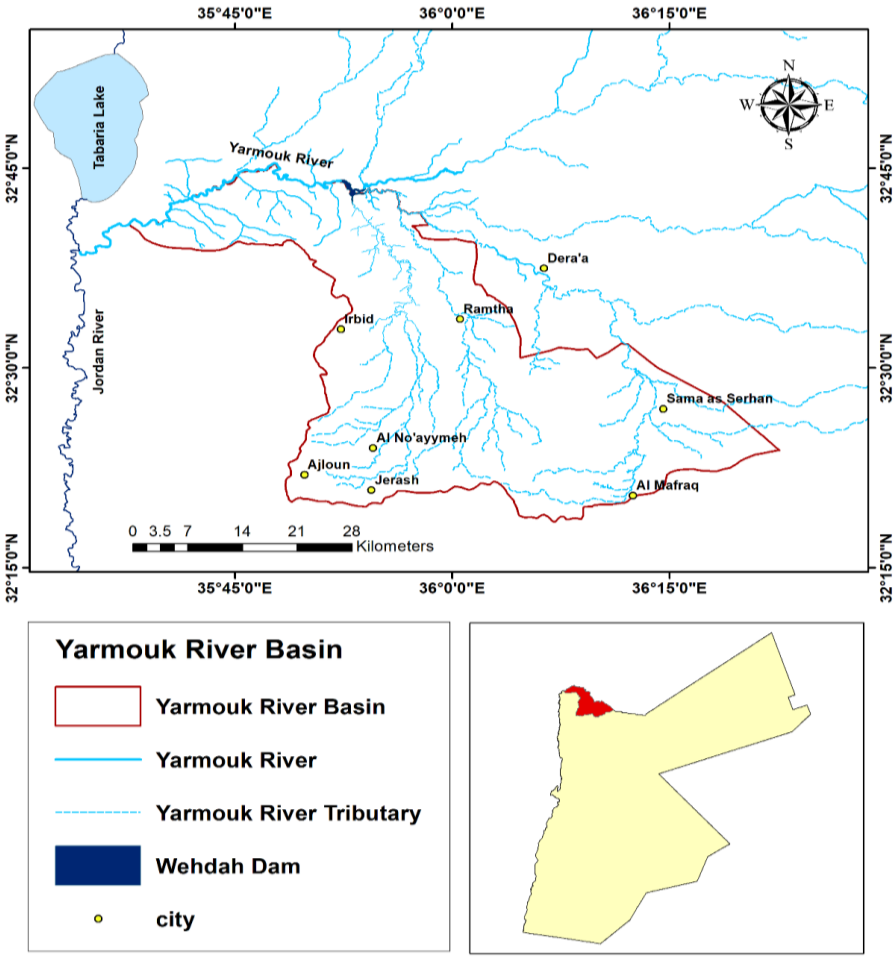

Figure 2 Location of the Yarmouk River Basin and its tributaries, the Yarmouk River, Wehdah Dam, and the main cities.

The Yarmouk flows along the Syria-Jordan border and into the Lower Jordan River. It is considered the main tributary of the Jordan River, and its historic flow was estimated at 480 MCM but is now dramatically reduced. The Yarmouk River provides about 50% of the Jordan River water flow (Avisse et al. 2020). The elevation is about -200 m in the Jordan Valley and about 1150 m in the Basin's upper boundary, RasMunif. The main wadi's included in the study area are Wadi al Shallalah, Wadiar Raggad, Wadi Al Showmar, Al Ghadir Al Abyad, and Shaqq al Barid. The main cities in the Yarmouk Basin in Jordan are Ramtha, Ajloun, Jerash, and Al Mafraq. These cities belong to the four main governorates: Mafraq, Irbid, Jerash, and Ajloun. There are five treatment plants, i.e., Al Akaidar Treatment Plant, Wadi al Shalalah Treatment Plant, Mafraq Treatment Plant, Wadi Hassan Treatment Plant, and Ramtha Treatment Plant in the Basin. The treated wastewater is used for irrigation. The main dam on the Jordanian side of the Yarmouk Basin is Wehdah Dam, which is used for irrigation. Numerous dams and canals in Syria have been built on the river's tributaries in the upper part of the Basin to enhance surface water availability (Obeidat et al. 2019). Figure 2 shows the location of the Yarmouk Basin and its tributaries, the Yarmouk River, Wehdah Dam, and the main cities.

2.2 Determining population growth, quantities, and quality of groundwater wells

In this study, population growth in Jordan for four governorates (Mafraq, Irbid, Jerash, and Ajloun) and the Yarmouk Basin was determined for the years 1979 and 2017 in terms of identifying the basin objects as (a) Hashemite Kingdom of Jordan governorates (b) Yarmouk Basin governorates (c) Communities that are located within the entire Yarmouk River Basin-Jordan and (d) Communities population. These data were determined at the Water, Energy, and Environment Center of the University of Jordan and in cooperation with the relevant Yarmouk Basin water managers. Population and the amount of water provided to the entire Yarmouk Basin were determined. Physicochemical parameters for the Al-Mukheiba wells were also analyzed (Table 1).

Population growth was computed according to the demographic records provided by the Jordanian Department of Statistics (DoS 1997; DoS 2017), as well as the water supply from the basin wells for the same targeted years was computed based on the open files of the Ministry of Water and Irrigation (MWI 1997; MWI 2017b). The total population in the entire Yarmouk Basin was computed, where part of these governorates is located there. The Basin's groundwater wells were used to extract quantities of water supply using ArcMap10.8.1. The number of running wells between 1997 and 2017 was computed to study changes in water quantities and the number of open wells compared to changes in population growth.

Water analyses were conducted in the laboratories of the University of Jordan, and the Ministry of Water and Irrigation, according to the Standard Method (American Public Health Association 2012). The analysis was carried out on Al-Mukheiba wells because these wells are an essential water source in the Yarmouk River Basin and provide domestic water needs. The studied parameters were pH, EC, TDS, DO, NO_2, and NO_3. Table 1 shows the parameters and method number used for analyses.

2.3 Identifying water supply-demand and quality issues

This study builds a network of expertise and knowledge exchange, sharing its findings, generic data, and best practice examples, where information shared by local related stakeholders, experts, and scientists is the basis to bridge the gap between supply and demand and protecting the water resources of the Yarmouk Basin. The following criteria have been implemented to identify water supply-demand and quality issues:

1 Meetings and interviews were conducted to enhance collaboration between the project team, relevant water managers, and decision-makers in identifying water issues and driving forces for preserving the Yarmouk River Basin.

2 Collecting the required data relevant to the Basin and research objectives, these data were compiled from the Department of Statistics, Ministry of Water and Irrigation (MWI), Ministry of Agriculture, Agriculture Department of Mafraq Governorate, and Directorate of Agriculture of Irbid Governorate.

3 Evaluating the collected data to ensure its compatibility with supply-demand describes water shortages and the balance of demand-supply across the Basin.

4 Determining water supply, demand, and quality issues in cooperation with the related water managers.

Table 1 Parameters and method number of analyses as per American Public Health Association (2012).

Parameter	Symbol	Method	Number
Potential of Hydrogen and Dissolved Oxygen	pH, DO	Meter at Field	SM4500-H+B, and 4500 OG
Electrical Conductivity	EC	Laboratory Method	SM 2510B (Ref: CHI-EC)
Total Dissolved Solids	TDS	Calculation by Analysis	1030 E
Nitrite, Nitrate	NO_2, NO_3	Ion Chromatography	SM 4110B

Table 2 Population of governorates in Jordan for the years 1997 and 2017

Governorate	Jordan Governorate Population 1997	Jordan Governorate Population2017	% Growth Rate 1997 to 2017
Mafraq	196,381	580,000	5.66
Irbid	830,901	1,867,000	4.13
Jerash	135,663	250,000	3.10
Ajloun	105,046	185,700	2.89
Total/%	1,267,991	2,882,700	4.2

Table 3 Population, well supply uses, and the number of running wells in the Yarmouk River Basin for the years 1997 and 2017

Yarmouk Basin Population 1997	Yarmouk Basin Population 2017	Yarmouk Basin Domestic Supply 1997	Yarmouk Basin Domestic Supply 2017	Yarmouk Basin Irrigation Supply 1997	Yarmouk Basin Irrigation Supply 2017
639,992	1,526,375	11 MCM (35 Wells)	21.5 MCM (62 Wells)	28.8 MCM (110 Wells)	33.3 MCM (125 Wells)

3 Results and Discussion

3.1 Influence of population growth on the groundwater responses

The influence of population needs on groundwater supply wells responses for 1997 and 2017 (Table 2) shows that Yarmouk River Basin was forceful in terms of population changes. Results presented in Table 2 summarized the population of the governorates in Jordan for the years 1997 and 2017; these data revealed that the population has significantly changed as Mafraq increased from 196,381 to 580,000 inhabitants, Irbid from 830,901 inhabitants to 1.87 million inhabitants, Jerash from 135,663 to 250,000 inhabitants, and Ajloun from 105,046 to 185,700 inhabitants. The overall population of Jordan in four governorates increased from about 1.27 to 2.88 million inhabitants. It is clear (Table 2) that the increases in growth rate percentages over twenty years were concentrated in Mafraq at 5.66%, followed by Irbid at 4.13%, then Jerash at 3.10%, and Ajloun at 2.89%. The reason for this is the influx of Syrian Refugees residing in the Mafraq governorate due to its proximity to the Syrian border (UNHCR 2015). These numbers put pressure on water resources, especially groundwater, to meet the population's needs, where groundwater wells are the primary source in Jordan for water use (MWI 2016; Shammout et al. 2021).

Results presented in Table 3 show the population, water supply uses, and the number of running wells in the Yarmouk River Basin for the years 1997 and 2017. The population of four governorates in the Yarmouk Basin increased from about 639,992 inhabitants to 1.53 million inhabitants. Comparison of the population with water supply, wells numbers, and uses for the years 1997 and 2017 showed significant changes, as evidenced by the observed increase in the population and the substantial increase in water supply for domestic water and irrigation. The quantity of domestic supply wells for the Yarmouk Basin in 1997 was 11 MCM with 35 running wells; in 2017, it was 21.5 MCM with 62 running wells. The quantity of irrigation supply wells for the Yarmouk Basin in 1997 was 28.8 MCM with 110 running wells, while in 2017, it was 33.3 MCM with 125 running wells. Parts of the quantities of these supply wells are also supplied to the Jordan valley to meet the population's domestic and irrigation needs. Hence, without proper management of water resources, the deterioration of the Basin will also increase (MWI 2016; Shammout et al. 2021).

Table 4 revealed the coordinates, water use, water kind, location, and average values of physicochemical analysis of Al-Mukheiba wells. The average pH 7.5, EC 895 µS/cm, TDS 501 mg/L, DO 7.60 mg/L, NO_2 <0.2mg/L and NO_3 <0.5 mg/L was reported. The physicochemical parameters were within the permissible limits as per the National Standards (2015).

Table 4 Coordinates, water use, water kind, location, and average values of physicochemical analysis of Al-Mukheiba wells for the year 2017

Well Name	X WGS 84	Y WGS 84	Water Use	Water Kind	Location	pH	EC (µS/cm)
Al-Mukheiba	35.686173°	32.703759°	Drinking	Groundwater	Irbid	7.5	895
Well Name	TDS (mg/L)	Depth (m)	Wtl (m)	T (©)	DO (mg/L)	NO_2 (mg/L)	NO_3 (mg/L)
Al-Mukheiba	501	1238	11.08	37	7.60	<0.2	<0.5

Table 5 Main water supply-demand and quality issues in the Yarmouk River Basin

Issues	Problems
Water Management	(a) Difficulties in law enforcement, (b) Conflict among sectors and lack of a single responsible authority, and (c)Lack of public awareness and extension programs.
Groundwater Supply-Demand	(a) Decrease in base flow and aquifer recharge in the Basin, (b)Transboundary upstream abstractions resulted in flow decrease in the river, (c) Uncontrolled population growth and activities related to their needs, (d) Over-pumping of groundwater for drinking has affected groundwater quantity, (e) Higher demand for groundwater for domestic uses compared to supply, (f) Pressure on groundwater by the agricultural sector, and (g) Uncontrolled uses as drilling illegal wells.
Quality	(a) Wastewater quantity is increasing with population growth, and the development of sewage systems is highly needed so that water can be reused for agricultural purposes, (b) Land degradation due to overgrazing, deforestation, and urbanization also affects the flood pattern, (c) Unplanned urban growth, and improper land use prevent rainfall from recharging groundwater aquifer, and (d) Over-pumping has exceeded the safe yield limit and thus affected groundwater quality.

3.2 Water supply, demand, and quality issues that may help decision-makers in water resources management

During this study, water supply demand and quality issues were discussed through meetings and interviews with stakeholders in the Yarmouk Basin regarding water management, groundwater supply demand, and Quality. Table 5 shows the primary water supply demand and quality issues in the Yarmouk Basin. The problems attributed to these issues are difficulties in enforcing the law, the conflict between water sectors, the lack of a single responsible authority, and the lack of awareness and extension programs on water issues and water resources management. Another problem associated with the decreased base flow and diminished aquifer recharge of the Basin is the flow in the Yarmouk River is also declining due to upstream abstractions. Also, problems are associated with the growing population activities and their needs. Over-pumping groundwater for drinking has affected groundwater quantity. Compared to supply, high demand for domestic uses creates pressure on groundwater. Further, the agricultural sector and uncontrolled uses such as drilling illegal wells also increase pressure on groundwater. Quality issues are related to the amount of wastewater quantity that increases with population growth, and there is a need to develop sewage systems. Moreover, land degradation due to overgrazing, deforestation, and urbanization affects the flood pattern, and unplanned urban growth and improper land use prevent rainfall from recharging groundwater aquifer (Chow et al. 1988; MWI 2016; Abualhaija et al. 2019; Shammout and Abualhaija 2019; Shammout et al. 2022), and over-pumping has exceeded the safe yield limit and thus affected the quality of groundwater.

Therefore, stakeholders indicated that administrative actions must focus on water technology scenarios, such as reducing losses in the water distribution network, inter-basin water supplies, and reducing consumption against water supply to secure water supplies to meet the population's demand. Moreover, enforcing the law to stop drilling illegal wells will also positively affect.

Jordanian decision-makers face challenges in securing more water for a growing population and agricultural needs. The above results regarding identifying the main water supply, demand, and quality issues of the Yarmouk Basin are of great importance. The results of the study demonstrate that this definition is an important approach for managing water resources because it is greatly influenced by population growth and needs, which can assist water resources managers and decision-makers in sustainable management and finding new sustainable solutions and scenarios for future water supply challenges (Shammout et al. 2013).

Conclusions

The Yarmouk River Basin is crucial for many domestic and irrigation uses. The significant problems related to the Basin's water resources arise from the pressure to meet human needs that generate increasing imbalances between the demand and supply of water. Groundwater wells were identified in terms of uses and quantities related to the targeted years of this research, 1997-2017. The amounts of water used and the number of running wells were calculated compared to population growth and needs. The issues of water supply, demand, and quality were identified in cooperation with related actors in the Yarmouk River Basin.

A significant trend of changes in the population of the Yarmouk Basin River from 1997 until 2017 has been observed. Comparing the population to the water supply and the number of wells also showed significant changes, as evidenced by the substantial increase in water supplies for domestic and irrigation water uses. The analyzed water parameters were within the ideal detection limits as per the National Standards. Water supply, demand, and quality issues were identified and showed that water management, supply-demand, and quality are critical to understanding and coping with the challenges of water supply shortages as they are influenced by population growth and needs. The population has more than doubled in twenty years, and decision-makers must consider this to develop future scenarios and research to manage water supply and demand.

Conflicts of Interest

The authors declare no conflict of interest.

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Optimization of Total Flavonoid Extraction From the *Helicteres hirsuta* Lour. Roots by Bath Ultrasound Assisted method and cytotoxic activities of these Flavonoids

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ABSTRACT

This study was carried out to optimize the various approaches to analyze the effects of various variables on the total flavonoid content extraction from the roots of *Helicteres hirsuta* L. The existence of various compounds in the methanol fraction was accessed by using LC-MS/MS analysis. The results of the study identified the ideal parameters such as times (30 minutes); methanol solvent concentration (50%); ultrasonic frequency (12 Hz); and material/solvent ratio [1:30 (w/v)] for extracting the highest total flavonoids from the roots of *H. Hirsuta*. The study's results suggested that the total flavonoid value was 3.52684 (mg Catechin/g extract). The verified experiment obtained an actual value of 5.205 (mg Catechin/g extract). Further, the results of the study suggested the presence of 20 compounds of a flavonoid nature (66.667%) appearing in the purified methanol fractional extract. These compounds can inhibit DPPH free radicals at 50%, with an IC_{50} value of 536.760 g/mL, and they also have inhibitory activity on the growth of cancer cell lines with IC_{50} values ranging from 115.81 and 219.17g/mL. The human leukemia cell line (HL-60) exhibits the most significant cytotoxic response to a methanol extract from *H. hirsuta* root with an IC_{50} value of 115.81 g/mL.

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1 Introduction

Helicteres hirsuta Lour contains many bioactive compounds that have therapeutic effects on many diseases; hence it is widely used in various herbal and allopathic medicine. All parts of *H. hirsuta* are frequently used in treating people who often have aches and pains, back pain, insomnia, blue skin, and even those with a tired heart (Pham et al. 2016). Recently, different parts of *H. hirsuta* are also utilized in traditional medicine to treat liver cancer (Chin et al. 2006). Similarly, Didna et al. (2007) also established the cytotoxic activity of the *H. hirsuta* extracts.

Many studies have been conducted on the effects of various factors on extracting various active components from different parts of *H. hirsuta* (Pham et al. 2015, 2016, 2017; Jain et al. 2014). Chin et al. (2006) extracted the active components from *H. hirsuta* in Indonesia and obtained six lignans (Chin et al. 2006), while Quang et al. (2018) recovered 12 compounds from *H. hirsuta* collected from Binh Phuoc (Quang et al. 2018). Several significant pharmacological effects of *H. hirsuta* include Antioxidant (Loganayaki et al. 2013; Jain et al. 2014; Phạm et al. 2015, 2016; Hieu et al. 2019), analgesic (Yen et al. 2017), antibacterial, and cytotoxic activities against various *in vitro* cancer cell lines have been demonstrated so far (Pham et al. 2017, 2020; Duyen and Phuoc 2016).

In recent years, flavonoids have drawn much interest for their potential to protect against various chronic illnesses such as cardiovascular disease, neurodegenerative diseases, and many cancers (Vijayan and Tsou 2010; Noorjahan and Saranya 2018; Ahmed et al. 2016). A study on flavonoid extraction from different parts of *H. hirsuta* and its Antioxidant activity has been conducted by Jain et al. (2014) and Phạm et al. (2015, 2017), and reports show that the best solvent for the extraction of flavonoid molecules is methanol. The ideal extraction conditions were tested in Thua Thien Hue to extract the flavonoids in the roots of *H. Hirsuta*, to obtain the maximum flavonoid extract content as a foundation for testing their biological activity. This study was carried out to optimize based on the surface response method (RSM) by Design Expert 11 software to predict the optimization by the expected function method to select the values of the four-factor (solvent concentration, ultrasonic time, material/solvent ratio, and ultrasonic wave frequency) at which total flavonoid collection from the *H. hirsuta* roots leaves was highest.

2 Materials and methods

2.1 Materials plant

The root samples of *H. hirsuta* Lour. (*H. hirsuta* L.) were collected from the Linh Mu pagoda, Kim Long ward, Hue city, Thua Thien Hue province, washed under the water faucet and air-dried in the shade. To be employed in further experiments, the root powder was kept in Polyethylene bags, and stored at room temperature, avoiding light and moisture (Figure 1).

2.2 Experimental design

Total flavonoids were extracted from the roots of *H. Hirsuta* (d < 1 mm) by bath ultrasound-assisted method at 60^{o}C in a methanol solvent (pH = 5) at range 30, 50, and 70 (v/v), ultrasonic time of 30, 50, and 70 minutes with material/solvent ratio is 1:10, 1:20 and 1:30 g/mL (w/v), ultrasonic wave frequency is 10, 12, 14 Hz. The outcomes of the preceding experiment determine the conditions for the subsequent experiments. After surveying the single factors, all four factors that influenced the total flavonoid in *H. hirsuta* roots extract were used to evaluate the influence of these mutual influence of each pair of single factors. Using Design Expert software version 11, the RSM surface response approach was designed for the trials. A quadratic polynomial model displaying the total flavonoid was produced using the regression of analysis approach based on experimental data.

Figure 1 The root samples of *H. hirsuta* L. (A. Roots; B. Root powder)

2.3 Determination of total flavonoid content

The amount of the total flavonoid extracted from the *H. hirsuta* root was determined according to the description by Pham et al. (2017). Catechin standard flavonoid solution (sigma) was diluted to concentrations of 45, 90, 180, 360, and 720 g/mL using methanol 70%.

A series of test tubes containing 2 mL of distilled water and 0.15 mL of $NaNO_2$ was prepared, and then 0.15 mL of Catechin standard (concentrations 45, 90, 180, 360, and 720 µg/mL) was mixed into each test tube, shake well and allow to stand at room temperature for 6 min. Add 0.15 mL $AlCl_3$, shake well, and let stand for 6 min. This was followed by adding 2 mL NaOH and 0.7 mL of distilled water, shaking thoroughly, and letting stand for 15 min. The reaction solution was measured photometrically at 510 nm ($OD_{510\ nm}$) by UV-Vis (U2900 Hitachi, Japan). Each treatment was replicated three times. The results of the $OD_{510\ nm}$ value were recorded, and a calibration line was drawn using Excel 2010. The total flavonoid content from the root extract of *H. hirsuta* was calculated based on the Catechin linear regression equation utilizing the following formula:

$$M = \frac{V_1 * m_1 * n}{V_2 * m_2 * 1000} \left(^{mg}/_{g}\right) \quad (1)$$

Here: V_1: initial extract volume, m_1: total flavonoid content calculated based on Catechin standard curve (µg/mL), n: number of dilutions, V_2: sample volume used for reaction (mL), m_2: initial sample mass (g), 1000: conversion factor from mg to µg.

2.4 Determination of Antioxidant activity

The Antioxidant activity of the methanol extract of *H. Hirsuta* roots was performed based on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method described by Long et al. (2020). The methanol extract was initially diluted into ratios of 1; 0.5; 0.25; 0.125; 0.0625, and 0.03125 mL; control Ascorbic acid solution into concentrations 0.05; 0.67; 0.10; 0.20; 0.50 and 1 mg/mL, and 0.2 mM DPPH solution mixed in 70% ethanol used for the reaction. Free radical scavenging of DPPH was estimated by adding 1 mL of the test sample and 1 mL DPPH (0.2 mM) into each test tube at each dilution, mixing well and allowing it to stand for 30 min in the dark, and then observations were taken at 517nm. For the control, ascorbic acid, similar steps were performed. The formula calculates the result of free radical scavenging of DPPH:

$$\%SC = \frac{OD_c - OD_m}{OD_c} \text{ x } 100 \quad (2)$$

Here: ODm: Valuesoptical density of the test sample; ODc: Valuesblank optical density

standard curve was constructed based on percent inhibition of free radical DPPH obtained at different concentrations. The IC_{50} value was calculated (at their roots extract or Ascorbic acid inhibited 50% free radical DPPH).

2.5 *In vitro* cancer cell line culture

The cytotoxic activity of isolated flavenoids was estimated against the selected cancer cell lines at the Institute of Biotechnology, Vietnam Academy of Science and Technology. The cancer cell lines were grown in monolayers in DMEM medium (Dulbecco's Modified Eagle Medium) containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate adding 10% fetal bovine serum-FBS (Gibco, Invitrogen). After 3-5 days, cancer cells were transplanted on the same media grown in a 1:3 ratio and kept in CO_2 incubators at 37°C and 5% CO_2.

2.6 Cytotoxic assay

2.6.1 Cytotoxic assay for monolayer cultured cancer cell lines

The experiment was conducted to ascertain the total cellular protein content based on the value OD (Optical Density) obtained with stained Sulforhodamine B (SRB, Sigma-Aldrich, USA). The amount of SRB bound to each protein molecule determines the observed OD value, and more cells (and consequently, protein) have a higher OD value. This approach is used by Monks et al. (1991). The method performs as described by Long et al. (2020).

2.6.2 Cytotoxicity assay to suspension cancer cell line (HL-60)

Tim Mosmann's histology method (1983) examined the suspension cell line (HL-60) cytotoxicity *in vitro* (Mosmann 1983). Tetrazolium salt was utilized as a reagent in a colorimetric assay to assess the development of cell survival and detection abilities. The active cell's mitochondria are firmly attached to the reagent's tetrazolium ring. The yellow color of MTT changed to formazan purple under the influence of the dehydrogenase enzyme in cells. The estimations method was performed as described by Long et al. (2020). The % inhibiting cell growth would be determined through the following formula:

$$\%\ \text{Alive cells} = \frac{[OD_{\text{reagent}} - OD_{\text{day 0}}]}{[OD_{\text{negativecontrol}} - OD_{\text{day 0}}]} \text{ x } 100 \quad (3)$$

$$\%\ \text{inhibited cells} = 100 - \%\ \text{alive cells} \quad (4)$$

2.7 Chromatographic and Mass Spectrometry Conditions

The methanol extract (1 µg) was diluted in 500 µL of 70% methanol and 500 µL of formic acid, vortex-mixed, and centrifuged at 14000 rpm/10 min. The supernatant (10 µL) was injected into the LC-MS/MS system for analysis.

Table 1 The levels of experimental design are based on factors

Factor	Variable	Lower level	Base level	Upper level	Interval
A: Time	X_1	30	50	70	20
B: Solventconcentration	X_2	30	50	70	20
C: Ultrasound frequency	X_3	10	12	14	2
D: Ratio of raw material:solvent	X_4	1:10	1:20	1:30	10

The LC-MS/MS assays of the methanol extract were performed for compound identification on the machine Exion LCTM-X500R QTOF (Sciex, USA) with an electrospray ionization (ESI) source. The implementation process was on a Hypersil GOLD Dim. 150x2.1, 3µ (Thermo Scientific, USA) column, at the 30°C column temperature. The mobile phase in chromatography consisted of 0.1% formic acid in water (A), and 0.1% formic acid in acetonitrile (B) was used in the following gradient elution method: 1 min: 98% (A): 2% (B); 20 min: 2% (A): 98% (B) và 25 min: 2% (A): 98% (B). The flow rate was 0.4 mL/min, and using the supernatant 2 µL was injected into the column.

The mass spectrometry was carried out in negative ionization multiple-reaction monitoring (MRM) mode. The source parameters were as follows: the capillary voltage is -4500 V, TOFMS with TOF start mass, and TOF stop mass is 100 and 2000 (Da), respectively, while that for TOFMS/MS is 50 and 2000 (Da). The Collision Gas (CAD) pressure was 7 psi. The mass spectrometer data of compounds were searched for comparison on the NIST2017 spectrum library of the American Academy of Science and Technology (https://chemdata.nist.gov/).

2.8 Statistical analysis

The experimental values are expressed as the mean of the measurements over the three experimental replications plus the standard deviation. The mean was compared using Duncan's test and the ANOVA analysis of variance. The analytical values were statistically significant, $p < 0.05$, based on Excel 2010 and IBM SPSS Statistics 20.

3 Results and discussion

3.1 Optimization of suitable conditions for total flavonoid extraction

Following research on the impact of each univariate element on total flavonoid extraction, optimization of these factors' reciprocal influence in the extraction process is necessary. This work forecasts the optimization issue by the "expected function" technique using the RSM surface method to choose the best values of four influencing parameters at maximum total flavonoid content. The regression analysis is based on experimental data collection of a quadratic polynomial model displaying the total flavonoid produced. The collected data were assessed using ANOVA.

The experiment was designed to predict the maximum total flavonoid content. The four single elements studied in this study are time, solvent concentration, material/solvent ratio, and specific ultrasonic frequency from X1 to X4 were employed as the four variables. The upper and lower-level values of every single factor are presented in table 1. The surface approach has 27 conditional trials designed, including 3 in the center. Table 2 displays each experiment's total flavonoid content findings (Table 2).

Table 2 shows that the flavonoids found in *H. Hirsuta* roots extract ranged from 2.426 to 5.768 mg (mg Catechin/g extract). Tables 3 and 4 demonstrate that the model's constituent parts exhibit a high significance level; most second-order values have $p < 0.05$. The above notation indicates that four quadratic values (X_1^2, X_2^2, X_3^2, and X_4^2) participating in all models show a high confidence level of over 95% and that the linear factors (X_1-X_4) and interactive factors (X_1X_2, X_1X_3, X_1X_4, X_2X_3, X_2X_4, and X_3X_4) do not participate in the regression equation because $p > 0.05$ (Tables 3 and 4).

The regression equation derived represents the relationship between total flavonoid with independent variables based on the Box-Behnken model as follows:

$$Y = 5.41 - 1.31\ X_1^2 - 0.8286\ X_2^2 - 0.9172\ X_3^2 - 0.6657 X_4^2 \quad (1)$$

Here: Y: Total flavonoid content (mg Catechin/g extract)

The setup regression equation has a highly significant statistical difference, as demonstrated by the Fisher F test model value (F = 3.6100) and the low probability p-value (p = 0.0158), as represented in Table 3.

Alternatively, the correlation coefficient R^2 is used to evaluate how well the model fits. According to the model analysis results in table 3, the model's R2 value collection is 80.830%; R2 - (adj) = 58.460%; and all p values exhibit high statistically significant differences. An acceptable model is required when R^2 is at least 80% (Xiao and Yao 2008). It is ideal for investigating the agreement between the actual data and the theory to produce

Table 2 Experimental design for the matrix of four factors affecting

No	Expression X_1 Time (min)	X_2 Solvent concentration (%)	X_3 Ultrasound frequency (Hz)	X_4 Ratio of raw material:solvent (w/v)	Y Total flavonotid content (mg Catechin/g extract)
1	50	50	10	1:10	3.269±0.008
2	30	50	10	1:20	3.812±0.199
3	50	30	10	1:20	3.116±0.003
4	70	50	12	1:10	4.140±0.123
5	50	50	14	1:30	3.821±0.125
6	70	50	10	1:20	4.946±0.015
7	30	50	12	1:10	3.991±0.544
8	50	50	10	1:30	3.808±0.005
9	50	50	12	1:20	2.877±0.009
10	70	70	12	1:20	3.171±0.021
11	70	50	14	1:20	3.294±0.007
12	50	30	14	1:20	3.416±0.004
13	50	50	14	1:10	3.442±0.020
14	30	70	12	1:20	3.092±0.055
15	50	50	12	1:20	3.283±0.199
16	30	30	12	1:20	3.866±0.800
17	50	50	12	1:20	3.439±0.001
18	70	30	12	1:20	2.426±0.289
19	50	70	14	1:20	3.191±0.444
20	50	30	12	1:10	3.443±0.003
21	30	50	14	1:20	2.855±0.286
22	70	50	12	1:30	3.822±0.015
23	50	70	12	1:10	5.048±0.024
24	50	30	12	1:30	3.712±0.007
25	30	50	12	1:30	5.301±0.015
26	50	70	12	1:30	5.176±0.007
27	50	70	10	1:20	5.768±0.145

results that are not statistically significant (based on the Lack of Fit test). There is no statistical significance, as evidenced by the data in table 3, where F (3.2900) and p (0.2556) values are both > 0.05. Therefore, the model created based on the variables chosen for our experiment is appropriate and exhibits excellent between the experimental and anticipated values (Table 3).

The regression equation can hypothetically predict the total flavonoid value collection from the *H. hirsuta* root. Figure 2 depicts the reciprocal effect between time, solvent concentration, material/solvent ratio, and ultrasonic wave frequency using the ultrasonic tank method and the total flavonoid of the *H. hirsuta* roots extract. Previous studies show that the total flavonoid extraction efficiency rose along with the extraction duration, ultrasonic frequency, and raw material/solvent ratio. To a certain extent, though, if we keep boosting these elements, the extraction efficiency tends to drop (Figure 2).

Table 3 Analysis of variance (ANOVA) of the regression equation

Source	Sum of squares	Degrees of freedom	Mean square	F Value	P Value	
Model	14.3200	1	1.0200	3.6100	0.0158	significant
X_1	0.1245	1	0.1245	0.4399	0.5197	
X_2	0.0001	1	0.0001	0.0005	0.9833	
X_3	0.4506	1	0.4506	1.59	0.2311	
X_4	0.2633	1	0.2633	0.9299	0.3539	
X_1X_2	0.0580	1	0.0580	0.2048	0.6590	
X_1X_3	0.3997	1	0.3997	1.4100	0.2578	
X_1X_4	0.0074	1	0.0074	0.0262	0.8740	
X_2X_3	0.2177	1	0.2177	0.7690	0.3977	
X_2X_4	1.3300	1	1.3300	4.6800	0.0513	
X_3X_4	0.4282	1	0.4282	1.5100	0.2423	
X_1^2	9.1800	1	9.1800	32.4100	0.0001	
X_2^2	3.6600	1	3.6600	12.9300	0.0037	
X_3^2	4.4900	1	4.4900	15.8500	0.0018	
X_4^2	2.3600	12	2.3600	8.3500	0.0136	
Residual	3.4000	1	0.2831			
Lack-of-Fit	3.2000	10	0.3203	3.2900	0.2556	not significant
Pure Error	0.1947	2	0.0974			
Total	17.7200	26				

R - Sq =80.830%; R^2 - (adj) = 58.460%

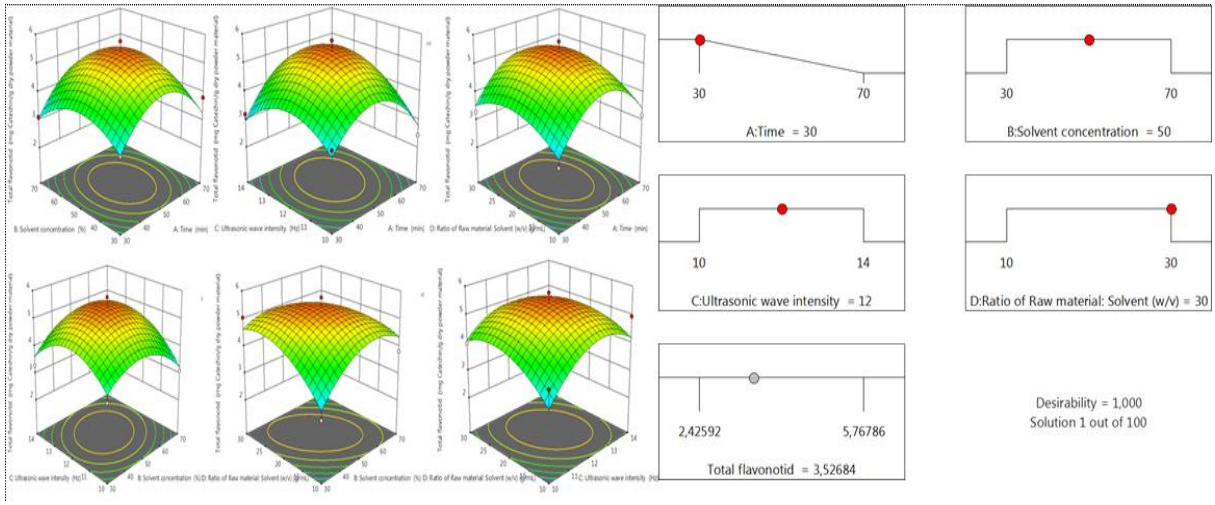

Figure 2 Expected function, response surfaces in 3D, and ranges of conditional values for optimal total flavonoid content

We needed to conduct experiments with conditions like time ultrasonic (30 minutes), methanol solvent concentration (50%), ultrasonic frequency (12 Hz), and raw material/solvent ratio (1:30 (w/v)) to obtain the total flavonoid content from the root of *H. hirsuta* under the condition as predicted by theoretical calculations. As a result, the total flavonoid content collection is 3.52684 (mg Catchin/g extract) (Figure 2). Meanwhile, the experiment's total flavonoid content under optimal conditions was 5.205 (mg Catechin/g extract), more significant than the model's theoretical calculation (Table 5). Research by Pham et al. (2017), the results indicated that the sample/solvent ratio had the most substantial impact on bioactive compounds and the Antioxidant power of *H. Hirsuta* with the optimum extraction conditions, including a temperature of 60°C, time of 35 min, the ratio of 1:100 g/mL

Table 4 Significance levels of regression coefficients

Factor	Coef	SE Coef	95% CI Low	95% CI High
β_o	5.4100	0.3072	4.7500	6.0800
X_1	0.1019	0.1536	-0.2328	0.4365
X_2	0.0033	0.1536	-0.3314	0.3379
X_3	0.1938	0.1536	-0.1409	0.5284
X_4	0.1481	0.1536	-0.1865	0.4828
X_1X_2	0.1204	0.2660	-0.4593	0.7000
X_1X_3	0.3161	0.2660	-0.2636	0.8958
X_1X_4	-0.0431	0.2660	-0.6227	0.5366
X_2X_3	0.2333	0.2660	-0.3464	0.8130
X_2X_4	-0.5758	0.2660	-1.1600	0.0038
X_3X_4	-0.3272	0.2660	-0.9069	0.2525
X_1^2	-1.3100	0.2304	-1.8100	-0.8096
X_2^2	-0.8286	0.2304	-1.3300	-0.3266
X_3^2	-0.9172	0.2304	-1.4200	-0.4152
X_4^2	-0.6657	0.2304	-1.1700	-0.1637

(sample/solvent) in 40% (v/v) methanol solvent. The highest total phenolic and flavonoid levels were 16.87 mg GAE/g and 17.55 mg CE/g, respectively (Pham et al. 2017). These results obtained two times higher flavonoids than our study when extracting on roots samples of *H. Hirsuta* collection Thua Thien Hue based on bath ultrasound-assisted method.

3.2 Assessment of the existing compounds in the methanol fraction

The results of LC-MS/MS analysis identified negative ions $[M+H]^-$ with an m/z value corresponding to each compound was recorded by high-resolution mass spectrometry analysis, and a negative ion measurement mode was obtained by LC-MS/MS analysis of ion fragmentation in negative ion measurement mode obtained the primary m/z fragmentation as presented in Table 5. The retention periods for the compounds ranged from 5.45 to 18.62 minutes at the detection wavelength of 350 nm (λ= 350 nm). The results of comparing the LC-MS/MS spectra of the compounds collected in the methanol fraction from roots of *H. hirsuta* with the standard LC-MS/MS data spectrum on the NIST/PubChem data spectrum bank with the identified natural active substances. Results of the study showed the presence of thirty compounds with similarity ranging from 95 to 100%, of which 20 compounds were of a flavonoid nature (66.667%), 7 compounds were of a nature phenolic (23.333%), 1 polyphenol compound (3.333%) and 2 alkaloids (6.667%) (Figure 3 and Table 5).

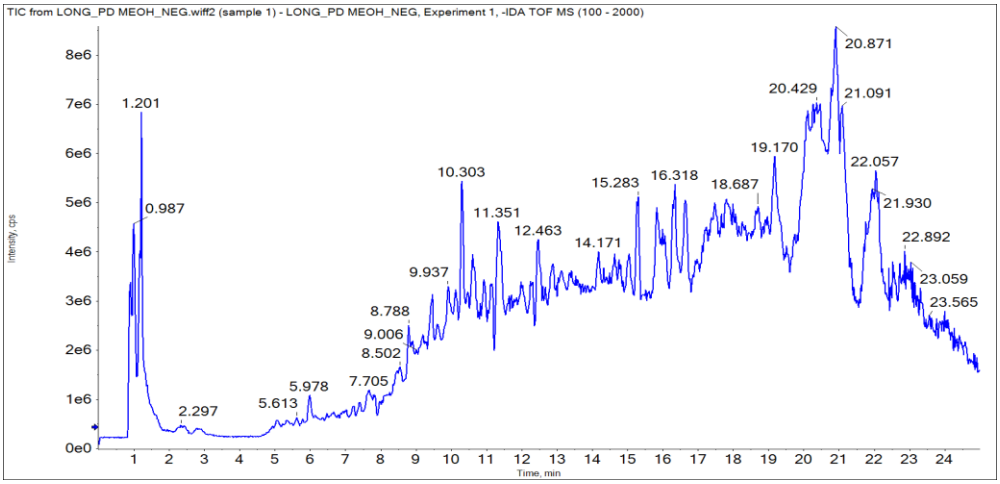

Figure 3 GC-MS spectral chromatogram analysis of segment extract methanol from the root of H. *hirsuta*

Table 5 The results analysis LC-MS/MS

No.	Retention Time	Precursor Mass [M-H]-	Library NIST/PubChem	Molecular formula	MS/MS Spectrum	Library Score
1	5.45	325.11	4'-Acetoxy-7-hydroxy-6-methoxyisoflavone*	$C_{18}H_{14}O_6$	124.0059; 195.0434; 223.0385	100
2	9.92	447.09	Luteolin 7-glucoside*	$C_{21}H_{20}O_{11}$	151.0038; 174.9570; 227.0336; 229.0477; 255.0274; 256.0350; 284.0308; 285.0386; 300.0236; 327.0463; 405.2091	100
3	9.96	285.04	Fisetin*	$C_{15}H_{10}O_6$	163.0033; 258.0412; 285.0407; 286.0445; 287.0469	97.80
4	10.06	431.10	Apigenin 7-glucoside*	$C_{21}H_{20}O_{10}$	241.1427; 268.0355; 269.0437; 311.0521	100
5	10.15	301.03	Quercetine*	$C_{15}H_{10}O_7$	121.029; 151.002; 107.011; 93.033; 139.039	98.40
6	10.36	187.10	Azelaic acid*	$C_9H_{16}O_4$	57.0339; 80.0251; 95.0495; 97.0649; 123.0806; 125.0961; 169.0837	99.60
67	10.59	461.07	Scutellarin*	$C_{21}H_{18}O_{12}$	59.0134; 85.0269; 99.0090; 113.0231; 213.0529; 241.0487; 283.0225; 284.0317; 285.0388	99.20
8	10.60	285.04	6,7,3',4'-Tetrahydroxyflavone*	$C_{15}H_{10}O_6$	183.0109; 197.0273	97.10
9	10.95	161.02	4-Hydroxycoumarin*	$C_9H_6O_3$	163.0375; 121.0275; 164.0414; 122.0315; 119.0481	95.10
10	11.16	593.13	Poncirin*	$C_{28}H_{34}O_{14}$	85.0292; 153.0197; 161.0620; 195.0300; 287.0923	100
11	11.74	315.05	6-Methoxyluteolin*	$C_{16}H_{12}O_7$	136.9884; 227.035; 228.0414; 243.0292; 300.0269;	97.30
12	12.87	299.05	Hispidulin*	$C_{16}H_{12}O_6$	79.9558; 183.0109; 239.0728	98.60
13	12.19	285.04	16.alpha.-Hydroxyestrone*	$C_{18}H_{22}O_3$	93.0341; 107.0132; 143.0486; 154.0403; 159.0446; 163.0013; 171.0430; 173.0631; 211.0387; 214.0267; 229.0492; 239.0329; 243.0277; 255.0276	98.10
14	13.57	285.05	Kaempferol*	$C_{15}H_{10}O_6$	93.0341; 107.0132; 143.0486	99.60
15	14.80	595.28	Neoeriocitrin*	$C_{27}H_{32}O_{15}$	151.0036; 135.0453; 459.115; 287.0554; 152.0076	93.70
16	16.57	297.15	Ricinoleic acid*	$C_{17}H_{14}O_5$	119,0496; 155,1060; 183,0106; 184,0186; 297,2312	97.90
17	17.32	593.27	Vitexin 4-O-glucoside*	$C_{27}H_{30}O_{15}$	78,9583; 152,9946; 241,0100; 277,2152; 315,0459; 413,2071	76.50
18	17.84	577.26	Apigenin 7-O-neohesperidoside*	$C_{27}H_{30}O_{14}$	63,9615; 71,0128; 80,9642; 85,0285; 94,9798; 101,02344	95.20
19	18.62	297.15	6,4'-Dimethoxy-7- hydroxyisoflavone*	$C_{17}H_{14}O_5$	79.9553; 119.0487; 155.9855; 170.0031; 183.0104	99.70
20	10.95	161.02	6-Hydroxycoumarin*	$C_9H_6O_3$	133.0274; 143.8884	98.00
21	6.15	153.02	3,4-Dihydroxybenzoic acid**	$C_7H_6O_4$	65.0027; 81.0335; 91.0182; 108.0206	98.50
22	7.01	181.05	p-Hydroxyphenyllactic acid**	$C_9H_{10}O_4$	72.9920; 107.0494; 119.0487; 135.0436; 134.0363; 163.0389	95.00
23	7.31	137.02	3,4-Dihydroxybenzaldehyde**	$C_7H_6O_3$	65.0027; 81.0337; 92.0259; 93.0339; 108.0207; 109.2085	99.60
24	8.00	163.04	trans-2-Hydroxycinnamic acid**	$C_9H_8O_3$	119.0501; 162.8392	100
25	10.32	359.07	(R)-rosmarinic acid**	$C_{18}H_{16}O_8$	72.9923; 96.9599; 135.0438; 179.0339; 197.0439	98.00
26	10.95	181.05	3,4-Dihydroxyhydrocinnamic acid**	$C_9H_{10}O_4$	93.0339; 121.0281; 122.0359; 123.0433; 136.9204	100
27	15.97	194.14	2,4,6-Trichlorophenol**	$C_6H_3Cl_3O$	194.9178; 158.9412	100
28	10.31	179.03	6-Fluoro-4-hydroxycoumarin***	$C_9H_5FO_3$	136; 136; 187.9	100
29	10.21	144.04	2-Hydroxyquinoline****	C_9H_7NO	146.0599; 147.0627; 118.0647; 128.049; 117.0566	100
30	13.73	293.21	Myristyl sulfate****	$C_{14}H_{30}O_4S$	293.1789; 294.1808;295.1748	100

Note: *: flavonoid/ flavone/ flavonoid glycoside; **: phenolic; ***: polyphenol; ****: alkaloid

Table 6 Flavonoid content and DPPH free radical scavenging activity of methanol extract from roots of *H. hirsuta*

Concentration of extraction (mg/mL)	Total flavonoid content (mg Catechin/g extract)	% free radical scavenging activity SC
0.03125	0.201 ± 0.514	14.692 ± 5.011
0.0625	0.397 ± 1.424	24.582 ±2.007
0.125	0.773 ± 1.710	41.308 ±2.061
0.25	1.497 ± 1.711	55.396 ±2.296
0.5	3.123 ± 3.832	73.044 ±2.318
1	5.205 ± 2.090	88.066 ±1.112

Table 7 IC_{50} values of methanol extract of roots *H. hirsute* and ascorbic acid control

Sample name	Equation	R^2	IC_{50} (µg/mL)
Extract methanol	y = 21.696ln(x) + 87.110	0.995	536.760± 0.021
Acid ascorbic	y = 26.678 ln(x) + 212.4	0.983	0.002 ± 0.001

3.3 Biological activities of methanol fraction from roots of *H. hirsuta* L.

3.3.1 Antioxidant activity

The onset of cancer and other illnesses may be influenced by cell damage brought on by free radicals. Antioxidants are recognized as compounds with the capacity to bind free radicals, thereby reducing the damage that causes the development of several diseases in people (Liu et al. 2014). The study of DPPH free radical scavenging activity yielded diverse findings in the methanol fraction from roots of *H. Hirsuta* when it was diluted to different quantities (tables 6 and 7). The examined data demonstrated statistically significant differences with $p < 0.05$ (Duncan's test), and the maximum DPPH free radical scavenging activity was reported at 1 mg/mL (5.205 mg Catechin/g extract) being 88.06% in the methanol fraction extract from the roots of *H. hirsuta* with an IC_{50} value of 536.76 g/mL. Ascorbic acid, a free radical scavenger frequently used as a standard, yielded a considerably better result than this (Tables 6 and 7).

3.3.2 Cytotoxic activity

The study on the cytotoxicity to some cancer cell lines of methanol extract of *H. hirsuta* roots *in vitro* conditions shown in table 8 revealed that the cytotoxicity to the cell lines was at the average level with IC_{50} values ranging from 115.81 – 219.17 µg/mL. The best inhibitory effect on the human leukemia cell line (HL-60) with IC_{50} = 115.81µg/mL (Table 8). However, the best inhibitory action against the human leukemia cell lines (HL-60) and human liver cancer cell line (HepG2) was observed at 79.83% (HL-60) and 79.22% (HepG2), respectively, at the concentration of 200 g/mL methanol extract. Next is Human carcinoma of the mouth cell line (KB) with inhibitory potencies of 77.46%. The human

Table 8 Effects of the *H. hirsuta* methanolic roots extract against the various cancer cell lines

Ext. Con. (µg/mL)	Inhibitory effect of *H. hirsuta* extract methanol roots extract on cell lines (%)									
	MCF-7	SK-LU-1	HepG2	Hela	SW480	MKN-7	KB	SK-Mel-2	LNCaP	HL-60
200	48.67 ± 1.12	45.62± 2.11	79.22 ± 1.37	58.31 ± 1.52	69.08 ± 1.28	62.27 ± 1.01	77.460± 2.26	65.72 ± 1.01	71.20 ± 2.62	79.83 ± 1.47
100	29.15 ± 0.94	23.45± 1.06	43.37 ± 1.27	36.52 ± 1.07	43.83 ± 1.45	30.82 ± 1.65	46.32 ± 2.50	47.15 ± 1.25	42.08 ± 2.13	64.04 ± 2.03
20	3.23 ± 0.50	11.94± 0.29	18.66 ± 1.10	12.57 ± 1.03	18.84 ± 0.17	13.02 ± 1.91	25.12 ± 1.27	12.48 ± 1.08	19.14 ± 1.75	23.72 ± 1.78
4	1.50 ± 0.42	1.68 ± 0.31	7.24 ± 0.46	1.15 ± 0.17	4.23 ± 0.71	2.11 ± 0.49	4.39 ± 1.65	2.68 ± 0.44	3.01 ± 0.82	4.13 ± 0.49
0.8	-1.42 ± 1.21	-0.64 ± 0.14	1.54 ± 0.82	-1.04 ± 0.37	2.15 ± 0.36	-0.27 ± 0.12	1.21 ± 0.09	0.47 ± 0.27	0.17 ± 0.19	0.53 ± 0.12
IC_{50}	197.86± 1.16	219.17± 1.75	119.04± 246	162.300±1.27	133.13±2.47	159.69 ±1.160	117.73±1.62	137.87±1.32	131.45±1.05	115.81±1.54
Con. (µg/mL)	Inhibitions of Ellipticine on cell lines									
	MCF-7	SK-LU-1	HepG2	Hela	SW480	MKN-7	KB	SK-Mel-2	LNCaP	HL-60
IC_{50}	0.42±0.03	0.51±0.04	0.45±0.03	0.39±0.03	0.44±0.02	0.41±0.05	0.37±0.03	0.41±0.04	0.38±0.03	0.48±0.03

Note: The concentration of Ellipticine used in the test was 10 -2-0.4-0.08 µg/mL; Ext. Con. = Extract Concentrations

breast cancer and the human lung carcinoma cell lines (SK-LU-1) displayed the lowest activity levels with inhibitory potencies of 48.67% and 45.62%, respectively (Table 8).

Research on the oxidative and cytotoxic activity of extracts from different parts of *H. Hirsuta* collected in Vietnam has been studied by many researchers. Research by Duyen and Phuoc (2016) shows that two extracts, i.e., petroleum ether (PE) and dichloromethane (DC), are showing cytotoxic activity against the Hep-G2 cell line, with percentage CS values less than 50%. Two samples with active expression were selected for further testing to find the IC_{50} value. The IC_{50} value of PE extracts was 28.29 µg/mL, and DC extracts were 30.30 µg/mL. The methanol (MeOH) fraction has not shown cytotoxic activity against the Hep-G2 cell line (Duyen and Phuoc 2016). However, according to this study, the 200 µg/mL concentration of *H. hirsuta* roots methanol extract exhibited the most substantial inhibitory effect against the human leukemia cell line HL-60 (79.83%), followed by the human liver cancer cell line HepG2 (79.22%). Thuy (2018) showed that the Antioxidant activity of ethanol extracts (IC_{50} = 60.83µg/mL) was higher than that of chloroform extract (IC_{50} = 74.58µg/mL). However, the HepG2 hepatotoxic activity of chloroform extracts (IC_{50} = 9.17µg/mL) was more potent than that of alcohol extract (IC_{50} = 19.96µg/mL). All of these results are significantly superior to the findings of this study. According to a study in Indonesia, *H. Hirsuta* can fight against cancer cells, especially liver cancer (Chin et al. 2006).

Conclusion

This study determined the optimal conditions and factors affecting the extraction of total flavonoids from *H. hirsuta* roots collected from the Thua Thien Hue province of Vietnam. The methanol solvent content (50%), ultrasonic wave frequency (12), the material/solvent ratio (1:30 (w/v)), and time ultrasonic (30 minutes) are the ideal conditions for producing the highest concentration of total flavonoids from *H. hirsuta* roots (3.52684 mg Catechin/g extract). The experiment's total flavonoid content under optimal conditions was 5.205 (mg Catechin/g extract), which was greater than the theoretical calculation of the model. The best linear regression equation to get the maximum total flavonoid content from the root of *H. hirsuta* (Y mg Catechin/g extract) is: Y = 5.41– 1.31 X12 – 0.8286 X22 – 0.9172 X32 – 0.6657X42 (1)

From the methanolic fraction extracted from the roots of *H. hirsuta* total of 30 compounds have been identified, of which 20 were flavonoid in nature (66.667%) based on the analysis of the results using the LC-MS/MS method. The DPPH free radical scavenging activity of the methanol fraction extracted from the roots of *H. hirsuta* was high (IC_{50} = 536.760 g/mL). The inhibitory effect against various cancer lines was moderate (IC_{50} fluctuated between 115.81 and 219.17 g/mL). The human leukemia cell line (HL-60) exhibits the highest cytotoxic response to a methanol extract from *H. hirsuta* root with an IC_{50} value of 115.81 g/mL.

According to the findings, the root of *H. hirsuta* may be used as a therapeutic herb for its Antioxidant and cancer cell-inhibiting properties. More research should be done to find biologically active substances and their pharmacological mechanisms of action.

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Conflict of interest

All authors declare that they have no conflict of interest

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Effects of Regenerative Agriculture Technologies on the Productivity of Cowpea in the Drylands of Embu County, Kenya

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ABSTRACT

Cowpea *(Vigna unguiculata)* is an important indigenous multi-purpose crop grown in arid and semiarid areas of Sub-Saharan Africa (SSA). The cowpea has nutritional and economic value, especially for smallholder farmers in dry lands. However, poor farming practices have declined cowpea productivity over the years. Low soil nutrient replenishment exacerbates the situation, leading to low soil fertility. Uptake of regenerative agriculture (RA) technologies is critical to building more resilient ecosystems that improve soil fertility and agricultural productivity while mitigating climate change effects. This study was carried out to evaluate the impact of the uptake of RA technologies on the productivity of cowpea in the dry lands of Embu County, Kenya. A survey involving 400 farming households was conducted using a semi-structured questionnaire. Descriptive statistics and a stochastic log-linearized Cobb-Douglas production function were used for the data analysis. The study results showed that RA technologies commonly used by farming households were: cereal-legume intercrop, mulching, minimum tillage, crop rotations, pasture cropping, organic agriculture, and compost manure. The findings also revealed that inputs, farm size, labour cost, and used manure amount positively influenced cowpea productivity. The results also showed that cereal-legume intercrop, crop rotations, pasture cropping, and organic agriculture significantly influenced cowpea productivity, while minimum tillage showed a negative relationship. Therefore, the current study's results recommend that the uptake of RA technologies should be scaled to scale up cowpea productivity in dry lands. The study contributes to determining appropriate technologies for cowpea production in arid and semiarid areas. These results will help the government, policymakers, and other inventors to make the right decisions while disseminating or introducing innovations in dry areas.

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1 Introduction

Cowpea *(Vigna unguiculata)* belongs to the Family of Fabacea. It is one of the most important annual legume crops in the world. The crop is ranked the second most important legume crop in Sub-Saharan Africa and third in Kenya (Gupta et al. 2019; Njonjo et al. 2019). It's a multi-purpose indigenous crop grown in arid and semiarid areas (Owade et al. 2020a) whose grains and leaves are utilized for human consumption and as livestock feeds, respectively (Owade et al. 2020a). Cowpea leaves contain many essential nutrients, such as vitamins and minerals, that have the potential to improve household nutritional status and food security (Owade et al. 2020b). Most households in dry lands depend on cowpea for food, income, soil management, and also as a source of animal feeds (Gewa et al. 2021).

Cowpea is a drought-tolerant crop that performed well in SSA, especially in dry lands where water stress and low soil fertility are evident, unlike other legumes. In Kenya, 85% of cowpea production areas lie in the Eastern region, characterized by arid and semiarid conditions (Njonjo et al. 2019). The crop is commonly grown in mixed farming systems dominated by sorghum and millet intercropping (Nelson et al. 2021). This crop suits the farming system due to its shade tolerance and early maturity characteristics (Saidaiah et al. 2021). The crop also helps in ecosystem management by improving soil fertility through nitrogen fixation. It gives an alternative to using inorganic fertilizers, especially to resource-constrained farmers in dry areas (Nyaga and Njeru 2020). Despite the various economic importance and nutritional benefits, the crop has been neglected and has a limited value chain (Mfeka et al. 2019).

The productivity of cowpea is reportedly declining globally, with farmers realizing about 260 kg/ha despite increasing land under cowpea production (Njonjo et al. 2019). In East Africa, Kenya holds the most prominent land under cowpea production (FAOSTAT 2019), albeit a low productivity trend attributed to extreme weather conditions and poor application of recommended agronomic practices (Kephe et al. 2021). According to Elrick et al. (2022), regenerative agriculture (RA) technologies offer solutions to these problems and opportunities to scale up productivity, profitability, and household food security while ensuring environmental sustainability.

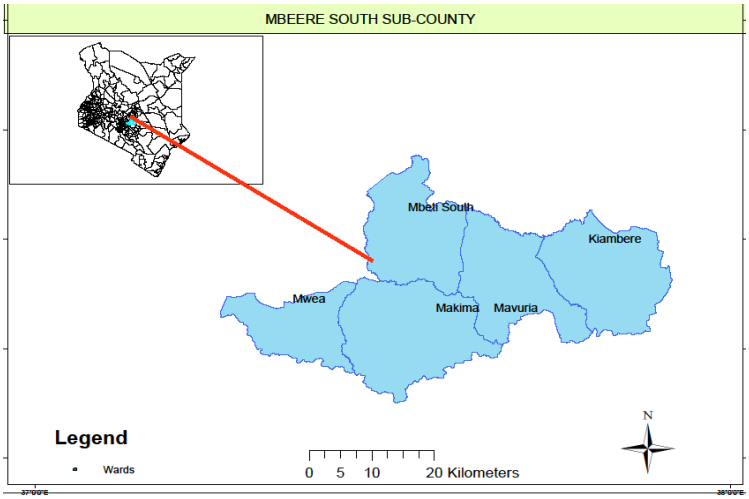
The regenerative agriculture approach uses soil conservation at the entry point to regenerate and contribute to numerous provisions, regulations, and ecosystem-supporting services to improve environmental, social, and economic dimensions (Schreefel et al. 2020). This approach is anchored to the four principles, i.e., integrated pest management practices, advances in plant breeding, soil fertility practices, and integrated crop-animal systems (Schulte et al. 2022). RA emphasizes not or low using synthetic fertilizers or pesticides. It is suggested as an alternate food production method with less adverse environmental and social effects or none at all. It aims to improve soil health and restore highly degraded soil fertility and land productivity through external inputs, utilization of on-farm inputs, integration of crops with livestock, and reducing or eliminating of tillage (Newton et al. 2020). Practices that underpin RA include intercropping cereal crops with legumes, mulching, cover cropping, agroforestry, controlled traffic, pasture cropping, minimum tillage, crop rotations, organic agriculture, and use of compost manure (Lal 2020). These innovations have been suggested to offer opportunities to farmers, especially in dry lands. RA technologies can improve ecosystems by regenerating degraded soils, scaling productivity, increasing household incomes, and boosting food security. However, no RA practice fits all the practices in different soils and agroecological zones (Lal 2020). Thus farmers should adopt more than one technology simultaneously, allowing the use of closely related practices.

The process of adopting innovations remains an important aspect in most developing countries. In literature, innovation and technologies have been used interchangeably (Worku 2019). The uptake of agricultural innovations is influenced by the farmer's perceptions, personality, and social characteristics (Rosario et al. 2022). Scholars have used three models to describe the adoption process, i.e., innovation-diffusion, economic constraint, and perception of adoption (Dissanayake et al. 2022). The diffusion of innovation model considers if the technology is technically and culturally relevant to users. The economic model considers the affordability of the technology to local users, while the adoption model's perception considers the various aspects of the technology that influence farmers' adoption behavior (Ikehi et al. 2022). Demonstrating that, despite the innovator's best efforts to develop innovations that can have a favorable impact on production, farmers will nevertheless view the technologies differently (Dissanayake et al. 2022). Thus, researchers need to collaborate with farmers to discover issues that may hinder the uptake of the innovations they come up with (Ikehi et al. 2022). This can be achieved through farmer training and creating awareness in every stage of technology development. Based on these theoretical models, regenerative agriculture (RA) technologies were disseminated to farmers in the study area. However, the effects of various technologies on cowpea productivity haven't been documented. Therefore, this study is designed to evaluate the impact of the uptake of regenerative agriculture (RA) technologies on cowpea productivity in the dry lands of Embu County at the household level.

2 Materials and methods

2.1 Study area

The study was conducted in Mbeere South Sub County, Embu County, Kenya. The area is a Lower Midland (LM4)



agroecological zone with hot and dry conditions suitable for drought-tolerant crops and livestock keeping. Pigeon peas, sorghum, millet, green grams, and cowpeas are the crops commonly grown in the area (Kiboi et al. 2019; Muthee et al. 2019). The Sub County is located on the South-Eastern slopes of Mt Kenya at an altitude of 700M to 900M above sea level. The rainfall is bi-modal, with long rains from mid-March to June and short rains from mid-October to February, with annual rainfall ranging between 700mm to 900mm. The mean annual temperatures range from 20.7^{0}C to 22.5^{0}C with a latitude of 0°46′S and a longitude of 37°39′E (Wafula et al. 2022). The sub-county covers approximately 1,312 km^2 with a population of about 163,476 (KNBS 2019). Cowpea productivity has declined over the years following poor farming methods that lead to nutrient mining. This has led to low agricultural productivity posing a threat to household food security as most of the households in the area rely on rain-fed small-scale agriculture.

2.2 Experimental design

The research design for the study was a cross-sectional survey. The study targeted approximately 27,274 rural-based farming households in Mbeere South Sub County (KNBS 2019). The following Cochran formula has been used to estimate the sample size:

$$n_{0=}n_{0=}\frac{Z^2PQ}{d^2}=\frac{(1.96)^2(0.5)(0.5)}{(0.049)^2}=400 \quad (1)$$

Where n_0 = required sample size, Z = (1.96) t value from normal table, p = (0.5) probability of success, q = (0.5) probability of failure and d= (0.049) desired level of precision

The respondents were chosen using a purposeful multistage stratified sampling method with probability proportionate to size. Mbeere South Sub-County was purposively selected based on its semiarid characteristics, its potential in cereal and pulse production, and interventions on RA. In the first stage, all five wards in the selected Sub-County were selected. The second stage involved choosing one sub-location randomly from each ward, and the final step involved choosing one village randomly from each sub-location. Using a sample frame obtained from the ward agricultural offices, a probability proportionate to size sampling approach was utilized to determine the total number of families to be questioned in each village.

2.3 Data collection and statistical analysis

A semi-structured questionnaire was used to collect data from 400 respondents. The questionnaire had three sections, i.e., demographic characteristics of the respondents, information on cowpea production, and information on RA. Using the Open Data Kit (ODK) software, the questionnaire was programmed into an electronic format and pre-tested for validity and reliability, and all the reported mistakes were fixed before it was distributed to the respondents. Trained enumerators did the daily data uploading and administration of the surveys.

Data collected were analyzed using Statistical Package for Social Sciences (SPSS version 27) computer program. Basic descriptive statistics, such as frequencies and percentages, were performed. The effects of RA technologies on cowpeas productivity were evaluated, and a stochastic log-linearized Cobb-Douglas production function was used (Isaboke and Musyoka 2022). The production function is expressed as;

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \cdots + \beta_n X_n + \alpha_1 Z_1 + \cdots + \alpha_n Z_n + \epsilon \quad (2)$$

Considering the natural logarithm, the production function is expressed as;

$$\ln Y = \ln\beta_0 + \beta_1 \ln X_1 + \beta_2 \ln X_2 + \beta_3 X_3 + \cdots + \beta_n \ln X_n + \alpha_1 Z_1 + \cdots + \alpha_n Z_n + \epsilon \quad (3)$$

Where Y=Cowpea yield produced in Kilograms, $\beta 0$= intercept, X_1 to Xn = inputs used in production, β_1 to βn = parameter estimates of the explanatory variables, α_1 to α_n= coefficients of RA technologies, $z_1 to zn$= RA technologies, ln= natural logarithm, and ε is the error term.

3 Results

3.1 Socioeconomic characteristics of the households

Table 1 shows the household's socioeconomic characteristics and reports that most household heads were aged between 31-50 years (44.25%) while the youths comprised 24.50% only. Further, most households were headed by males (59.75%), and females directed a few (40.25%). Most (80.75%) of the studied respondents were married, while only 19.25% were unmarried. Results of the study also suggested that most household heads (50.00%) attained only a primary level of education, and only (5.25%) attained post-secondary education. Regarding farming experience, 37.75% of the household heads had farming experience between 10-20 years, and most households (45%) owned 2-5 acres of land. The main occupation for most households (86%) was crop farming. In addition, a few household heads (39.00%) also engaged in the off-farm activity.

3.2 Main Regenerative Agriculture technologies used by farming households

The leading RA technologies commonly used by farming households in the area of study are summarized in Table 2. The results of the study revealed that cereal-legume intercrop (69.75%), mulching (74.50%), minimum tillage (30.50%), use of compost manure (26.50%), pasture cropping (70.00%), crop rotations (93.75) and organic agriculture (77.75%) are the some common RA technologies which have been used by the respondents.

Table 1 Socioeconomic characteristics of the households

Variable	Group	Frequency	Percentage (%)
Age (years)	18-30	98	24.50
	31-50	177	44.25
	More than 50	125	31.25
Gender	Male	239	59.75
	Female	161	40.25
Marital status	Married	323	80.75
	Not married	77	19.25
Education level	None	74	18.50
	Primary	200	50.00
	Secondary	105	26.25
	Post-secondary	21	5.25
Main occupation	Crop farming	344	86.00
	Crop and livestock	30	7.50
	Salaried worker	8	2.00
	Self-employed	18	4.50
Experience in farming (years)	Less than 10	127	31.75
	10-20	151	37.75
	More than 20	122	30.50
Off-farm occupation	Yes	156	39.00
	No	244	1.00
Total land holding (acres)	Less than 2	59	14.75
	2-5	180	45.00
	More than 5	161	40.25

Table 2 Descriptive results for RA technologies commonly used by farming households

Technology	Frequency	Percentage (%)
Cereal legume intercrop	279	69.75
Mulching	298	74.50
Minimum tillage	122	30.50
Use of compost manure	106	26.50
Pasture cropping	280	70.00
Crop rotations	375	93.75
Organic agriculture	311	77.75

3.3 Effects of Regenerative Agriculture technologies on the productivity of cowpeas

The combined effect of RA technologies and inputs on cowpeas productivity was estimated using a stochastic log linearized Cobb-Douglas production function. Multiple regression procedures in SPSS software were used for model development. The results of Cob-Douglas multiple regressions (Table 3) showed that the model gave an R-square value of 0.7625, which implies that the explanatory variables explained 76% of variations in cowpeas productivity in the study area. The F value (100.31) was highly significant at 1 % (0.000). The tolerance value for each variable was computed to test the significance of regression coefficients. The results revealed t-values greater than 0.1 for significant variables suggesting an increased difference between the null hypothesis and the variables. VIF values for all the explanatory variables were below 5, implying that multicollinearity between the variables was not significant.

Four inputs, i.e., cost of seeds, cost of labour, farm size, and quantity of manure, were included in the production function. Farm size was statistically significant at 1% with a factor of 0.706, while the amount of manure used was significant at 5% with a factor of 0.042. These data suggested that increasing land size under cowpea production by 1% would increase cowpea productivity by 70.6%. On the other hand, increasing the quantity of manure used in cowpea production by 1% increases cowpea productivity by 4.2%. The cost of seeds and labour was also not significant. Seven RA technologies were introduced in the production function to estimate their effects on cowpea productivity (Table 3).

Cereal-legume intercrop, minimum tillage, and pasture cropping were significant at 1%, while organic agriculture and crop rotations were positive and significant at 5%. On the other hand, mulching and the use of compost manure were insignificant. The coefficient of cereal-legume intercrop was positive (0.122) and significant at a 1% level (t=3.704, p=0.000), implying that farmers who

Table 3 Log linearized Cobb-Douglas Multiple Regression Results for Effects of Regenerative Agriculture Technologies on cowpeas Productivity

Variables	Parameters	Beta	SE	t-Value	P-Value	VIF
Constant	β_0	1.820	5.217	0.350	0.727	
Inputs						
Lncost of seeds(Ksh)	β_1	-0.029	0.013	-1.12	0.263	1.05
Lncost of labour (Ksh)	β_2	0.011	0.000	0.45	0.654	1.04
Ln farm size	β_3	0.706	1.307	24.00	0.000***	1.37
Ln manure in Kgs	β_4	0.042	0.018	5.210	0.011**	1.09
RA Technologies						
Cereal-legume intercrop	α_1	0.122	2.114	3.70	0.000***	1.70
Crop rotations	α_2	0.038	3.899	1.44	0.028**	1.13
Mulching	α_3	-0.017	1.759	-0.68	0.499	1.05
Minimum tillage	α_4	-0.078	1.705	-2.84	0.005***	1.19
Pasture cropping	α_5	0.164	2.130	4.90	0.000***	1.70
Organic agriculture	α_6	0.058	2.021	2.06	0.040**	1.24
Use of compost	α_7	-0.012	1.886	-0.43	0.669	1.21
R-squared						0.7625
Prob>F						0.000
Mean VIF						1.25

***significant at 1% and **significant at 5%.

intercropped cowpeas with cereals realized 12.2% higher cowpeas yield compared to non-users of this technology. The results further revealed that rotating cowpeas with other crops would affect cowpea productivity positively (0.038) and significantly (0.028), suggesting that increasing land under crop rotations by 1% would increase cowpea yield by 3.8%. Minimum tillage had a negative coefficient (-0.078) and was significant at a 1 % level (t=-2.843, p= (0.005), implying that practicing minimum tillage decreases cowpeas yield by 7.8%. Further, Pasture cropping was positively significant at a 1% level (t=4.995, p=0.000) with a coefficient of 0.164, implying growing cowpeas together with pasture crops would increase cowpeas yield by 16.4%. Further, the results indicated that practicing organic agriculture positively (0.058) and significantly (t=0.058, p=0.040) influenced cowpeas productivity at a 5% level. Suggesting that increased use of organic inputs in cowpeas production by 1% increases yield by 5.8%.

4 Discussion

4.1 Main Regenerative Agriculture technologies commonly used by farming households

The leading RA technologies used by farming households were cereal-legume intercrop, crop rotations, organic agriculture, minimum tillage, mulching, pasture cropping, and compost manure. These findings corroborate with Mpanga et al. (2021), who noted that smallholder farmers commonly adopt these technologies, especially those in dry areas in Kenya, where extreme weather events dominate.

4.2 Effect of the use of Regenerative Agriculture technologies on the productivity of cowpea

Cowpeas productivity was hypothesized to be a function of factor inputs and RA technologies. The combined effect was estimated using a stochastic Log linearized Cobb-Douglas production function. The calculated results revealed that the quantity of manure used positively influenced cowpea productivity. The findings resonate with Islam et al. (2021), who noted that manure application at the recommended rates significantly affected agricultural productivity. Drylands are usually water-stressed, and using organic manure helps increase microbial activity and water retention capacity, leading to increased grain yield. In addition, organic inputs help improve soil quality and reduce the use of chemical fertilizers, which ensures environmental quality to mitigate climate change effects (Kareem et al. 2021).

Farm size has significantly and positively influenced cowpea's productivity. This indicates that farmers with large farms and adopting RA technologies were likely to realize more cowpea yield per unit area than those with smaller farms, which could be associated with more space to practice innovations and increased plant population. These results are similar to those of Moronge and Nyamweya (2019), who noted that farm size influences adoption as large land gives space to experiment and practice innovations, which could lead to increased productivity. According to Shah et al. (2021), farm size and the process of technology uptake have a relationship with agricultural productivity.

Intercropping cereals with legumes in the study area showed a positive and significant association with the production of cowpeas. Intercropping implies that adopters of this technology had a 1.2% higher cowpea yield than non-adopters. According to descriptive data, 71.3% of the respondents (Table 1) used cereal legume intercrop technology. Intercropping has been proven to have many advantages on cropping systems, including ecological balance, more utilization of resources, enhancement of crop productivity, and sustainability in agricultural production (Maitra and Gitari 2020). A study by Weih et al. (2021) on the effects of intercrop components on yield stability showed that cereal legume experiments had higher yield stability than sole crop experiments.

The relationship between crop rotations and cowpea productivity was positive and significant, indicating that rotating cowpeas with other crops, especially nonlegumes, could increase cowpea productivity. Moreover, rotating pulse crops with cereal crops increases economic returns and reduces nitrogen fertilizer use. The study of Liu et al. (2020) on crop rotation of pea and lintel crops with wheat in semiarid areas suggested that crop rotation positively affected crop productivity. This enhancement in crop productivity was associated with the combined effects of nitrogen benefits between a cereal and a pulse and also gave soil water conservation benefits from branched and deep-rooted legumes that allow for water and nutrient uptake during stressful conditions (Zhao et al. 2022). In addition, rotating cereals and pulses help in pest and disease control, improve soil health, and significantly influence grain yield (Darai et al. 2021).

The results also revealed that uptake of minimum tillage reduced yield by 7.8%, suggesting that cowpea farmers who adopted minimum tillage were likely to get lower yield than non-adopters. The findings of Jena (2019) minimum tillage did not positively impact yield improvement on the adopter. The lower yield can be associated with the agroecological conditions of the study area. The impacts of new innovations on agricultural productivity are highly dependent on rainfall, soil types, inorganic inputs used, and socioeconomic drivers such as returns from production (Mwaura et al. 2021). However, the brighter side of minimum tillage is labour saving. According to Jena (2019), there is significant labor saving from minimum tillage, especially for women. Women provide more farm labour than men in most developing countries. Thus, minimum tillage has a labour-saving impact on farming households headed by women.

Pasture cropping also significantly influenced cowpea's productivity, and farmers who planted cowpeas with a pasture crop were more likely to have a 1.6% yield increase than those who did not. Pasture cropping involves planting a cereal or legume crop into a living perennial pasture, and adopters of this innovation gain more profits (Cougnon et al. 2022). Pastures help control pests, diseases, and weeds and serve as livestock feed (Luna et al. 2020). They also help in ecosystem sustainability by reducing nitrate leaching, controlling soil erosion, and improving water infiltration (Martin et al. 2020). As RA attempts to ensure environmental sustainability, cultivating cowpeas alongside pastures will increase agricultural productivity while restoring degraded soils.

Further, the findings show that the uptake of organic agriculture significantly affects cowpeas productivity in the study area, and the use of organic inputs in the production of cowpeas will increase yield by 0.5%. In comparison between organic and conventional agriculture, it was reported that net returns from organic agriculture were higher than those from traditional farming (Durham and Mizik 2021). The higher returns were attributed to fewer financial inputs as organic farming heavily relies on ecosystem service providers such as biological weed and pest control. Earlier empirical evidence suggests that organic farms are more profitable, environmentally friendly, and produce nutritious food with low chemical residues (Soni et al. 2022).

Conclusion

In conclusion, the current study's findings showed that cereal-legume intercrop, pasture cropping, organic agriculture, mulching, and crop rotations were highly adopted, while minimum tillage and use of compost manure had low uptake. Further, this low uptake could be associated with unfavorable weather conditions in the area of study as well as limited knowledge of the benefits that come with the use of these technologies among cowpea farmers. However, minimum tillage had a significant negative association. This information will help the government and other inventors make appropriate decisions while disseminating or introducing innovations to farmers in dry areas. Thus, farmer exposure through knowledge dissemination could increase the adoption of appropriate technologies to increase cowpea productivity and household food security. Further inventors need to find ways to deal with farmer perceptions and economic well-being that come with innovations to encourage the utilization of new opportunities. The findings are vital in implementing programs to improve agricultural productivity and household food situations in Kenya and other similar environmental countries.

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Estimation of Carbon pool in various agricultural crops in peatlands of West and Central Kalimantan, Indonesia

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ABSTRACT

Tropical peat is an important natural ecosystem, and its natural state plays an important role in climate regulation. These peatlands globally provide vital environmental benefits, especially in case of their enormous carbon storage potential. Peat land also functions as a source of livelihood for the community, especially for agricultural activities, and this will lead to the potential loss of carbon stock in peatlands. This study examines plants' potential to create Carbon to offset carbon dioxide emissions and different land use types. The study focused on Central and West Kalimantan, Indonesia. Peat soil samples were collected from various types of land from 0-15, 15-45, and 45-100 cm depth and analyzed for physical and chemical parameters. The cylinder chamber method with infrared gas analysis model EGM-4 was used to measure CO_2 emissions. Plant carbon sequestration was measured in a 6.25 m^2 plot in the study sites of Central Kalimantan. The study showed that type of commodity and period of management affect the carbon content in peat with different land uses, and it is affected by soil bulk density, organic matter content, and CO_2 emission. In the case of study crops, oil palm, rubber, corn, and mustard emit the highest CO_2. Further, corn crop has the highest potential to fix carbon dioxide and produces more Carbon per hectare than the Carbon emitted from corn-planted under peatland conditions. The study indicated that the type of commodity and the time of its management affected the carbon content in peat with different land uses, and carbon content got the change with soil bulk density and soil organic matter content.

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1 Introduction

Worldwide, the peatlands reached up to 400 million ha; about 350 million ha of this is subtropical, and the rest is tropical (Strack et al. 2008; Page et al. 2011). In addition, according to Maltby and Proctor (1996), approximately 31–46 Mha of peatland (or 10–12% of the total peatlands) are found in tropical countries such as Southeast Asia, South America, Africa, Central America, the Caribbean region, Mainland Asia, Australia and Pacific regions (Rieley and Page 2016). According to Anda et al. (2021), peat area in Indonesia reaches 13.43 million ha, and it is mainly spread over Sumatra (5.5 million ha), Kalimantan (4.54 million ha), Papua (3.01 million ha), and several other areas. Further, in West Kalimantan, the recorded peat area was about 1.55 million ha, consisting of 1.02 million ha of less than 3 m depth and 0.53 million ha with a depth of > 3 m. While in Central Kalimantan, the peat area reaches 2.55 million ha, consisting of 1.86 million peat with a depth of < 3 m and 0.69 million ha of peat with a depth of > 3 m (Anda et al. 2021).

In total recorded Indonesian peatland, peat forest covers 12.31 million ha, including 2.34 million ha of conservation forest, 1.02 million ha of protection forest, and 8.95 million ha of production forest (Wahyunto et al. 2010). Peat land available for the plantations is 1.42 million ha, for agriculture 1.23 million ha, and 4.66 million hectares for other uses (Bappenas 2010). About 3.74 million acres (25.1%) of Indonesia's peatland have been degraded or overrun with plants (Wahyunto and Dariah 2014).

Tropical peats are vital in biodiversity, climate regulation, and human health (Joosten 2015; Wildayana 2017). In their natural state, peatlands provide globally important environmental services, primarily related to climate change due to their enormous Carbon (C) storage capacity (Page et al. 2011). This peatland also regulates water flow (water storage, filtration, and water resources), protect against natural stresses (prevention of erosion and flooding), provides macro-climate stability, helps in recreation and education, and is also a source of natural resources and biodiverse. Other ecological functions of peatland are sediment retention, nutrient detention, and microclimate stability (Maltby 1997; Rieley et al. 2008). In Central Kalimantan, peatlands are used to plant horticultural and food crops.

Further, Kalampangan Village of Central Kalimantan is a center for peat-grown vegetables. Sjarkowi (2005) found that sweet corn, green mustard, tomatoes, and long beans were the most suitable plants among Kalampangan farmers. The peatlands of West Kalimantan is ideal for producing food crops, horticulture crops, rubber, and oil palm. The Slamet River area in Siantan Hilir is a production center for horticultural crops, while the Siantan Hulu area is a production center for aloe vera plants. Plantation crops such as rubber and oil palm are the major crops of the Ambawang river area of Mempawah.

The construction of drainage facilities to reduce the depth of the groundwater table is an essential requirement in the utilization of peat land into agricultural land. The decrease in the depth of the groundwater table also results in changes in the upper peat conditions from anaerobic to aerobic conditions. Under aerobic circumstances, oxidation of Carbon occurs, which produces CO_2 and releases it into the atmosphere. This release of Carbon dioxide from the soil is also the result of the respiration process, namely the decomposing of organic molecules into energy, water, and CO_2 in cells. Further, this CO_2 release process results from root respiration, microbial respiration in the rhizosphere, respiration from the decomposition of litter and organisms, and soil organic matter oxidation (Luo and Zhou 2010; Moyano et al. 2009). Environmental factors such as the depth of the groundwater table, temperature, humidity, and pH of peat soil significantly influence the amount of CO_2 emissions released from peatland (Jauhiainen et al. 2001; Hooijer et al. 2006; Strack et al. 2008; Agus et al. 2010). If Carbon is released through carbon dioxide emissions in large quantities and lasts for a long time, then this, in addition to threatening the existence of the peat function as a carbon repository, is also a source of greenhouse gases whose contribution reaches 48% (Pirkko and Nyronen 1990).

Various agricultural production systems and uses of peatland also affect the groundwater level, soil's physical, chemical, and biological qualities, and air temperature, which can be affected the peatlands' CO_2 emissions. Information related to the CO_2 emissions from peatland comes from the forests, open land (Jauhiainen et al. 2005), and agricultural land (Hatano et al. 2004) of the Kalimantan region, while the information regarding the CO_2 emissions from peatlands utilized for oil palm, rubber, biennial aloe vera, and seasonal corn and mustard greens production in the central Kalimantan and west Kalimantan have not been estimated in any previous study.

Various biological and abiotic factors also affect the CO_2 emissions from peatlands. Among the various studied abiotic factors, the depth of the groundwater table is one of the most influential abiotic factors that remarkably affect CO_2 emissions. Decreased groundwater table depth also correlates with increased peatland CO_2 emissions (Hooijer et al. 2006; Fahmuddin and Subiksa 2008; Jauhiainen et al. 2012). Each type of plant requires a specific depth of groundwater table for its normal growth and development (Fahmuddin and Subiksa 2008; Morrison and Page 2012). Therefore, different types of plants in the same peatland have differences in CO_2 emissions, and it is related to their need for groundwater depth.

Peatlands emit and store Carbon simultaneously, but the final amounts of carbon emission depend on various natural conditions and human intervention. Changing the depth of the groundwater table to the optimal depth of plants can minimize the emissions of

CO_2 from peatlands. Selecting plant species that can produce Carbon to offset peatland CO_2 emissions is also crucial. Selecting plants that can create Carbon will preserve a balance between CO_2 emissions and carbon sequestration. In peatland areas, the carbon-producing potential of some plants is still unknown. Therefore, this study aimed to evaluate the plants' potential to create Carbon to offset CO_2 emissions. Further, this study also intends to assess the effect of various types of peat land uses on the amount of CO_2 emissions and sequencing Carbon to replace Carbon lost through CO_2 emissions.

2 Materials and Method

2.1 Study area and Design

The current study was carried out in the peatland area of Central Kalimantan and West Kalimantan, Indonesia. The research was conducted in the Central Kalimantan region in the Kalampangan Village, Sebangau District, located 20 km southeast of Palangka Raya Municipality (Figure 1a). Since 1980, Kalampangan Village has been a transmigration center for horticulture crops. Land use in Kalampangan village is determined by the duration (period) of managed agricultural land and plant type. The land management of Kalampangan village is separated into two categories, i.e., freshly managed land (land maintained for five years or less) and long-managed land (land managed for ten years). Green mustard (*Brassica campestris*) and sweet corn (*Zea mays*) are the major crops frequently grown in both land groups. As per the crop duration, study area land uses are divided into four categories, i.e., mustard greens cultivation in 5-year-managed land, mustard greens in 10-year-managed land, sweet corn in 5-year-managed land, and sweet corn in 10-year-managed land.

In West Kalimantan, various annual, perennial, and plantation crops have been selected for this study. Corn (*Zea mays*) was chosen to symbolize annual crops, while oil palm (*Elaeis guineensis*) and rubber (*Hevea brasiliensis*) represented perennial crops. Major corn farms are in Rasau Jaya, aloe vera fields in Siantan Hulu, and oil palm and rubber fields in the Ambawang river area (Figure 1b). The four types of crops which determined the land use in the study area are corn, aloe vera, oil palm, and rubber.

2.2 Peat-sampling

Undisturbed peat samples were obtained from 30 to 40 cm depths. The undisturbed peat samples were collected using a metal ring of 5 cm in height and diameter. In contrast, the customized Eijkelkamp peat drill was used for the collection of disturbed samples collections. Peat samples from all land use types were obtained from 0-15, 15-45, and 45-100 cm depth in each replication. In the case of mustard greens and corn crops, peat sampling was carried out between the plants, while in the case of aloe vera cultivation, peat sampling was carried out between plant spacing in rows. The peat sampling was carried out 1.5 m from the plant trunk in oil palm and rubber cultivating the land. Peat samples for total bacterial analysis were taken from a 10-20 cm depth.

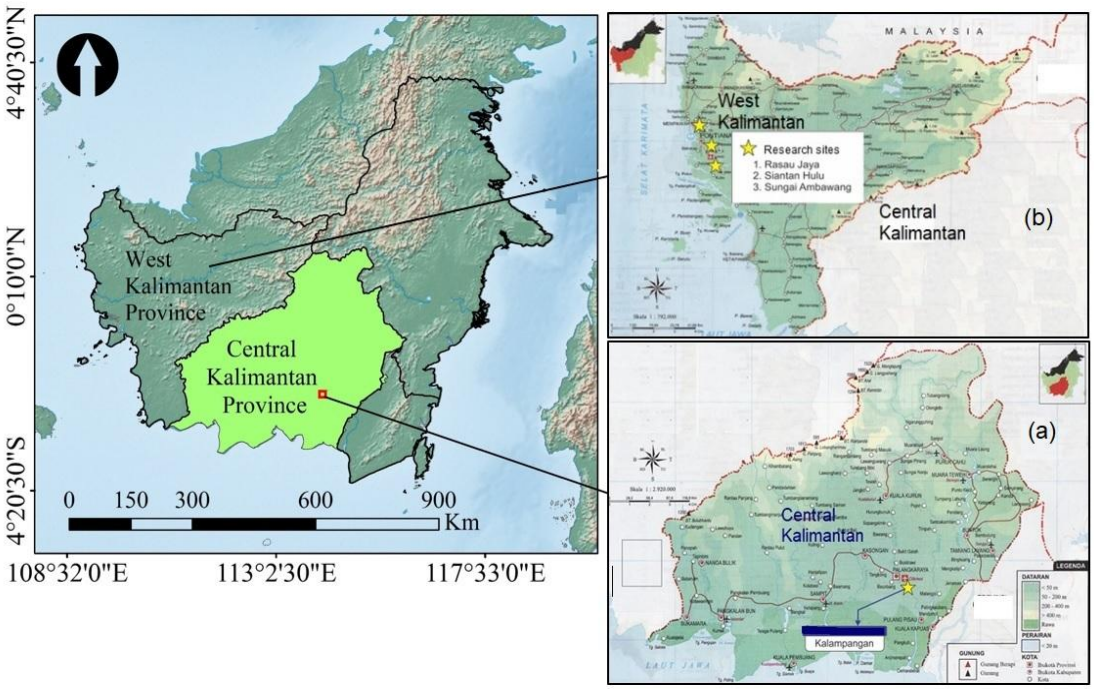

Figure 1 geographical location of the study areas

2.3 Plant-sequestered carbon

Carbon sequestration by plants is limited by the Carbon produced by the plants and does not consider the Carbon that comes from land cover. Observation of plant carbon fixation and biomass production was computed for each farmer in 5 plots of 2.5 m × 2.5 m or 6.25 m2 representing each land use type.

For mustard and corn biomass estimation, the complete plant was uprooted, kept in a paper bag, and incubated in a hot air oven at 60°C for 48 hours, or until its weight stabilized. Dry biomass can estimate organic matter, and organic Carbon is extracted from the total organic matter.

2.4 Estimation of CO_2 Emission

The infrared gas analysis method measured peatland CO_2 emissions (model EGM-4, P.P. system, Hitchin, U.K.). The CO_2 analyzer recorded the CO_2 emissions from the ground from the first second to the 81st second at three sites per replication (Jauhiainen et al. 2001). The CO_2 estimation chamber is set 50 cm away from the rows of mustard greens plants to measure CO_2 emissions. While the distance between the chamber sites and the mustard plant stem is around 10 cm. In corn fields, the chamber is set between rows of corn 60 cm apart, and the distance of the chamber is 15 cm from the corn stem. In such close planting rows, it is impossible to avoid the chamber wall being beyond the root radius, even though the roots of plants grown in peat do not extend too far from the planting hole. In *Aloe vera* fields, the planting distance is 80 cm x 100 cm, and the chamber is positioned between planting rows, so it is believed that the chamber is still inside the root radius. In oil palm and rubber fields, the chamber is placed at a distance of 1.5 m from the stem of the plant, and it is estimated that the chamber is within the radius of root spread because the oil palm and rubber fields used in this study are ten years old, so the roots of the rubber plant have spread far from plant stems.

CO_2 emissions recorded at 81 seconds are CO_2 emissions released by peatlands with units of g $CO_2/m^2/hr$. Measured CO_2 emission data are validated by looking magnitude of r^2 from the regression between time and CO_2 concentration in the atmosphere. The data is considered valid if the value of r^2 is at least 0.98. Along with the CO_2 emissions measurement, the groundwater table depth, air temperature, and soil temperature were also recorded at 10, 20, 30, and 40 cm depth. Conversion CO_2 emissions from units of g $CO_2/m^2/hr$ from measurement to units of ton $CO_2/m^2/y$, as follow:

$$CO_2 \text{ emission (ton } CO_2/m^2/y) = \text{ g } CO_2/m^2/hr \text{ x 24 hr x 365 days}$$

The conversion results only provide a rough idea, likely higher than the actual annual emission value. This is because the measurement of CO_2 emissions is only carried out for a short duration, so it cannot represent the rainy and dry seasons, which are closely related to the depth of the groundwater table. In addition, in the current study, the measurements were only carried out during the day, when the temperature was higher than at night.

2.5 Analysis of Peat Properties in the Laboratory

Peat soil analysis was conducted at the Soil Science Laboratory, Faculty of Agriculture, Gadjah Mada University, Indonesia. The analytical method depends on the parameters analyzed, and standard methods were used for peat analysis (Table 1).

Table 1 Peat soil analysis method

Parameter	Method
Water content (% weight)	Collected soil dried in an oven at 80°C for 24 hr*
Bulk density (gr/cm^3)	Ring Sample**
Carbon	Based on bulk density, percentage of organic matter content, and percentage of C-organic; Accounted for 58% of the total plant organic matter
Percentage of peat organic matter	Air-dried peat was dried in a kiln at 80°C for 24 hr, then burned in a 600°C muffle furnace for 4 hr. The percentage of organic matter is calculated from the percentage of ash content.
Percentage of C-organic	Calculated 58% of peat organic matter percentage
Total plant organic matter	Calculated 98% of dry plant weight
C-organic plant	Calculated 58% of total plant organic matter

*Water content was determined by drying, and peat was placed in an oven at 80°C for 24 hours. The calculation results are expressed in percent (%), namely as follows:

WC = ((WC wet soil - WC oven dry soil) / WC oven dry soil) x 100% (WC=water content)

**peat bulk density was measured by calculating the volume of soil contained in the metal ring under wet and dry conditions, namely after being heated in a heating oven at 105°C for 24 hours. Peat volume values are determined in dry unit weight and are expressed in units of weight per volume of peat (g/cm3).

Table 2 Average content of organic matter and organic Carbon (C-organic) in peat soil of various land use

Land use type	Content of organic matter	C-organic of Peat Soil	
	(%)	(%)	(ton C/ha/m)
Central Kalimantan			
Mustard green, land 5 yrs	98.68	57.23	782.19
Mustard green, land 10 yrs	98.01	56.85	876.38
Corn, land 5 yrs	98.65	57.22	810.06
Corn, land 10 yrs	98.19	56.95	817.82
West Kalimantan			
Corn	98.51	57.13	802.13
Aloe vera	98.06	56.87	929.89
Palm oil	98.80	57.30	820.85
Rubber	99.09	57.47	748.82

2.6 Data analysis

To find out the differences in the characteristics of peat based on the type of land use, depth of the peat, and the differences in the amount of CO_2 emissions released, the data were analyzed using variance fingerprints and then Duncan's test. Meanwhile, to compare the amount of CO_2 emissions by various types of land use during the study period of the first year with the second year, the data were analyzed using the t-test.

3 Results

3.1 Carbon Content

The content of organic matter and organic Carbon (C-organic) in peat soil from various types of peat land use in Central Kalimantan and West Kalimantan is presented in Table 2.

Table 2 shows that the average organic matter in Central Kalimantan peat soil was 98.01–98.65%, while it was reported to be 98.06–99.09% in West Kalimantan. According to Andriesse (1988), the high proportion of organic matter indicates the differences in bulk density of peat between the types of land use. It depends on the nature or condition of the peat material, including its maturity, compression, and peat soil water content. Based on bulk density, the percentage of organic matter content and the percentage of C-organic content was calculated and recorded 782.19 to 876.19-ton C/ha/m C-organic content in the peat soil samples collected from the Central Kalimantan, while a minor variation was reported in the peat soil samples collected from the West Kalimantan, where it was recorded 748.82 to 929.89-ton C/ha/m (Table 2).

3.2 CO_2 Emissions

The land use type also affects the rate of CO_2 emissions. Based on the analysis of variance in CO_2 emissions by four types of peat land uses in Central Kalimantan, the study's results revealed a significant difference in the second year of study, and a reduction was reported in the rate of CO_2 emission. The results of the average value of CO_2 emissions by the different types of land use in Central and West Kalimantan are presented in Table 3.

Based on the average CO_2 emissions in the first year, the highest CO_2 emissions value of 0.81g $CO_2/m^2/hr$ was recorded from the sweet corn (10 years of land use) in Central Kalimantan. The lowest emission of 0.56g $CO_2/m^2/hr$ was recorded from sweet corn (5 years of land use) in the same region. Further, a significant difference was reported in the ten years of land use for mustard greens and corn than in the five years of land use of sweet corn CO_2 emissions value (Table 3). In the case of the second year CO_2 emission rate, a sharp decline was recorded in all four land uses and two crop types. In mustard greens (10 years of land), the average CO_2 emissions value was recorded 0.82g $CO_2/m^2/hr$ in the first year, while it dropped to 0.52 $CO_2/m^2/hr$ in the second year. Similarly, 0.56g $CO_2/m^2/hr$ in the first year for the sweet corn (5 years land) was reported, and it fell to 0.29g $CO_2/m^2/hr$ in the second year.

The analysis of variance shows that CO_2 emissions from all four forms of peatland use in West Kalimantan differ significantly in both years of investigation. The average value of CO_2 emissions showed that rubber plantations showed 1.26g $CO_2/m^2/hr$ CO_2 emissions in the first year of the study, significantly higher than oil palm, aloe vera, and corn fields. Like the first year, in the second

Table 3 Average CO_2 Emissions in four land types of Central and West Kalimantan Peatland

Land use Types	CO_2 Emission (g $CO_2/m^2/hr$)		
	Y1	Y2	Average
Central Kalimantan			
Mustard green, land 5 yrs	0.74±0.11[b]	0.72±0.11[b]	0.73±0.07
Mustard green, land 10 yrs	0.82±0.05[b]	0.52±0.08[c]	0.67±0.08
Corn, land 5 yrs	0.56±0.03[c]	0.29±0.06[d]	0.43±0.07
Corn, land 10 yrs	0.81±0.04[b]	0.77±0.04[b]	0.79±0.03
West Kalimantan			
Corn	0.30±0.03[d]	0.39±0.01[d]	0.35±0.02
Aloe vera	0.64±0.09[bc]	0.72±0.04[b]	0.68±0.05
Palm oil	0.78±0.08[b]	1.15±0.18[a]	0.97±0.12
Rubber	1.26±0.16[a]	1.18±0.04[a]	1.22±0.07

The mean followed by the same letter in the same column is not significantly different based on Duncan's test (p =5%*) compared to Year 1 and Year 2 average emissions

Table 4 Total C emissions and emissions from the decomposition of conversion results in four types of peat land use in Central and West Kalimantan

Landuse types	C emission (ton C/ha/yrs)				
	Y 1		Y 2		Average emission from decomposition
	Total Emission	Emission from decomposition	Total Emission	Emission from decomposition	
Central Kalimantan					
Mustard green, land 5 yrs	17.68±2.64	13.97±2.09	17.06±2.61	13.48±2.06	13.72±1.66
Mustard green, land 10 yrs	19.59±1.33	15.48±1.05	12.42±0.76	9.81±0.60	12.64±0.44
Corn, land 5 yrs	12.66±1.04	10.00±0.82	6.83±1.38	5.40±1.09	7.70±0.14
Corn, land 10 yrs	19.35±1.09	15.29±0.86	18.32±0.90	14.47±0.71	14.88±0.30
West Kalimantan					
Corn	7.17±0.83	5.66±0.65	9.25±0.16	7.31±0.13	6.49±0.33
Aloe vera	15.29±2.22	12.08±1.75	17.20±0.90	13.59±0.71	12.83±0.71
Palm oil	18.63±1.95	14.72±1.54	27.47±4.47	21.70±3.53	18.21±2.09
Rubber	30.10±4.66	23.78±3.68	28.19±0.99	22.27±0.79	23.02±1.48

year also, rubber plantations had the highest CO_2 emission (1.18g $CO_2/m^2/hr$), which was immediately followed by the oil palm plantations (1.15g $CO_2/m^2/hr$). In contrast, the Aloe vera and corn fields had significantly lower CO_2 emissions than the rubber plantations (Table 3). In contrast to the Central Kalimantan, all tested crops except rubber have higher CO_2 emissions in the second year in the West Kalimantan, but in t-test, it is not statistically different. Results presented in Table 4 convert the total CO_2 emissions from g $CO_2/m^2/hr$ to ton C/ha/yr and conversion of emissions from decomposition. Mustard green and corn plants with more prolonged use produce more significant CO_2 emissions and emissions resulting from decomposition.

In Central Kalimantan, ten years of land use show the highest total emission and lowest decomposition for both crops in the first year of study. In the second year, all crops and land uses except for five-year uses of corn have similar emissions, and average decomposition was recorded. In West Kalimantan, the highest total emission and decomposition were recorded from the rubber plants, while the lowest was reported from the corn crops for both years.

3.3 Plant-sequestered carbon

Production of plant biomass shows the ability of plants to sequester Carbon stored in the form of biomass. Dry plant biomass and C-

Table 5 Dry biomass production and C-organic production obtained from four types of peatland use in Central Kalimantan

Land use types	Dry biomass production (ton/ha/y)			C-organic production (ton/ha/y)		
	Y1	Y2	Average	Y1	Y2	Average
Mustard green, land 5 yrs	2.72±0.09	2.48±0.10	2.60±0.08	1.55±0.05	1.41±0.06	1.48±0.05
Mustard green, land 10 yrs	3.34±0.06	2.98±0.05	3.16±0.09	1.90±0.04	1.69±0.03	1.80±0.05
Corn, land 5 yrs	16.72±0.17	15.03±0.03	15.87±0.39	9.50±0.10	8.54±0.02	9.02±0.22
Corn, land 10 yrs	26.35±0.03	23.95±0.12	25.15±0.54	14.98±0.02	13.61±0.07	14.30±0.31

Table 6 CO_2 emissions from decomposition, C fixing by plants, and the difference between C emissions and C fixing in four types of peat land use in Central and West Kalimantan

Land use types	CO_2 emissions from decomposition (ton C/ha/y)	C fixing by plants (ton C/ha/y)	Difference between C emission and C-fixing (ton C/ha/y)
Central Kalimantan			
Mustard green, land 5 yrs	13.72±1.66	1.48±0.04	-12.24±1.69
Mustard green, land 10 yrs	12.64±0.23	1.80±0.12	-10.84±0.32
Corn, land 5 yrs	7.70±0.14	9.02±0.04	1.32±0.17
Corn, land 10 yrs	14.88±0.30	14.30±0.04	-0.58±0.33
West Kalimantan			
Corn	6.49±0.23	11.66*	5.17
Aloe vera	12.83±0.71	Nd	nd
Palm oil	18.21±2.09	2.44**	-15.77
Rubber	23.02±1.48	2.56***	-20.46

Here nd = no data and references * Average C mooring in Central Kalimantan, **Reference value as per Rogi (2002), and *** Reference value as per Fahmuddin and Subiksa (2008)

organic production obtained from all four types of peat land use were only measured in Central Kalimantan during the first and second-year research periods and are presented in Table 5.

In dry biomass and C-organic production, the highest dry biomass production and C-organic production were recorded from the five and ten years of land uses for the corn crop for both years (Table 5). Table 5 shows that corn crop for ten years of land uses shows the highest dry biomass production and C-organic production, and it was recorded at 26.35±0.03 ton/ha/y and 14.98±0.02 ton/ha/y respectively, for the first year, while 23.95±0.12 ton/ha/y and 13.61±0.07 ton/ha/y, respectively for the second year. The land followed its uses for the five years for corn crops; other crops showed at par results and no significant difference. In corn, other part biomass was recorded at 46.34% for the stem, 24.74% for leaves, 16.74% for cornhusk, and 12.18% for root.

Table 6 shows the difference between C released by peatlands through CO_2 emissions and Carbon fixed by plants through photosynthesis and stored in plant biomass in four types of peat land use in Central Kalimantan by excluding organic C from land cover. Among the four types of land uses, the highest CO_2 emissions from decomposition (14.88±0.30 ton C/ha/y) and C fixation by plants (14.30±0.04 ton C/ha/y) were recorded from the ten years of the land uses corn crop, and the difference between these two was recorded -0.58±0.33 ton C/ha/y. In contrast to ten years of the land uses, five years of land uses for corn crops have shown the lowest value of CO_2 emissions from decomposition (7.70±0.14 ton C/ha/y) and C fixation by plants (9.02±0.04 ton C/ha/y), and the differences were recorded as 1.32±0.17 ton C/ha/y. In the case of Mustard green, no significant difference was recorded between both types of land use and study years (Table 6).

Using secondary data for C fixation by plants, the difference between the amount of C released by peat lands and the ability of plants to produce Carbon in four types of crops in West Kalimantan have been presented in Table 6. Based on research data and existing reference information on the C fixation, the difference between C emissions and C fixation of the four crops or land types in West Kalimantan was evaluated, and the highest CO_2 emission (23.02±1.48 ton C/ha/y) was recorded from the rubber plantation while lowest (6.49±0.23 ton C/ha/y) was reported from the corn plant. In contrast, the highest C fixation (11.66 ton C/ha/y) was recorded in the corn crop, while the lowest was in the palm oil plantation (Table 6).

4 Discussion

4.1 Carbon Content

The level of the carbon content depends on the duration of the land uses; in Central Kalimantan, there is a tendency for the more prolonged the land is used, the more C-organic content increases. In Central Kalimantan and West Kalimantan, the difference in C-organic contained in various types of peat land uses seems to be influenced by the kind of commodity and the time it has been managed. The results of this study agree with the findings of Devi et al. (2019), who found that the maturity level of peat soil is strongly related to the length of time the peatland has been used, and the degree of maturity of the peat is related to bulk density. Similar types of findings have been reported by Adji (2017). Further, the value of total C-organic estimated in this study for both Central Kalimantan peat and West Kalimantan peat was higher than the results reported by Page et al. (2002).

4.2 CO_2 Emissions

CO_2 emissions by mustard greens and corn fields in Central Kalimantan are lower than the results of Hatano et al. (2004), which claims that vegetable fields release CO_2 emissions in the range of 0.23 - 1.02g $CO_2/m^2/hr$, with a range of 0.61-0.75 m groundwater depth. This is more than the 0.22g $CO_2/m^2/hr$ released from unmanaged agricultural land (Jauhianien et al. 2004). The analysis of variance shows that CO_2 emissions from four forms of peatland use in West Kalimantan differ significantly in both years of investigation. The average value test showed that rubber plantations released 1.26g $CO_2/m^2/hr$ (110.38 ton CO_2/ha/yr) in the first year, and it was recorded as 1.18g $CO_2/m^2/hr$ (103.37 ton CO_2/ha/yr) in the second year. For rubber plantation, the results of this study show higher emissions than the research conducted by Jamaludin et al. (2020), which is 42.6 tons CO_2/ha/year, and Wakhid et al. (2017), which is 51.60 tons CO_2/ha/year. According to Kusin et al. (2015), using nitrogen fertilizers, especially in oil palm plants, may significantly contribute to greenhouse gas emissions and loss of carbon content. Therefore, applying nitrogen fertilizers is likely to increase C emissions from the conversion of rubber plantations to oil palm plantations. However, this seems slightly different at the study site, where emissions from rubber plantations are greater than from oil palm plantations.

The average CO_2 emissions from all land uses in the first year were not substantially different from the second year. In the second year also, the rubber plantations released the highest CO_2, followed by oil palm, aloe vera, and corn fields. These differences in the rate of CO_2 emissions might be due to the variations in water table depth, which was reported 24.33 and 19.33 cm for corn crops in the first and second year, respectively. In comparison, 87.25 cm and 68.59 cm were reported for rubber fields in the first and second years, respectively. These results agree with the findings of Evans et al. (2021), who stated that greenhouse gas emissions from peatlands drained for agriculture are strongly related to groundwater depth and that raising the groundwater table can be achieved without reducing productivity in land use. Halving the groundwater depth in drained agricultural peatlands could reduce emissions equivalent to more than 1 percent of global anthropogenic emissions. According to Luo and Zhou (2010), decreasing groundwater level will make the peat soil aerobic, increasing soil microorganism activity to decompose organic matter, producing CO_2 and harmful organic acids. Jauhiainen et al. (2001) also reported that CO_2 emissions from peatlands in West Kalimantan are strongly correlated with groundwater table depth.

In the current study, oil palm and rubber land produced more CO_2 emission for both years than Melling's (2005) study, which found that oil palms emitted 0.64g $CO_2/m^2/hr$, sago palms 0.49g $CO_2/m^2/hr$, and forests 0.92 g $CO_2/m^2/hr$. Similar results were also recorded by Fahmuddin and Subiksa (2008).

Accumulated emissions may be higher or lower than the annual emissions after conversion. It might be due to short-term emission measurements that do not reflect CO_2 emissions emitted during rainy or dry seasons and are directly tied to groundwater table depth. In addition, in this study, CO_2 emissions are measured only during the day, without considering temperature changes between day and night. Luo and Zhou (2010) state root respiration increases with temperature in response to temperature sensitivity. Root respiration increases exponentially with temperature, but biochemical reactions slow it because substrates like glucose, oxygen, and CO_2 cannot diffuse at high temperatures.

During the conversion of emissions from mustard greens, corn, and aloe vera, the period of no crop is ignored, so this is also one of the reasons for the significant error in the conversion. Likewise, emission measurements from oil palm and rubber fields that are ten years old have spreader root systems extensively. Hence, the measured CO_2 emissions are likely not only the result of peat decomposition but are also expected to originate from root respiration. With these limitations, presenting CO_2 emission data in units of g $CO_2/m^2/hr$ from instantaneous measurement results is considered the most appropriate because it is not conversion data.

4.3 Carbon-Fixation

Results related to the carbon fixation study showed significant variances in organic C produced by various crops; this variation is assumed to be driven by numerous factors, including population per unit (hectare), plant fertility, and corn species with varying traits or sizes of stems and leaves.

Using secondary data for C anchorage by plants, the difference between the amount of C released by peatlands and the ability of

plants to produce Carbon in four types of peatland use in West Kalimantan is presented in Table 6. These results show that corn can provide a value of 5.17 tons of C/ha/yr, which means the level of emissions from corn fields can be overcome by metabolic processes in the plant, other than that corn plants do not require excessive drainage or merely require a depth of groundwater table not far from the soil surface.

According to Rogi (2002), oil palm can store more than 80 tons C/ha, but this amount is reached after 10-15 years of growth, so the average amount of Carbon held by oil palm plants is around 60.5 tons/ha equivalent to 2.44 ton C/ha/yr, assuming one oil palm production cycle of 25 years. The ability of rubber plants to sequester Carbon is not much different from oil palm plants. Fahmuddin and Subiksa (2008) found that rubber plants store 56.45 tons C/ha for 25 years or 2.26 tons C/ha/yr.

The ability of plants to produce biomass varies on the type of crop. Based on the metabolic mechanism, corn plants are C4 plants, and these plants can create higher biomass due to their high photosynthetic efficiency. Table 6 shows the difference between C released by peatlands through CO_2 emissions and C fixed by plants through photosynthesis and stored in plant biomass in four types of peat land uses in Central Kalimantan by excluding organic C from land cover.

Conclusion

Results of the study can be concluded that the type of commodity and period of management significantly affected the carbon content, soil bulk density, and organic matter content in peat with different land uses in Central and West Kalimantan. Further, among the studied crops, the highest CO_2 emissions were recorded from the perennial crops (oil palm and rubber), followed by seasonal crops (corn, mustard greens), and this CO_2 emission is positively and significantly associated with the depth of the groundwater table. Results of the study also suggested that plant biomass production is also associated with carbon dioxide fixation.

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Productivity and profitability of commercial broiler chickens under various farming conditions

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ABSTRACT

Broiler farming plays a vital role in fulfilling global protein requirements. Although broiler farming is considered profitable, profitability might be affected by factors such as genetics, feed quality, and management practices. In the current study, the productivity and profitability of commercial broiler farming were studied under various farming conditions, such as farm size, location of the farm, and mortality of the broilers. Data were collected through farmers' interviews and farm record books and processed and analyzed to determine the productivity and profitability of broiler farming. Productivity and profitability did not differ significantly across farm sizes and locations. The results of the study reported mortality as a factor affecting productivity and profitability in broiler farming. It was manifest that mortality adversely affected the productivity and profitability of broiler farming. A significant positive relationship was recorded between mortality and feed conversion ratio. Moreover, mortality was negatively correlated with the gross margin of broiler farming, meaning that the low gross margin was due to the high mortality at broiler farms. The farms were more profitable when the mortality was <5%, compared to >10%. It is recommended to reduce the mortality percentage of broiler chickens as minimum as possible, preferably <5%. Good quality chicks, better management, and the prevention of diseases might play an important role in keeping the mortality rate at a minimum level in broiler farming.

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1 Introduction

Poultry is the fastest-growing agricultural sub-sector and is essential in supplying meat and eggs worldwide. Thousands of people are engaged in this sub-sector to support their livelihoods (Kabir et al. 2016) and contribute to enriching the global economy (Hamid et al. 2016). Among poultry enterprises, broiler farming is widespread due to its quick returns, and such farmers have been profitable by rearing the broilers (Chowdhury and Chowdhury 2015). Recently, contract broiler farming has been initiated, where farmers are assured of getting a reasonable product price by following the rules of contracted feed companies (Saha et al. 2021).

Although broiler farming is profitable, several risk factors and challenges are associated with this sub-sector (Islam et al. 2014). The biosecurity of farms sometimes falls due to improper planning and management. Despite regular vaccination and medication, diseases and deformities might occur at broiler farms, which might cause a loss in broiler farming (Akintunde et al. 2015). Moreover, there are natural calamities and disasters that affect the productivity and profitability of broilers.

Among the factors affecting the productivity and profitability of broiler farming, mortality is a significant concern in achieving profitability in broiler farming. Farm data analysis from different aspects needs to be analyzed to find out the factors affecting the productivity and profitability of broiler farming. Since broiler mortality is common in broiler farming, it may adversely affect the productivity and profitability of broiler farming (Chauvin et al. 2011). The study of how mortality affects productive performances and gross returns has received less attention. The extent of losses in productivity and profitability of broiler enterprises at the level of farmers needs to be investigated. The current study aimed to investigate mortality at broiler farms and its effects on productivity and profitability. In this regard, the relationship between mortality rate and productive or economic parameters in broiler farming was investigated in the present study. The current study findings will help to understand the productivity and profitability of broiler farming under various farming conditions.

2 Materials and Methods

2.1 Data collection

The current study was conducted from January to December 2019. A total of 436 broiler farmers who reared the Ross 308 broiler strain were included from the Bogura, Rangpur, and Dinajpur districts of Bangladesh. The selected farms were of various sizes and ranged from 200 to 9,200 broilers per farm. A structured and pretested questionnaire was used to collect data. Data related to production (live weight of broilers, daily weight gain, and feed conversion ratio [FCR]) and economic parameters (gross cost, gross return, and gross margin) were collected from all the selected broiler farms.

2.2 Rearing and management

Commercial broiler strain Ross 308 was reared at a stocking density of 9 birds/m^2 for five weeks. Standard commercial broiler diets (starter, grower, and finisher) were used *ad libitum*. The farms were regularly monitored and supervised by poultry production specialists.

2.3 Productivity and profitability

The productivity and profitability of broiler farming were calculated according to Sarkar et al. (2008). The record of chick weight, amount of feed intake, mortality, and final body weight were considered for the productivity analysis of broilers. Then, daily weight gain and FCR were analyzed. The cost of chicks, feed, vaccines, medicines, and miscellaneous costs was investigated for the gross cost analysis.

2.4 Productivity and profitability at various farm sizes

To determine the effects of farm size on the productivity and profitability of broiler farming, the farm size was divided into three groups:<2000, 2001–5000, and >5000 broilers/farm. The productive and economic parameters were calculated according to the farm size category.

2.5 Mortality analysis

To know the adverse effects of mortality on economics in broiler farming, the mortality was categorized into three groups: <5%, 5–10%, and >10%. The productive and economic parameters were calculated according to the category of mortality rates.

2.6 Data analysis

Means and standard deviations are used to represent data. Percentage mortality data were arcsine transformed before analysis, and analysis was performed based on the transformed data. A one-way analysis of variance (ANOVA) using MS Excel was used to determine the significant differences between means. Differences between treatments were analyzed using Tukey's honestly significant difference test, and the significance level was declared based on a P <0.05.

3 Results and Discussion

Although broiler chickens could yield a reasonable return and are considered profitable, farmers often face challenges in gaining the expected productivity and profitability through broiler farming. Several factors influence the productivity and economics of

broilers, such as the strain of the broiler, feed quality, management, and the market price of broiler chickens (Bandara and Dassanayake 2006; AL-Masad 2010; Rana et al. 2013; Baracho et al. 2019). The current study looked at broiler productivity and profitability across various farm sizes, locations, and broiler mortality rates. The mean values of the productive performances and economic analysis of the studied farms, irrespective of farming conditions, are presented in Table 1. The study's results revealed that a broiler consumed 3.12 kg of feed and achieved a body weight of 1.88 kg. The daily weight gain of the broiler was 55.24 g, with an FCR of 1.66. Overall economic parameters of gross cost, gross return, and gross margin (Tk./kg broiler) were 108.37, 114.99, and 6.62, respectively. Regardless of farming conditions, overall mortality was 10.39%. The gross margin obtained from broiler farming was not high, but the FCR of the broilers might have coincided with the previous study. The FCR of broilers was reported to be 1.64 in Bangladesh (Husna et al. 2017). The FCR has improved in the poultry industry over time due to improvements in strains, feed quality, management, and nutritional biotechnology (Havenstein et al. 1994; Islam et al. 2016). Regardless of seasons or farm sizes, improved management interventions may increase productivity (Kawsar et al. 2017; Kawsar et al. 2018). One of the major challenges of the broiler industry is earning profitability and financial stability. In many countries, there is a fluctuation in the cost of chickens, feed, and the sale price of broiler chickens. Even with high productivity, profitability might not be achieved due to the lower demand for broiler chickens (Fouzder et al. 2021). Therefore, the expected level of gross margin might not be achieved due to the instability of gross cost and gross return.

Tables 2 and 3 show the results under various farm sizes and locations, respectively. The feed intake (kg/bird), live weight (kg/bird), and FCR ranged from 3.03–3.25; 1.83–1.93; and 1.65–1.72, respectively, under various farm sizes and locations. The gross margin (Tk./kg broiler) ranged from 4.51 to 7.04. These

Table 1 Productivity and profitability of broiler chickens regardless of farming conditions

Parameters	Mean ± Standard deviation
Rearing period (days)	34.16 ± 2.82
Feed intake (kg/bird)	3.12 ± 0.31
Live weight (kg/bird)	1.88 ± 0.16
Daily weight gain (g)	55.24 ± 5.14
Feed conversion ratio	1.67 ± 0.162
Mortality (%)	10.39 ± 5.14
Gross cost (Tk./kg)	108.37 ± 9.75
Gross return (Tk./kg)	114.99 ± 3.51
Gross margin (Tk./kg)	6.62 ± 9.86

Data are expressed as mean ± standard deviation.

Table 2 Productivity and profitability of broilers at various farm sizes

Parameters	<2000 broilers/flock	2001–5000 broilers/flock	>5000 broilers/flock
Rearing period (days)	33.83 ± 2.81[a]	34.84 ± 2.77[a]	35.18 ± 2.27[a]
Mortality %	10.21 ± 4.91[a]	10.70 ± 5.63[a]	11.91 ± 5.22[a]
Feed intake (kg/bird)	3.12 ± 0.31[a]	3.10 ± 0.32[a]	3.25 ± 0.33[a]
Live weight (kg/bird)	1.88 ± 0.17[a]	1.86 ± 0.15[a]	1.93 ± 0.18[a]
Daily weight gain (g)	55.9 ± 5.07[a]	53.76 ± 5.18a	54.76 ± 3.14[a]
Feed conversion ratio	1.66 ± 0.16[a]	1.67 ± 0.18[a]	1.69 ± 0.09[a]
Gross cost (Tk./kg)	108.21 ± 9.08[a]	108.67 ± 11.42[a]	109.25 ± 5.67[a]
Gross return (Tk./kg)	115.17 ± 3.54[a]	114.54 ± 3.34[a]	115.46 ± 46[a]
Gross margin (Tk./kg)	6.95 ± 9.37[a]	5.87 ± 11.22[a]	6.21 ± 3.91[a]

Data are expressed as mean ± SD.A similar superscript means there are no significant differences among treatments within the same row ($P< 0.05$)

Table 3 Productivity and profitability of broilers at various farm locations

Parameters	Bogura	Rangpur	Dinajpur
Rearing period (days)	32.81 ± 2.09[a]	32.96 ± 1.98[a]	34.99 ± 2.97[a]
Mortality %	11.19 ± 5.73[a]	10.66 ± 5.30[a]	10.08 ± 4.89[a]
Feed intake (kg/bird)	3.17 ± 0.31[a]	3.03 ± 0.27[a]	3.14 ± 0.32[a]
Live weight (kg/bird)	1.85 ± 0.16[a]	1.83 ± 0.16[a]	1.91 ± 0.16[a]
Daily weight gain (g)	56.63 ± 4.91[a]	55.5 ± 5.04[a]	54.78 ± 5.19[a]
Feed conversion ratio	1.72 ± 0.19[a]	1.67 ± 0.17[a]	1.65 ± 0.15[a]
Gross cost (Tk./kg)	111.54 ± 11.34[a]	109.57 ± 10.53[a]	107.08 ± 8.72[a]
Gross return (Tk./kg)	116.05 ± 3.58[a]	116.46 ± 3.31[a]	114.13 ± 3.30[a]
Gross margin (Tk./kg)	4.51 ± 11.73[a]	6.88 ± 11.15[a]	7.04 ± 8.68[a]

Data are expressed as mean ± standard deviation. A similar superscript means there are no significant differences among treatments within the same row ($P< 0.05$)

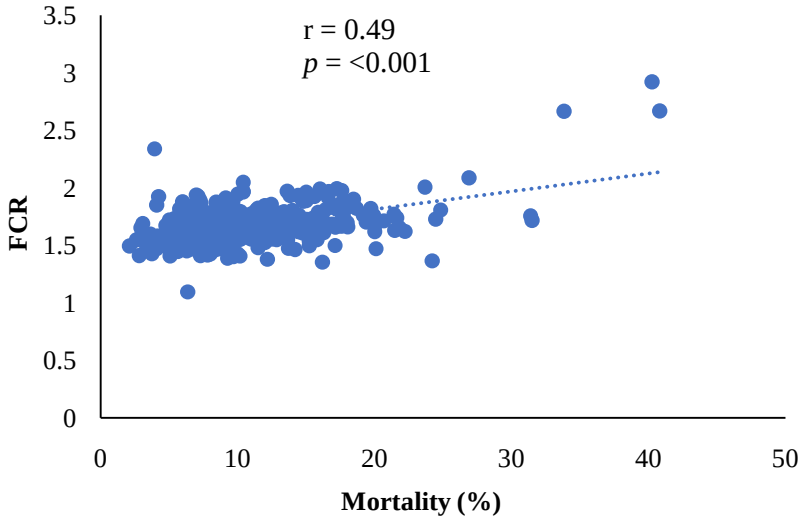

Figure 1 Correlation between mortality rate and feed conversion ratio in 436 independent broiler farms

results demonstrate no significant differences among the various farm sizes and locations. This could be due to the high mortality of broilers in different farm sizes and locations. Table 4 tabulates the results in which significant differences in the gross margin of broiler farming were found when the mortality rate was categorized and analyzed. In the present study, the effects of mortality on productive and economic performances were highlighted. According to Figure 1, broilers had a significant positive correlation between mortality rate and FCR value (r = 0.49, $P< 0.05$). The results manifested that FCR could be increased with the increased mortality percentage of broilers in the flock, as feed consumed by the dead birds was also included in the calculation of the FCR. In broiler farms, mortality occurs at various stages of the rearing period. Usually, mortality occurs during the first week of the rearing period due to poor management at the breeder and the types of housing (Yerpes et al. 2020).

Moreover, broiler chicks are more susceptible to mortality since the immune system is not well-developed at an early stage. Mortality after the brooding period is usually due to the outbreak of diseases on the farm (Delabouglise et al. 2019). If a broiler dies in a flock, the amount of feed consumed by the dead broiler is also calculated as part of the flock's cumulative FCR. In this circumstance, the broilers have consumed feed without contributing to the cumulative live weight of broiler chickens. The higher level of FCR depends on the occurrence of mortality at the final stage of the rearing period. Increases in FCR negatively correlate with broiler productivity (Ali and Hossain 2010).

We found a significantly negative correlation between mortality and gross margin in broiler farming (r = -0.61, $P< 0.05$; Figure 2), which indicates that the gross margin will be reduced with increased mortality and vice versa. Detailed information on how

Table 4 Productivity and profitability of broilers at various rates of mortality

Parameters	Mortality < 5%	Mortality 5–10%	Mortality > 10%
Rearing period (days)	34.41 ± 2.56[a]	34.09 ± 2.77[a]	34.22 ± 2.96[a]
Feed intake (kg/bird)	2.95 ± 0.24[a]	3.08 ± 0.28[b]	3.19 ± 0.33[c]
Live weight (kg/bird)	1.87 ± 0.15[a]	1.89 ± 0.16[ab]	1.86 ± 0.17[c]
Daily weight gain (g)	54.57 ± 4.66[a]	55.81 ± 4.95[a]	54.47 ± 5.43[a]
Feed conversion ratio	1.58 ± 0.11[a]	1.63 ± 0.12[ab]	1.73 ± 0.19[c]
Gross cost (Tk./kg)	101.64 ± 7.66[a]	105.84 ± 6.55[b]	113.18 ± 11.52[c]
Gross return (Tk./kg)	114.85 ± 3.88[a]	115.37 ± 3.78[a]	114.47 ± 1.30[a]
Gross margin (Tk./kg)	13.21 ± 7.74[a]	9.53 ± 6.97[ab]	1.30 ± 11.15[c]

Data are expressed as mean ± standard deviation; [a,b,c] Meaning significant differences among treatments within the same row (P< 0.05)

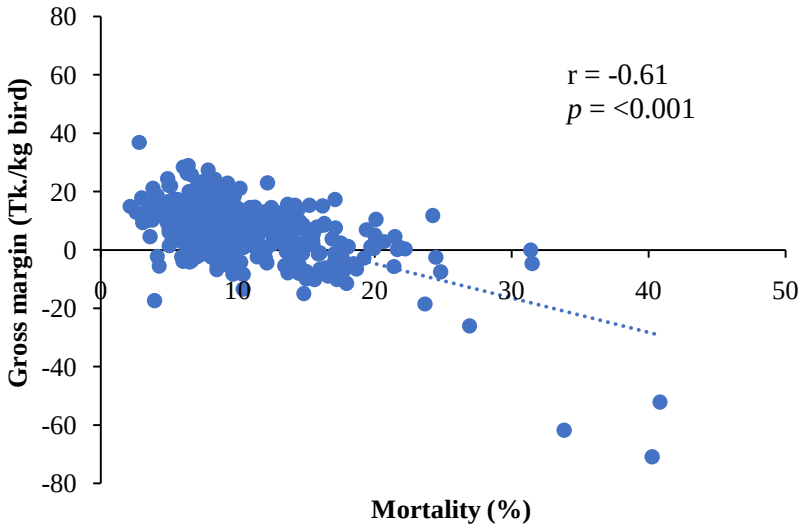

Figure 2 Correlation between mortality rate and gross margin in 436 independent broiler farms

mortality affects productivity and economics in broiler farming is shown in Table 4. The FCRs for the mortality rates of <5%, 5–10%, and >10% were 1.58, 1.63, and 1.73, respectively. The gross margin (Tk./kg broiler) was 13.21, 9.53, and 1.30 for the mortality as mentioned earlier rate. When mortality was compared between <5% and >10%, feed intake and live weight (kg/broiler) differed significantly. When mortality was >10%, feed intake increased, and live weight decreased. In farms where mortality was <5%, the FCR was significantly lower. In this situation, the gross cost (Tk./kg broiler) was significantly low, reflecting the farms' gross margin. The gross margin (Tk./kg broiler) was high in the farms where mortality was <5%, compared to the farms where mortality was >10%. In a case where the overall mortality was 10.39%, the gross margin (Tk./kg broiler) was 6.62. At farms with a mortality rate of around < 5%, the gross margin (Tk./kg broiler) was doubled by 13.21 compared to the mortality rate of 10.39%. To earn high productivity and profitability, the mortality rate should be kept as low as possible, preferably below 5%. The primary concern for earning a high gross margin is the outbreak of infectious diseases on farms and the unstable market price of broiler chickens (Delabouglise et al. 2016). Despite the availability of vaccines against most poultry diseases, outbreaks of diseases are common in broiler farming due to biosecurity issues. The outbreak of diseases represents high morbidity and mortality in broiler farming (Sahoo et al. 2022). Although prediction and monitoring of the market for broilers are demanding, mortality can be minimized through improved management and strict biosecurity. In these circumstances, the knowledge and skills of farmers need to be improved to make the farms more profitable (Paul et al. 2017).

Conclusion

The productivity and profitability of broilers in various farm sizes and areas were not found to be significant due to high mortality. Mortality adversely affected the productivity and profitability of the broiler chickens. The feed conversion ratio was high when the

mortality rate increased, while the gross margin was negatively correlated with the mortality rate. To achieve high productivity and profitability, the mortality rate at broiler farms should be kept as low as possible through improved management and strict biosecurity.

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Authors' Contribution

Sharif Uddin Khan collected the data from the broiler farms. Swapon Kumar Fouzder contributed to the writing of the manuscript. Prodip Kumar Sarkar planned and designed the study, analyzed the data, prepared graphs and tables, and wrote the manuscript. All the authors confirmed the data and the final manuscript.

Competing Interests

The authors declare no conflict of interest.

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IRIS-Stage 4 CKD in a Dog: Diagnostic Approaches and Staging of Chronic Kidney Disease: A Case Study

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Glomerular filtration

Hematological evaluation

IRIS staging

ABSTRACT

Chronic kidney disease (CKD) is a devastating disease of the kidneys that often arise from unresolved acute injury. As a chronic disease, CKD is challenging to diagnose, thus it needs a good combination of a comprehensive understanding of the kidney's anatomy and physiology and thorough planning for a framework of diagnostic tools to be utilized. This study is intended to provide the diagnostic planning used to determine CKD in an approximately 5-year-old intact male dog that was brought to My Vets Animal Clinic for a check-up visit. On presentation, the dog was emaciated, mildly dehydrated, halitotic, and infested with ticks. A complete blood count (CBC) indicated a normocytic, normochromic, non-regenerative anemia, and lymphopenia. The blood chemistry panel indicated azotemia, elevated symmetric dimethylarginine (SDMA), hypocalcemia, and hyperphosphatemia. Elevated SDMA level (64 µg/dL, reference value: 0-14 µg/dL) and hypercreatinemia (5.9 mg/dL, reference value: 0.5-1.8 mg/dL) indicated impaired glomerular filtration. Physical and clinical pathological findings signified the presence of CKD in this dog, with a stage-4 severity based on International Renal Interest Society (IRIS) CKD staging criteria. The prognosis of this case was highly guarded, and the dog eventually passed away on the sixth day of hospitalization. In a case with an uncertain outcome, accuracy in both diagnosis and staging of CKD in dogs will aid the therapy regimen planning, which may improve the patient's conditions.

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1 Introduction

Chronic kidney disease (CKD) refers to irreversible pathological alteration that most often arises from unresolved acute injury of the kidney. As a disease of chronicity, diagnosis can be challenging. Clinical symptoms is often subtle when results from diagnostic tools (*e.g.,* abdomen ultrasonography, clinical pathological evaluations) may indicate otherwise. Likewise, other chronic diseases such as metabolic disorders due to endocrinopathy and malignancy may present with the same symptoms as CKD. Therefore, it is important to rule out any other diseases, either being a different entity or concomitant, complicating disease, to diagnose CKD properly. This paper will discuss a case work-up of CKD in a dog, staged as a stage-4 CKD based on International Renal Interest Society (IRIS) CKD staging guideline.

2 Case Reports

A male, intact, mixed-breed dog was presented to My Vets Animal Clinic, BSD City, Indonesia. The dog was recently rescued by the client, approximately 2 weeks before the presentation, and had been brought to another veterinarian who diagnosed the dog with IRIS-stage-2 CKD. The dog had ongoing treatment with oral sodium bicarbonate, keto acids/analog (Aminoral®), and renal diet (Royal Canin Veterinary Diet® RenalTM dog formula).

Based on physical examination, cachexia (body condition score 2/9, Nestlé PURINA Body Condition System), mild dehydration (~6% based on slightly delayed skin turgor and tacky mucous membrane), and halitosis were identified. The characteristic "ammoniac breath" was suspected as uremic fetor. However, calculi were evident on the dog's teeth, hence it could not be ruled out as the possible cause of halitosis. Palpation of the body and auscultation of the heart and lungs were unremarkable, especially the urinary bladder, ruling out any post-renal urinary obstructions. Kidneys could not be appreciated due to thoracic cavity conformation. Because the clinical findings were not conclusive of any diseases with pathognomonic features, the use of other diagnostic tools was warranted. Analysis of complete blood count (CBC, IDEXX ProCyte Dx® Hematology Analyzer) and blood chemistry (IDEXX Catalyst One® Chemistry Analyzer, Chem 15®, SDMA test®) was done on the same day of presentation. Hemogram indicated normocytic, normochromic, non-regenerative anemia (RBC 4.42, reference range 5.65-8.87 x 10^6/µL; Hct 30.5, reference range 37.3-61.7%; Hb 10.2, reference range 13.1-20.5 g/dL; RDW 12.9, reference range 13.6-21.7%) and lymphocytopenia (0.80, reference range 1.05-5.10 x 10^3/µL). The blood chemistry panel indicated elevated serum symmetric dimethylarginine (SDMA, 64, reference range 0-14 µg/dL), creatinine (5.9, reference range 0-14 mg/dL), blood urea (>130, reference range 7-27 mg/dL), phosphate (>16.1, reference range 2.5-6.8 mg/dL) and decreased serum calcium (7.3, reference range 7.9-12.0 mg/dL). Both increased serum SDMA and creatinine indicated acute or chronic kidney injury. The dog, despite the serious increment in SDMA and creatinine levels, was relatively asymptomatic. Hyperphosphatemia and hypocalcemia, along with normocytic, normochromic, and non-regenerative anemia, also hinted at electrolyte disturbance and anemia of CKD, as discussed in the next section of this paper. Although the trends of BUN, creatinine, and SDMA levels in this dogs were not available, based on the serum SDMA and creatinine value, the dog was diagnosed with stage-4 CKD [Chronic Kidney Disease Guidelines, International Renal Society Interest (IRIS)].

Abdominal ultrasonography and blood pressure (BP) monitoring were conducted on the second day after the dog's initial presentation. Ultrasonography revealed narrowed renal medullar space and increased echogenicity of the renal cortex. No active inflammation was indicated, especially on the renal hilus connecting to the renal pelvis. The renal size was not unmeasured during the evaluation. Another abnormal finding from the ultrasonography was biliary sludge, covering up to 80% of the cystic lumen. BP monitoring revealed marked systolic hypertension (197, reference 90-140 mmHg; high-risk individuals, according to IRIS, are those with >40 mmHg above breed-specific blood pressure reference value). Macroscopic, chemical, and sedimentation urinalysis were performed the next day after the dog was admitted as an inpatient. The macroscopic feature of the urine was indicative of hypo- to isosthenuria (*i.e.,* clear urine with no turbidity, urine output was seen to be markedly increased, although quantification was not performed). Isosthenuria was then confirmed through refractometry (urine specific gravity of 1.012). Unstained urine sediment microscopic evaluation was performed, but it showed no apparent abnormalities, which ruled out lower urinary tract infection (UTI) and crystalluria.

During hospitalization, the dog was quiet, alert, and responsive. Polyuria and subsequent polydipsia were noticed, even though urine output quantification was not performed. The dog had postprandial vomiting for the first two days. The use of serotonin (5HT3) receptor antagoniston the first day of hospitalization, ondansetron (0.5 mg/kg IV q24h), was inadequate to suppress the emesis on the second day. Ondansetron was substituted with maropitant (1 mg/kg SQ q24h), and vomiting stopped the following days.

This dog was hospitalized for further evaluation and a timely treatment regimen. The treatment regimen was made specific to abnormalities found during diagnostic tests, though the use of some medications like sodium bicarbonate was not based on laboratory findings (*i.e.,* blood gas analysis), particularly bicarbonate and hydrogen concentration. Due to the mild nature of the dog's anemia, the use of hematopoietic hormone was postponed pending another CBC examination. The dog was treated

with a proprietary blend of calcium carbonate and chitosan (Yochito®) to treat hyperphosphatemia, antiemetics as previously described, and intravenous lactated Ringer's solution (500 mL per day). Other anecdotal drugs used in this case were proprietary keto acids (Aminoral®) and sucralfate (Inpepsa® 100 mg/ml). Other pharmacological agents were administered later, namely enalapril (0.56 mg kg-1 q24h) and ursodeoxycholic acid (Urdafalk® 250 mg, 1 capsule q24h), after hypertension and biliary sludge from abdominal ultrasonography were discovered, as mentioned previously. The dog died 6 days post-hospitalization. Postmortem evaluation was not conducted, thus making the cause of death undeterminable.

3 Discussion

3.1 Pathophysiology of CKD

CKD, as mentioned in the previous part, is characterized by the nearly irreversible damage of the kidney's functional units, nephrons. CKD, or chronic renal failure, is reported when the kidney loses one or more of its physiological functions including (1) metabolic waste excretion through urine formation, (2) acid-base regulation through reabsorption of bicarbonate and excretion of hydrogen, (3) conservation of water, (4) maintenance of intra- and extracellular electrolyte equilibrium, and (5) control of endocrine function, including renin-angiotensin-aldosterone (RAA) axis, calcitriol [1,25-(OH)2-D], and erythropoietin (Breshears and Confer, 2016). A nephron consists of an encapsulated glomerulus, proximal convoluted tubule, Henle's loops, distal convoluted tubule, and collecting tubule. Any disruptions in one or multiple parts of the nephron will subsequently cause renal failure. It takes a severely damaging insult to cause acute renal failure. This acute injury, if unresolved for more than three months, progresses into a chronic state of renal failure (Bartges 2012; Kovarikova 2015).

Damage to the kidney can happen both intrinsically (*e.g.,* renal ischemia, exposure to nephrotoxins) and extrinsically (*e.g.,* any circulation-related disorders that reduce kidney perfusion, any postrenal anomalies that may cause blockage of urine output). Decreased renal perfusion induces the kidneys into an ischemic state that, if it is severe and/or prolonged enough, causes acute necrosis of tubular cells which need high energy to meet the metabolic demand. The outer medulla of the kidney is at the greatest risk of hypoxia and subsequent necrosis due to the already hypoxic nature of the structure. Renal ischemia can be induced by non-steroidal anti-inflammatory drugs (NSAIDs), as any drugs of this class will cause a diminution of intrinsic prostaglandin E2 (PGE2) synthesis, an eicosanoid compound that is responsible for vasodilation of the afferent arteriole when the volume of circulating intravascular decreases (Wilson 2019). It should be noted that dehydration, or other causes of reduced intravascular volume, will cause vasoconstriction of the afferent arteriole which can result in the exacerbation of the renal ischemia and resultant uremic crisis due to the diminished intraglomerular pressure (DiBartola and Westropp 2014). Other nephrotoxic agents are more direct – drugs like aminoglycosides and mycotoxins such as aflatoxins may cause degenerative to necrotic damage of glomerulus and/or renal tubules (Yilmaz et al. 2018). The dog presented to My Vets Animal Clinic, BSD City, Indonesia was recently rescued, hence the cause of renal failure was not possible to be determined, although, according to Ross (2011), previous UTI, arthropod-borne infections (particularly ehrlichiosis, as tick infestation were apparent in this dog), prolonged dehydration, and ingestion of moderately nephrotoxic agents (e.g. chronic exposure of melamine, heavy metals, or organic compounds) may be attributed as the possible etiologies of early renal failure in this dog. Positive serological tests for *Ehrlichia* antibodies have been reported in multiple studies in Indonesian stray dogs (Bagus and Ardana 2017; Nesti et al. 2019; Putra et al. 2019), but further studies are imperative to determine the epidemiology of ehrlichiosis in Indonesian dogs to appropriately determine its risk factor. Serological testing for ehrlichiosis detection was not conducted in this dog, so this hypothesis is unproven. A recent retrospective study has indicated a 2.12 relative risk of CKD acquirement in dogs with ehrlichiosis, a 112% greater risk compared to those in the unexposed group (Burton et al. 2020).

During the initial phase of renal failure, there is latency when clinical signs are absent or minimal. If any kidney insults are removed, renal function will return rapidly. If the insult continues, renal failure enters its maintenance phase, indicating that a significant amount of injury has occurred in renal glomeruli and/or tubules. This phase can persist for 1-3 weeks until restoration happens. It is imperative, therefore, to promptly identify the type of injury (*e.g.,* nephrotoxins, renal ischemia due to decreased intravascular volume, nephrolith, or infection) and resolve the determined issue, although immediate removal of the inciting cause may not guarantee swift renal function restoration if an injury has progressed to the maintenance phase. Some etiological agents are more severe at inducing renal failure. For instance, lily and ethylene glycol induce severe kidney injury with very poor prognosis, even when a treatment like fluid therapy for correction of renal blood flow (RBF) is initiated. Serum creatinine and blood urea nitrogen (BUN) concentration usually will go down during the recovery phase because GFR is restored, ensuring normal diuresis in patients with clinical signs of oliguria or anuria (DiBartola and Westropp, 2014). However, most often than not, acute renal failure induces cellular maladaptation, causing an irreversible structural change in the nephrons (Breshears and Confer 2016).

As an organ responsible for fluid and electrolyte homeostasis, any disorders of the kidney will disturb this balance. This consequence

results from an orchestra of reduced GFR due to afferent arteriole vasoconstriction, leakage of filtered fluid across destroyed tubular epithelial cells into the interstitial space and/or occlusion of intraluminal space by dislodged damaged cells, casts or crystalloids, and/or reduction in glomerular permeability due to contraction of mesangial cells and its subsequent decrease in several fenestrations formed by podocytes, causing a significant reduction of effective filtrating surface area (DiBartola and Westropp 2014). Glomerulonephritis can also cause glomerular proteinuria, as intact glomeruli are responsible for the selective permeability of the filtrate so that protein greater than 70 kDa cannot be filtrated (Breshears and Confer 2016). Proteinuria, in renal failure, has been associated with further deterioration of the remaining renal function (Harley and Langston, 2012). Fluid disturbance was evident in this dog, and this abnormality is possibly attributable to the prevailing deterioration of kidney function. Proteinuria was detected based on a dipstick test (VET-10® urine test strips, KRUUSE). However, diagnostic acumen should be taken because proteinuria, especially in free-catch samples such as in this case, can be falsely positive in the presence of genital disease and other artifacts (*e.g.*, alkalized urine due to prolonged storage, presence of penicillin metabolites, microscopic hematuria). Urine sedimentation was done after initial dipstick evaluation and the result did not show active sediment (*i.e.*, presence of pus, erythrocytes, and/or casts). Therefore, proteinuria was concluded to be of intrarenal origin. Unfortunately, this finding was not evaluated further by the measurement of the urine protein-to-creatinine ratio. This evaluation is useful to determine the risk of any further renal damage that should be carefully addressed in the treatment regimen.

Potassium is a main intracellular cation and its concentration in extracellular space is tightly regulated. Patients with CKD may present with hypo- or hyperkalemia. Hypokalemia is usually associated with reduced renal reabsorption in the proximal tubules and abrupt reduction to elimination of sodium that results in activation of the RAA system and subsequent enhancement of potassium secretion from distal tubules (Palmer 2015; Polzin 2011). Renal failure, especially in stage-4 CKD, may cause renal hyperkalemia. Although common, hyperkalemia usually develops in patients with impaired renal excreting ability and/or concomitant disorders such as urine obstruction, adrenal insufficiency, and diabetes mellitus (Pak 2000). In humans, decreased renal function causes metabolic acidosis through increased retention of hydrogen cation and decreased reabsorption of bicarbonate anion, another renal metabolic disorder that may exacerbate hyperkalemia due to diminished potassium excretion by cortical collecting tubules in low blood pH (Breshears and Confer 2016; Krapf et al. 2008). Hyperkalemia-related clinical symptoms including muscle weakness and lethargy with clinical findings such as dysrhythmia and electrocardiograph (ECG) anomalies such as peaked T waves, increased PR intervals, and widened QRS complexes, indicating heart conductivity disorder (Parham et al. 2006). However, hyperkalemia can have a biphasic effect on conduction and excitation, dependent on the resting membrane potential level and the difference between the resting and the threshold potential. Indeed, the increased serum potassium level may initially speed up the ventricular repolarization due to less negativity of atrial myocytes' resting membrane potential and its subsequent elevated excitability threshold, then followed by a diminution in the cell membrane's depolarization ability due to increased resting membrane potential (Johns et al. 2011). ECG and serum potassium were not evaluated in this dog. However, the absence of arrhythmia based on heart auscultation indicated normal cellular electrical conductivity and possibly normal serum potassium level.

3.2 Other Consequences of CKD

There is a myriad of consequences that can happen in patients suffering from CKD. Reduction of RBF induces activation of the RAA axis which can cause systemic hypertension through increased heart preload volume and myocardial contractility exertion. Through this mechanism, other clinical consequences such as retinopathy, encephalopathy, myocardial hypertrophy, and further remaining renal function destruction ensue. Management of hypertension in CKD patients should be done and monitored periodically (Acierno et al. 2018). Due to the association between morbidity-mortality rate and hypertension, BP monitoring is critical. BP also can be used to determine the prognosis of CKD. This dog had severe systolic hypertension, indicating a high risk of multiple organ damage and further renal deterioration.

Tubular epithelial cells, especially the proximal convoluted tubule, synthesize cytochrome P450 enzyme (CYP27B1), and tubular degeneration and/or necrosis lessens the production of which substance is needed to hydroxylate 25-(OH)-D (calcidiol) into calcitriol. Calcitriol induces tubular and small intestinal calcium reabsorption. Phosphate, an anion that is mostly stored inside the bone, and to a lesser extent in the extracellular compartment, is mainly eliminated through renal excretion. In case of renal failure, serum phosphate is retained. Hypocalcemia and hyperphosphatemia may present with the sequelae of secondary hyperparathyroidism. Hyperparathyroidism leads to increased resorption of the bone, eventually worsening the hyperphosphatemia and hypocalcemia, as serum phosphate complexes with serum calcium. The blood chemistry panel of this dog indicated hypocalcemia and hyperphosphatemia, both of which are the causative agents of secondary hyperparathyroidism in CKD patients. Osteodystrophy and heterotopic ossification have been reported in CKD patients (Hruska et al. 2008; Polzin 2011; Stillion and Ritt 2009). This dog, however, was ambulatory with no gait anomalies, thus osteodystrophy was not indicated.

Metabolic acidosis is another clinical-pathological finding in patients with CKD. Renal function is essential to maintain the equilibrium of serum bicarbonates and hydrogen concentration. Bicarbonate is one of the main chemical buffers that are readily used to offset the fluctuation of hydrogen ions. Although the compensation is delayed in comparison to chemical buffer and respiratory compensation, renal compensation remains vital in maintaining blood pH value. In acidic blood, normal renal parenchyma, especially the proximal convoluted tubules, will increase their bicarbonate reabsorption and hydrogen secretion through Na^+/H^+ exchangers, H^+-ATPases, and Na^+/HCO_3^- co-transporters. Another key player of renal metabolic acidosis is the increased retention of acid anions (*i.e.*, uremic acidosis, marked by elevation of organic acid concentration such as phosphoric acid), contributing to the diminution of blood pH, marked by high serum anion gap (Ha et al. 2013; Kraut and Madias, 2010). When metabolic acidosis happens respiratory compensation entails where carbon dioxide is actively exhaled, thus decreasing the carbon dioxide partial pressure (PCO2). About 1.0 mmHg of PCO_2 will be reduced to offset the 1 mEq/L decrement of bicarbonate concentration. This compensatory mechanism starts immediately when pH drops and usually completes within hours, and it should be noted that this compensatory mechanism will eventually get overwhelmed if acidification continues (DiBartola 2012).

3.3 Staging and Sub-Staging of CKD

Once CKD has been determined as the definitive diagnosis, staging and sub-staging entail. Staging and sub-staging are done in accordance to the IRIS' chronic kidney disease guidelines that have been approved by the American and European Societies of Veterinary Nephrology and Urology (Kovarikova 2015; Polzin 2011). Based on laboratory results, the dog, in this case, was diagnosed with stage-4 CKD with a high risk of multiorgan damage due to severe systolic hypertension. Proteinuria had been screened with dipstick, and was not evaluated by UP/C, hence characterization of proteinuria in this dog is not available. To stage CKD, both serum creatinine and SDMA are used. IRIS' chronic kidney disease guideline recommends both parameters to be assessed together. However, in many instances where SDMA quantification is unavailable, staging based on fasted serum creatinine level is also possible. However, early detection may be impossible when serum creatinine level was used as a sole parameter of staging. Care must be taken that, if possible, serum creatinine level is measured on two separate occasions, all of which should be done in fasted and hydrated state, to rule out prerenal azotemia (Polzin 2011; Kovarikova 2015; Sargent et al. 2021). Staging of CKD in this dog was done immediately after the hematological evaluation dog was first presented. Sub-staging of this dog was conducted on the second day of hospitalization. Although UP/C was not evaluated in this case, the authors strongly encourage all clinicians to perform UP/C when presented with a confirmed case of CKD in their patients.

Table 1 IRIS stages of CKD in cats and dogs

Parameters	Dog	Cat
Creatinine (mg/dL)		
Stage 1	< 1.4	< 1.6
Stage 2	1.4 – 2	1.6 – 2.8
Stage 3	2.1 – 5	2 – 5
Stage 4	> 5	> 5
SDMA (µg/dL)		
Stage 1	> 18	> 18
Stage 2	18 – 35	18 – 25
Stage 3	36 – 54	26 – 38
Stage 4	> 54	> 38

SDMA has been gaining recognition as a novel endogenous biomarker that offers sensitivity in early renal failure detection in comparison with creatinine. Creatinine has been used both in human and veterinary medicine to assess renal function, in particular renal clearance. However, a steep curvilinear relationship between GFR and serum creatinine revealed no significant increase in serum creatinine until a significant diminution of GFR occurs, making creatinine a less sensitive renal biomarker to signify early renal failure. It is estimated that 75% of nephron loss will result in a value that is higher than the upper-limit value. Therefore, several measurements need to be taken to detect any subtle changes in serum creatinine levels (Kovarikova 2015; Hall et al. 2016). Serum creatinine is also affected by an extrarenal factor, especially the percentage of lean body mass and biological age of the patient. SDMA is an N-methylated form of arginine residue methylation, a post-translational modification that aids in its detection after release through proteolysis. SDMA has a renal clearance of up to 90% and even a slight elevation from its serum concentration reference range readily alerts for renal damage. Previously, its measurement had always been done by use of liquid-chromatography-mass spectrometry (LC-MS). Presently, SDMA assay is offered by a commercial veterinary diagnostic laboratory, IDEXX, through high-throughput immunoassay, thus offering less analytical variability. It should be considered, however, that SDMA may be affected by the patient's age and breed. One study indicated that a healthy, young dog had serum SDMA of up to 16 µg/dL, a concentration that otherwise will indicate mild elevation in adult dogs. It has been discovered that greyhounds have significantly higher mean serum SDMA concentrations in comparison to other dog breeds. Conclusively, it is advised to interpret serum SDMA concentration in both juvenile animals and greyhound dogs carefully, especially if other

Table 2 IRIS sub-stages of CKD in cats and dogs

	Dog	Cat
UP/C		
Proteinuria	Proteinuria	Proteinuria
Borderline proteinuria	Borderline proteinuria	Borderline proteinuria
Non proteinuria	Non proteinuria	Non proteinuria
Mean arterial blood pressure (dog, systolic; cat, diastolic) (mmHg)		
Minimum risk	< 150	< 95
Low risk	150 – 160	95 – 99
Moderate risk	160 – 179	100 – 119
High risk	≥ 180	≥ 120

diagnostic tools such as urinalysis and ultrasonography are at the clinician's disposal (Sargent et al. 2021). SDMA algorithm has been excellently provided by IDEXX and can aid in the early detection of renal failure[1].

Sub-staging of CKD facilitates the determination of prognosis and clinical reasoning necessary for pharmacological intervention that should be used on the patient. IRIS recognizes two sub-staging parameters, UP/C and BP. UP/C is a gold-standard test to determine risk factors associated with proteinuria and should be done after the dipstick test indicated a trace of increased protein in the urine. After urine sedimentation has been evaluated and the presence of active sedimentation has been ruled out, UP/C will help to distinguish the degree of proteinuria in the patient. BP, as already mentioned in the previously in this paper, needs to be monitored because renal failure will subsequently increase the activation of the RAA axis. Hypertension significantly increases the mortality rate in CKD patients. It is recommended that both parameters are evaluated at least twice over several weeks to accurately sub-stage the CKD (IRIS 2019).

3.4 Planning the treatment regime

Once staging and sub-staging have been done, choosing the right treatment regimen is the next thing a clinician should do. According to IRIS' Treatment Recommendations for CKD in Dogs[2], treatment in CKD patients is used to (1) impede the progression of CKD, hence the remaining renal function is spared, and (2) improve the life quality of the patient by correcting any CKD-associated clinical abnormalities. The former treatment goal is especially important in stage 1 and 2 patients, emphasizing the earlier the diagnosis, the better the prognosis. In CKD patients, it is important to identify any other intrarenal factors that potentially exacerbate renal failure. Identification and elimination of exposure to nephrotoxic agents, concomitant UTI, and/or urolithiasis, particularly nephroliths, and treatment of extrarenal abnormalities are important to preserve the remaining renal function and improve the life quality of the patient (Polzin 2011). In addition to CKD-associated clinical consequences, the dog, in this case, was also diagnosed with biliary sludge through abdominal ultrasonography. With this regard, the use of drugs unrelated to renal failure will also be discussed in this paper.

Correction of dehydration and maintenance of hydration ensure euvolemia and adequate renal perfusion. Therefore, it is important to provide an ample quantity of fresh water for the patient. However, it should be underscored that CKD patients might have impaired renal ability to appropriately concentrate the urine. It is of utmost importance, therefore, to quickly correct any clinical dehydration and/or hypovolemia with intravenous fluid administration. Lactated Ringer's solution (273 mOsm/L) was used (500 mL, dripped over 9 hours, q24h) to correct dehydration in this dog. Because the dog was still drinking, administered volume was estimated based on dehydration level and ongoing loss that was vomiting and polyuria. Vomiting happened twice a day for the first two days before finally being managed using a maropitant. It was noted, however, that the volume of fluid administered might have not been adequate because tacky mucous membranes and slightly delayed skin turgor were persistent throughout the dog's hospitalization. According to Polzin (2011), the use of crystalloid isotonic solution should be adjusted based on the patient's response. In other words, an increase in the volume administered would have been appropriate to correct the hydration status of the patient. It is also greatly suggested that ongoing loss (*e.g.*, urine output, vomiting, diarrhea) needs to be measured in the future, as it will help with the prudent calculation of fluid therapy that will be administered to the patient (DiBartola and Bateman 2012).

[1]IDEXX. https://ca.idexx.com/en-ca/veterinary/reference-laboratories/sdma/interpreting-your-sdma-results/
[2]IRIS. (2019). http://www.iris-kidney.com/pdf/IRIS-DOG-Treatment_Recommendations_2019.pdf

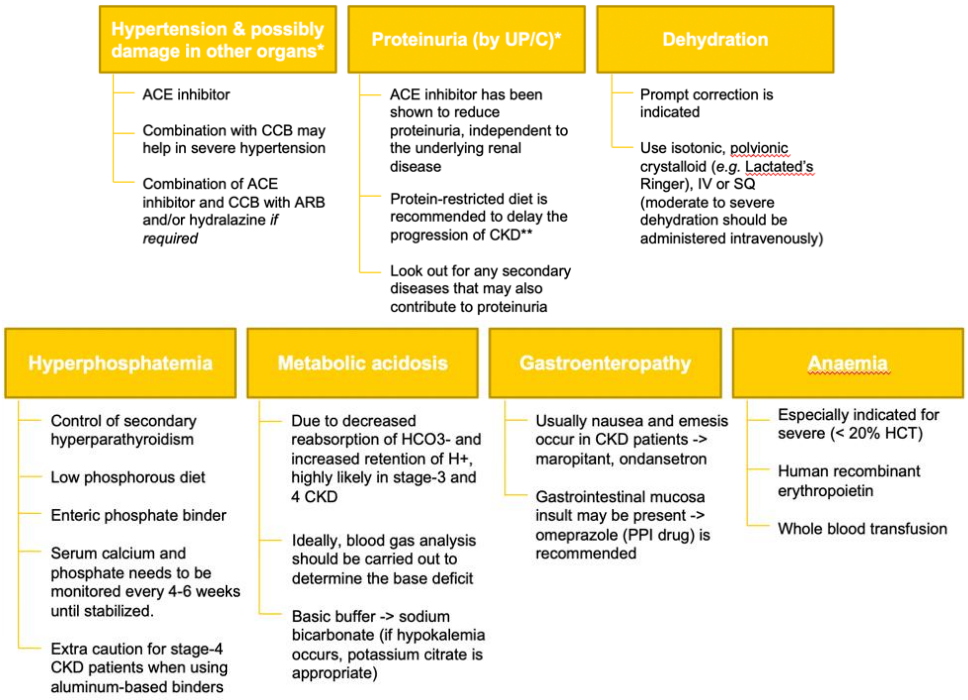

Figure 1 Approach to treatment of CKD patients (adopted from IRIS)
** In conjunction with low phosphorus dietary plan

Compared to human medicine, BP monitoring remains challenging for veterinarians on the account of differences in subject populations, measurement techniques, and handling methods. Hound dogs are found to naturally have higher arterial blood pressure than other dog breeds. In dogs, males have higher arterial BP, even when the males subjected to BP readings are already castrated, whereas intact females have lower BP. In contrast, neutered male cats have higher BP than females and even their intact counterparts (Acierno et al. 2018). In addition to those differences, the endpoint of antihypertensive drug administration in cats and dogs remains a moot point (Polzin 2011). Therefore, judicious use of antihypertensive drugs is necessary. ACEI is beneficial in reducing intraglomerular hemodynamics and its subsequent degree of proteinuria. Although ACEI can inhibit RAA system activation and its following elevation in systemic vascular resistance, its ameliorating effect is thought to be more attributed to its vasodilating effect in efferent arterioles, notwithstanding its lack of data to prove its efficacy and superiority against other antihypertensive agents in advanced CKD, at least in human (Hou et al. 2006; Ahmed et al. 2016). Nonetheless, the use of ACEI is recommended as the first-line antihypertensive drug according to IRIS, hence the use of enalapril (0.5 mg/kg q24h) in this dog. The efficacy of this drug in managing the dog's hypertension and suspected proteinuria could not be assessed. However, an increase in dosing frequency from q24h to q12h is recommended when enalapril is used, especially in severely hypertensive patients, and the addition of renal diet as an adjunct is shown to be more efficacious than the use of enalapril alone (Zatelli et al. 2016). Enalapril should never be used unless dehydration has been corrected and long-term monitoring is advised due to increased elimination half-life as the kidney plays a major role in the drug's metabolite excretion (Plumb 2011).

As mentioned in the previous part, hyperphosphatemia is detrimental due to its induction of secondary hyperparathyroidism and further exacerbation of renal failure. Secondary hyperparathyroidism and inhibition of calcitriol production will worsen the hypocalcemia state, if present, in CKD patients (Hruska et al. 2008; Stillion and Ritt 2009). In this case, the dog was treated with a proprietary blend of chitosan and calcium carbonate (q12h, with meal). In addition to the administration of the previously mentioned phosphate scavenger, the dog also received a renal diet that is low in phosphorus. Although hyperphosphatemia management in stage-3 and 4 CKD gives no merit in slowing down CKD progression, it can, at least, improve the patient's life quality. Aluminum (in hydroxide, oxide, or carbonate form), lanthanum (in carbonate form), and calcium (in acetate, carbonate, or citrate form) are used, although the use of aluminum-based phosphate binder is discouraged because of its nephrotoxicity (Segev et al. 2008; Polzin 2011).

Amelioration of renal metabolic acidosis is preferably done upon confirmation through blood gas analysis which can provide information regarding the patient's blood pH value and the serum

level of bicarbonate, so bicarbonate deficiency can be identified and calculated. The calculation is therefore advised by using the following formula[3]: [(n of the desired HCO3-) – (n of measured HCO3-)] x 0.5 x BW (kg), although patients with confirmed metabolic acidosis may require an additional dose of bicarbonate. When blood gas analysis is performed, it should be noted that aerobic handling of blood samples does not affect dissolved bicarbonate concentration while it affects carbon dioxide concentration (DiBartola 2012). Administration of bicarbonate has been used in human patients with a satisfactory result in the retardation of CKD progression and improvement of nutritional status (Bartges 2012). Sodium bicarbonate (500 mg, q12h) was used to treat presumptive metabolic acidosis in this dog, even though blood bicarbonate concentration is unknown. Care should be given to avoid overzealous alkalization of the blood (*i.e.*, not exceeding 19-23 mEq/L of bicarbonate), as no recommended dose of sodium bicarbonate in cats and dogs with CKD has been established (Zatelli et al. 2012). In an experimental setting, a dose of 0.01 g/kg body weight, divided into two daily administrations with a meal was seen to be beneficial for increasing serum bicarbonate levels in dogs with advanced CKD. However, considerations should be taken because the administration of sodium bicarbonate in this study used a proprietary amalgam of other substances such as calcium lactate gluconate, calcium carbonate, and chitosan (Martello et al. 2020).

Constant vomiting can exacerbate the existing fluid and electrolyte imbalances in CKD patients. This is particularly induced by uremia and the clinical presage is not limited to nausea and vomiting. Stomatitis, uremic fetor, gastrointestinal ulceration and hemorrhage, diarrhea, and hemorrhagic colitis are common alimentary tract signs in patients with stage-3 and 4 CKD. Reduced GFR also results in increased gastrin half-life, causing upregulation of hydrochloric acid by parietal cells of gastric fundus and corpus, consequently exacerbating gastrointestinal symptoms by directly insulting gastric mucosa (Santacoloma Osorio et al. 2017). Therefore, the use of gastric acid blockers and antiemetics is recommended in CKD patients. In this study, ondansetron (0.5 mg/kg, IV, q24h) and sucralfate (1 g/30 kg, q12h before meal) were used in this patient. However, ondansetron administration, in this case, may not be sufficient to suppress nausea and vomiting due to the under-frequency of drug administration (therapy regimen, in this case, was 0.5 mg/kg IV q24h, but its pharmacokinetic property, particularly its half-life and elimination rate, warrants for a constant rate infusion administration of 0.5 mg/kg/h up to 6 hours) (Plumb 2011). Sucralfate administration in CKD patients remains debatable due to the increased risk of aluminum toxicity, particularly in long-term use (Hemstreet 2001; Segev et al. 2008). Nevertheless, Polzin (2011) suggests that the use of sucralfate may be beneficial for gastric lining protection. Administration of a proton-pump inhibitor, omeprazole (0.5 mg/kg, q24h, IV), was started the day after initial gastrointestinal drugs were given. In addition, due to refractory postprandial vomiting, ondansetron was substituted with another antiemetic agent with a longer half-life and potentially more central action (neurokinin-1 antagonist compared to 5-HT3 antagonist), maropitant (1 mg/kg, q24h, SQ). Vomiting was resolved afterward.

Anemia, in this case, was treated with hematopoietic factor supplementation (Sangobion®) that contains ferrous gluconate. Iron supplementation has been associated with the alleviation of anemia by increasing the production of heme and subsequently hemoglobin (Naigamwaila et al. 2012). As anemia of chronic disease, erythrocytes in CKD patients are usually fragmented with/without the presence of Howell-Jolly bodies if a microscopic blood smear evaluation is performed. CBC finding characteristic of anemia of chronic disease is its poorly non-regenerative state as indicated by reticulocyte count and red blood cell distribution width (RDW) (Lippi et al. 2021). An erythropoiesis-stimulating agent such as human recombinant erythropoietin (not approved for veterinary use) and darbepoetin is indicated when anemia is severe enough, as manifested through hematocrit value (< 20%). Erythropoiesis-stimulating agent administration needs to be monitored for the risk of hypertension exacerbation in animals with CKD has been reported (Acierno et al. 2018).

The use of keto acids (Aminoral®) was another empirical therapy that was given to this dog. Keto acids or keto analogs are precursors of essential amino acids so through the process of amino acids transfer, ammonia (NH_3) can be consumed. In human medicine, with the addition of a low protein diet, administration of keto acids has been shown to impede the deterioration of renal function, though it should be noted that a review study suggested no significant differences in serum creatinine and blood urea nitrogen (BUN) between control (untreated) group and treatment groups (Jiang et al. 2016). Albeit that, in veterinary medicine, the study of keto acid administration remains polarized. According to a study conducted by Zatelli et al. (2017), supplementation of keto analogs might prevent the decrement of proteinuria (UP/C) and serum urea concentration, although the supplemented group did show improvement in terms of body condition score (BCS) and serum albumin. Another study conducted in dogs with stage-3 CKD indicated no improvement in terms of BUN and serum creatinine value (Linguori et al. 2018). Therefore, further studies are warranted before keto acid supplementation is recommended in dogs.

Conclusion

Appropriate diagnosis of CKD and its entailing staging and sub-staging play a crucial factor in the determination of a patient's life expectancy through meticulous treatment regimen planning and

[3]Dodam, J. (n.d). *Sodium bicarbonate*. https://www.vetstream.com/treat/canis/generics/sodium-bicarbonate

periodic monitoring. Not every drug recommended is necessary to be administered – sagaciousness to discriminate clinical and laboratory findings are advised.

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