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Present Status, Practices, Limitations, and Future Prospects of Organic Fruit Production in Nepal

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ABSTRACT

Organic fruit production in Nepal has significant potential due to the country's varied agro-climatic conditions and reliance on traditional agricultural practices. However, this sector faces considerable challenges, including high certification costs, limited market access, and inadequate infrastructure. Despite the increasing global demand for organic products and a growing interest among local consumers, only 0.3% of Nepal's agricultural land is certified organic. This review analyzes 27 studies that examine the current status, practices, limitations, and prospects of organic fruit cultivation in Nepal. The findings reveal that smallholder farmers comprise most organic fruit growers, primarily using traditional methods such as composting and animal manure, which align well with organic farming principles. However, the lack of certification limits their access to premium markets. To enhance organic fruit production in Nepal, this review emphasizes the need for policy reforms that simplify certification procedures, improve infrastructure, and strengthen market linkages. Increased involvement of cooperatives and non-governmental organizations (NGOs) can provide smallholder farmers with essential training, technical guidance, and resource access. Furthermore, raising community awareness through targeted initiatives will boost local demand, encouraging more farmers to adopt organic practices. Despite the existing challenges, the study highlights Nepal's strong potential to compete in the global organic marketplace. By addressing key barriers and promoting sustainable farming practices, Nepal can enhance environmental sustainability, improve rural livelihoods, and strengthen its organic fruit industry. This review also presents policy recommendations to foster a more robust and inclusive organic farming system in Nepal.

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1 Introduction

Over the past 50 to 60 years, agrochemicals' widespread use in fruit production has led to significant environmental and health hazards and socio-economic challenges (Pathak and Ram 2004). In response, organic farming practices have gained global interest as a viable agricultural method that minimizes environmental harm and offers substantial health benefits to consumers. By 2020, the global area dedicated to organic fruit production reached 1.1 million hectares, driven by increasing consumer demand and supportive policies for sustainable agriculture (Willer et al. 2022). Apples, kiwis, and citrus fruits are prominent in this sector, with FAOSTAT noting organic farming's integration into global agricultural systems. These trends highlight organic agriculture's growing importance in addressing environmental and market needs (FAO 2023).

In Nepal, organic fruit production is still in its early stages but is gradually gaining traction due to rising interest from farmers, consumers, and policymakers (Atreya et al. 2020). There is an urgent need for cost-effective input generation, optimization of agrochemical combinations, ongoing soil health improvements, enhanced produce quality, and eco-friendly technologies (Ram and Kumar 2019).

Globally, organic farming has experienced significant growth, fueled by robust consumer demand for sustainable agricultural practices (Granatstein et al. 2013). The organic food market has expanded rapidly, with fruits being one of the most popular organic products. Countries like the United States, European nations, and Australia have emerged as leaders in organic production, offering models that Nepal and other nations could emulate. However, Nepal's challenges, especially regarding infrastructure and market access, differ markedly from those experienced in more developed countries (Granatstein and Kirby 2016). Currently, there are no structured markets for selling organic produce. Global organic cultivation generally relies on demand and is often conducted on a contractual basis (Mondal 2012). Worldwide, fruit and vegetable biodiversity is declining, necessitating urgent conservation efforts. Genetic resources are under-conserved, which calls for national strategies, global partnerships, sustainable practices, and awareness campaigns for effective germplasm conservation (van Zonneveld et al. 2023).

For instance, organic production in Turkey started in the mid-1980s and has seen a significant increase in demand over the past decade, with the leading organic fruits being raisins, figs, and apricots. Turkey's diverse conditions offer great potential for organic agriculture, but challenges include loss of diversity, low soil fertility, and high certification costs (Gubbuk et al. 2004). The production of organic fruits worldwide is thriving due to increasing consumer demand, favorable policies, and robust infrastructure. Nepal's organic fruit sector faces high certification costs, limited market access, and a lack of farmer awareness. Insufficient infrastructure, fragmented supply chains, and inadequate government policies hinder growth. Addressing these issues is critical for Nepal to align with global trends and leverage its agro-climatic potential.

Despite technological advances since the early 1990s in conventional and Integrated Fruit Production (IFP), fruit growers remain hesitant to transition to the newer, less specialized organic fruit market, primarily due to increased risks of lower yields (Weibel 2002). Organic production of oilseeds, largely soybeans, saw a 28% increase, while vegetables, particularly those from Mexico, experienced an 88% increase. However, the production of vegetable oils, tropical fruits, and feedstuffs faced a 15% decline, including olive oil from Tunisia and palm oil from Latin America (Willer et al. 2024). Nevertheless, organic fruit production can be successful and rewarding if pest, disease, and weed control are effectively managed, soil fertility is maintained, and various management practices are implemented (Neeson 2008).

Organic farming suggests that organically grown foods may be nutritionally superior, containing higher levels of vitamins, minerals, and amino acids (Shaltout 2024). A review of 343 studies indicates that food produced under organic standards may have beneficial antioxidants and reduce exposure to harmful heavy metals (Baranski et al. 2014). This review examines the current status, traditional practices, and limitations of organic fruit production in Nepal while exploring its growth prospects. The analysis is based on 27 research articles, providing a comprehensive overview of the country's prevailing conditions, challenges, and opportunities for organic fruit producers.

2 Methodology for Systematic Review Using PRISMA Model

A systematic review followed PRISMA guidelines to evaluate the current status, practices, limitations, and future prospects of organic fruit production in Nepal. Researchers utilized Google Scholar and PubMed to search for relevant articles using key terms such as "organic fruit farming in Nepal" and "sustainable fruit production in Nepal." The review focused on peer-reviewed, English-language studies related explicitly to organic fruit production in Nepal while excluding non-relevant and non-open-access papers. The initial search resulted in 216 articles undergoing a multi-stage screening process. Firstly, 35 duplicates were removed, and 11 irrelevant materials were excluded. Title analysis led to the filtering out of 104 articles. Following this, an abstract screening eliminated 20 more articles. During the full-text screening, an additional 19 articles were excluded. Ultimately, 27 articles that met all inclusion criteria were selected for the final review, providing valuable insights into the landscape of organic fruit production in Nepal (Figure 1).

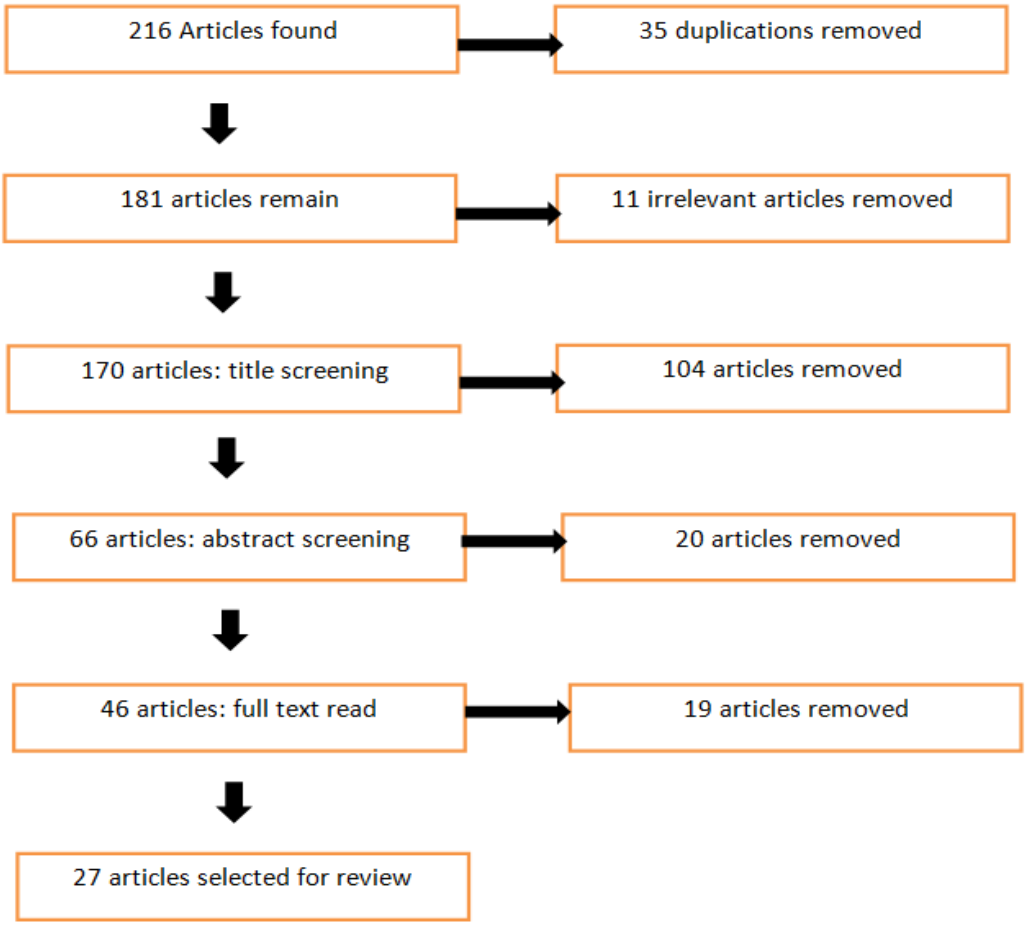

Figure 1 Flowchart of the methodology

3 Present Status of Organic Fruit Production in Nepal

3.1 Organic Farming in Nepal: An Overview

Nepal's diverse topography, varying altitudes, and climates create favorable conditions for cultivating various fruits, from temperate varieties like apples and pears to tropical fruits like citrus (Tamang et al. 2011). Despite this potential, organic farming in the country remains relatively underdeveloped. Only 0.30% of Nepal's agricultural land is certified for organic agriculture (Atreya et al. 2020). This low adoption rate can be attributed to several factors, including a lack of awareness, high certification costs, and limited market access (Chand et al. 2022). Organic fruit production in Nepal is primarily driven by smallholder farmers in remote hill and mountain regions, where traditional farming practices align closely with organic principles (Rokaya and Pandey 2023). In these areas, farmers rely on age-old techniques such as composting and using animal manure, making the transition to organic farming more feasible (Figure 2). However, the lack of formal certification prevents these farmers from accessing premium domestic and international organic markets (Sharma 2014).

3.2 Organic Fruit Varieties in Nepal

The geography of Nepal supports cultivating a diverse range of fruits under organic conditions. In temperate regions such as Mustang and Jumla, fruits like apples and pears are grown organically. Meanwhile, in lower-altitude areas, various tropical fruits, including specific citrus varieties, are cultivated organically (Hijazi 2021). One of Nepal's most popular organic fruits is citrus, particularly mandarins, which farmers practice sustainable agriculture in regions like Salyan (Dahal et al. 2023).

Organic apple production is particularly prominent in high-altitude areas, with Jumla benefiting from a cool climate ideal for growing these apples. However, two significant challenges to enhancing organic apple production are pest management and water scarcity (Neilsen et al. 2009). Additionally, inadequate storage facilities lead to substantial post-harvest losses, preventing farmers from realizing their expected economic benefits (Peck et al. 2009). Beyond apples and citrus, Nepal also produces organic pears, plums, and other stone fruits (Acharya and Atreya 2012). However, the production of these fruits is often limited; most are

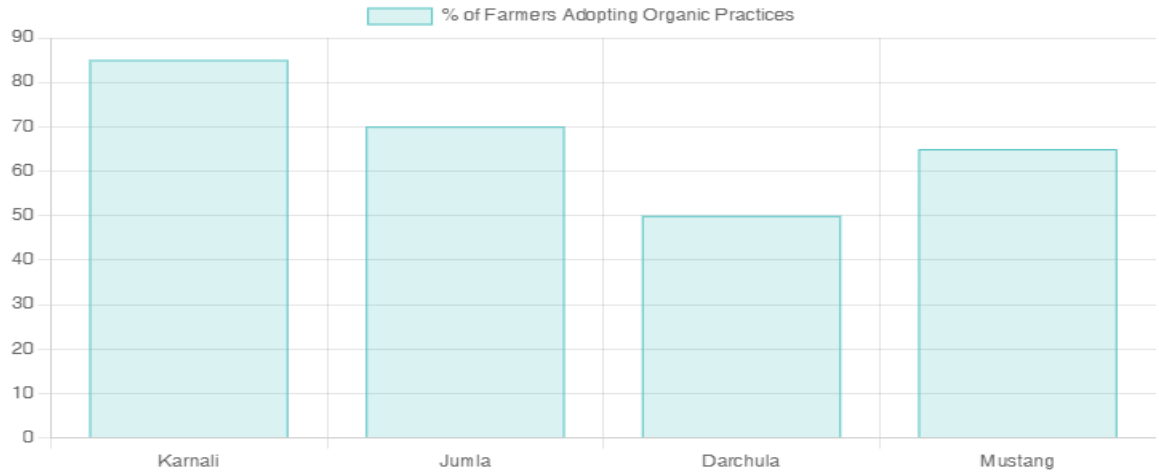

Figure 2 displays the percentage of farmers adopting organic farming in Karnali, Jumla, Dachula, and Mustang, showing Karnali with the highest adoption and Dachula with the lowest. Factors like policy support, awareness, and agricultural conditions influence these variations.

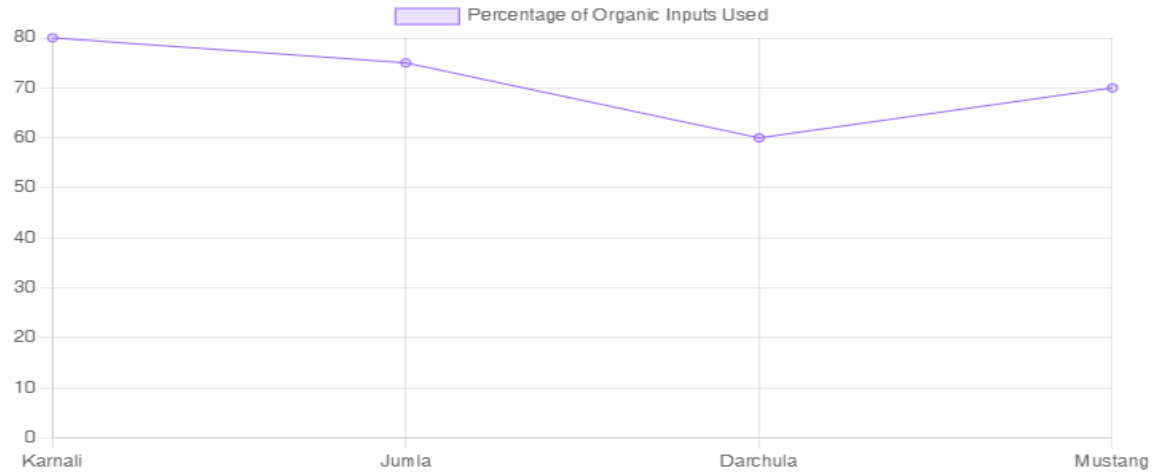

Figure 3 illustrates the percentage of organic inputs used by farmers in Karnali, Jumla, Dachula, and Mustang, showing a decline from Karnali to Dachula, with a slight increase in Mustang. This variation may reflect differences in resource access, farming practices, or regional policies supporting organic agriculture.

grown in small quantities, primarily for local consumption. The absence of proper certification further limits efforts to access wider markets (Shrestha 2021).

3.3 Certification and Market Access

Certification poses one of the biggest challenges for organic farmers in Nepal. While urban areas like Kathmandu are experiencing increased consumer demand for organic products, many rural farmers either lack awareness of the certification process or find it prohibitively expensive (Tamang et al. 2011). The high costs and complex bureaucratic steps in obtaining organic certification from agencies like Organic Certification Nepal (OCN) create significant obstacles for smallholder farmers (Chand et al. 2022). For many of these farmers, the benefits of certification seem distant. With limited market access and inadequate infrastructure, especially in remote areas, the steep certification costs often outweigh the potential for higher prices associated with certified organic products. Consequently, many farmers resort to selling through local informal networks, where there is usually no clear distinction between organic and conventional produce (Banjara 2016).

4 Practices of Organic Fruit Production in Nepal

4.1 Use of Organic Inputs

One of the fundamental principles of organic farming is the use of natural inputs. In Nepal, this often involves compost and livestock manure. Organic fruit production in the country heavily depends on local inputs, particularly for maintaining soil fertility (Figure 3). For example, Burmese grapes (*Baccaurea ramiflora*) have been

shown to benefit from organic fertilizers like Kazi and Payel, which help plants absorb nutrients, grow, and increase yields (Munna et al. 2021). Incorporating compost enhances tree growth, flowering, and yield over time, making compost-filled spaces more productive than untreated areas (Schupp and Mora 2004). Organic matter improves the soil's ability to retain water and nutrients, while lime helps maintain soil calcium levels. This highlights Nepal's widespread use of animal manure and compost to support soil health (Shrestha 2021). The organic approach to fruit production emphasizes natural reproduction methods, allows for artificial insemination, prohibits synthetic drugs, promotes nutrient conservation, and encourages the cultivation of diverse crops to improve soil quality (Chandran et al. 2019). Despite frequent organic inputs, access to these materials can be limited in remote areas (Granatstein and Kirby 2016). Transportation challenges in these regions hinder farmers' ability to obtain organic fertilizers, which affects their capacity to increase production. Effective organic nutrient management requires a long-term perspective, soil testing, compost incorporation, a holistic approach to agricultural ecosystem management, addressing calcium deficiency, and compliance with organic standards set by the Organic Materials Review Institute (OMRI) (Schupp and Mora 2004).

4.2 Pest and Disease Management

Managing pests and diseases is one of the most significant challenges faced by organic fruit producers in Nepal. Due to the limited availability of biopesticides and natural pest control methods, farmers often rely on traditional pest management practices (Weibel et al. 2002). For example, neem extracts and biopesticides such as *Bacillus thuringiensis* (Bt) and *Beauveria bassiana* (a fungal pathogen) are commonly used to control pests in fruit orchards (Hijazi 2021). However, the effectiveness of these methods can be inconsistent, highlighting the urgent need for more research into sustainable pest management techniques, especially for high-value fruits like apples and pears (Rozpara and Głowacka 2012).

Preventive methods are crucial for successfully growing organic fruit, but various regulations, registration issues, and complex production requirements hinder the effectiveness of alternative pesticides (Tamm et al. 2002). Many organic fruit farms face pest management challenges due to their geographical isolation, which limits access to modern pest control technologies. This isolation also restricts the exchange of knowledge regarding organic pest management practices, making it difficult for farmers to adopt new methods that could enhance yields. Diseases and pests, such as scab, sooty blotch, and fire blight, complicate organic fruit cultivation. To address these issues, effective control methods, resistant varieties, and natural predators are necessary (Tamm et al. 2002).

Some organic compounds that effectively manage pests include volatile organic compounds (VOCs) and natural plant products, which aid in integrated pest management (IPM). Certain VOCs, such as eucalyptol and methyl salicylate, deter pests like *Aphis punicae* and *Delia radicum* by attracting natural enemies, which can enhance biological control (Lamy et al. 2017; M'sakni et al. 2024). Plant oils, including pine and castor oil, demonstrate strong insecticidal effects, achieving up to 80% mortality in pests such as whiteflies (Böhme and Dimitrov-Skatov 2018). Additionally, plant-based compounds and semiochemicals like pheromones play a crucial role in altering pest behavior through strategies like "push-pull" and "lure and infect," which make pest control more effective (Alves and Ascari 2019; Shashank et al. 2024). These organic solutions contribute to sustainable agricultural practices. However, regulatory barriers limit access to innovative pest control methods (De Wilde et al. 2024; Muhammad and Umma 2024).

4.3 Water and Soil Management

Water and soil management are crucial for the success of organic fruit farming, particularly in water-scarce regions of Nepal. Farmers in these areas have adopted innovative techniques such as rainwater harvesting and using organic mulches to conserve soil moisture (Singh et al. 2018). These practices have proven effective in conserving water, improving soil health, and increasing the productivity of fruit crops (Granatstein and Kirby 2016). Improper cropping practices can harm water quality, while nutrient conservation helps protect water bodies. Integrated farming systems enhance sustainability, and organic farming improves animal health and product quality (Chandran et al. 2019). Organic mulches have been incredibly beneficial in regions like Karnali, where water resources are limited. These mulches help retain soil moisture, reduce soil erosion, and enhance soil structure, making them a key component of sustainable organic farming practices in Nepal (Shrestha 2021). Using organic mulches in water-limited environments improves soil moisture retention and crop yields. For dryland and semi-arid farming, mulches such as straw, spent mushroom compost, and grapevine pruning debris help prevent soil evaporation, make nutrients more accessible, and improve soil structure (Demo and Bogale 2024; Mairata et al. 2024). These mulches also support plant health and growth, increasing yields and enhancing physiological responses, particularly in grapevines (Mairata et al. 2024). Biodegradable mulching technologies are gaining popularity as long-term alternatives to plastic mulches. They provide similar benefits while being more environmentally friendly (Shcherbatyuk et al. 2024). Overall, organic mulches contribute to sustainable agriculture by conserving resources and enhancing crop productivity in water-scarce regions (Renzi et al. 2024; Shcherbatyuk et al. 2024).

5 Limitations of Organic Fruit Production in Nepal

5.1 Lack of Infrastructure and Market Access

One of the most frequently cited challenges in the literature is the lack of infrastructure and market access for organic fruit producers in Nepal (Tamang et al. 2011). Farmers in remote areas often encounter significant difficulties in transporting their produce to markets, and the absence of cold storage facilities further aggravates post-harvest losses (Peck et al. 2009). These infrastructure gaps hinder organic fruit farmers from scaling up their operations or accessing higher-value markets. In contrast, Turkey's export markets dominate while domestic demand grows. The country's diverse climate supports a wide range of organic crops, and its regulatory frameworks align with EU standards (Gubbuk et al. 2004); Nepal has possessed similar potential.

Another major limitation is the absence of organized supply chains for organic products. Farmers struggle to connect with consumers willing to pay a premium for organic fruits without effective distribution networks. This lack of market access not only limits the profitability of organic farming but also discourages farmers from transitioning to organic practices in the first place (Shrestha 2021).

5.2 Inadequate Government Support

Although the Nepalese government has acknowledged the potential of organic farming, current policies and support systems are still inadequate (Banjara 2016). Farmers often lack access to essential resources such as subsidies, training programs, and technical assistance to transition to organic farming successfully (Chand et al. 2022). Additionally, many short-term government initiatives focus on immediate gains rather than long-term sustainability. Like Pakistan, Nepal has significant potential for organic fruit production; however, limited awareness and other challenges must be addressed to establish a national organic certification organization and implement legislation that promotes growth (Baig and Raza 2022).

Organic farming in Nepal is gaining momentum due to its sustainability and health benefits. However, a small certified land area and inadequate policies hinder its development. Firm government decisions, stakeholder coordination, consumer awareness, and legal frameworks are necessary for its promotion (Banjara and Poudel 2016; Baral et al. 2020). The lack of coordination among various government bodies further complicates the situation. Farmers often navigate a maze of bureaucratic procedures without clear information about available resources or support programs (Banjara 2016). This lack of government engagement has left many organic farmers feeling isolated, with little incentive to invest in organic certification or expand their operations (Chand et al. 2022).

5.3 Low Consumer Awareness

Despite the increasing global demand for organic products, consumer awareness in Nepal remains low (Figure 4). A study conducted in the Lalitpur District revealed that only 40% of

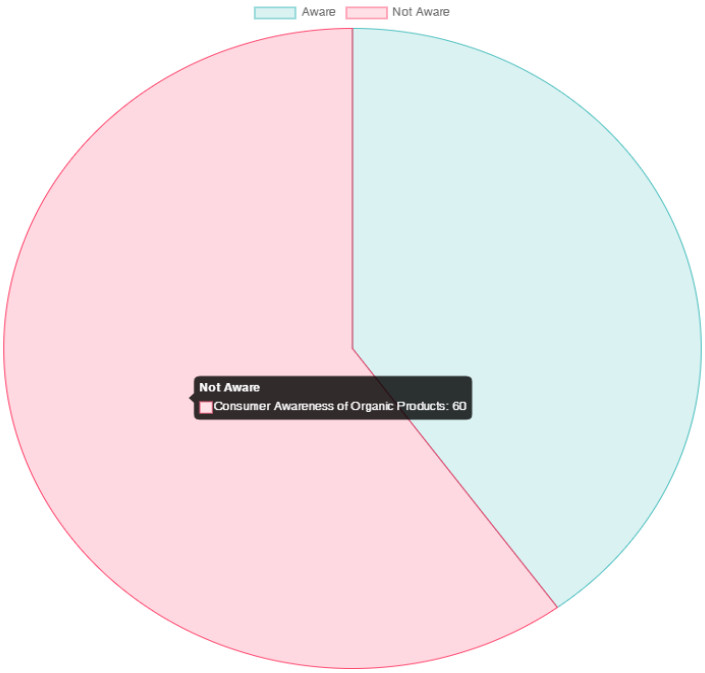

Figure 4 Consumer Awareness of Organic Products: The pie chart shows that 60% of consumers are unaware of organic products, highlighting the need for better education and marketing to promote them.

consumers are aware of organic produce, with many citing high prices as the primary reason for not purchasing organic fruits (Shrestha 2021). This lack of awareness limits domestic demand for organic products, discouraging farmers from investing in organic certification and production processes (Banjara 2016). Factors such as limited consumer awareness, small landholdings, the absence of a national policy, and the lack of organic certification hinder growth in the sector (Uma and Kumar 2025). Additionally, the global biodiversity of fruits and vegetables is declining due to insufficient conservation efforts, decreased pollinators, and an urgent need to preserve wild fruit species. School feeding programs and global partnerships are crucial in addressing this challenge (van Zonneveld et al. 2023).

6 Future Prospects for Organic Fruit Production

6.1 Growing Global and Local Demand

The global organic product market is rapidly expanding, creating significant opportunities for Nepalese farmers to access international markets (Granatstein et al. 2013). Fruits such as apples and citrus, which are already grown organically in regions like Mustang and Jumla, could be marketed as premium products if the challenges of certification and market access are addressed (Neilsen et al. 2009). Additionally, as urban consumers in Nepal become more health-conscious, the demand for organic fruits gradually increases, leading to a more substantial local market for organic produce (Shrestha 2021). Key factors influencing purchasing intentions include health awareness, knowledge of organic products, and availability (Upadhyay and Niraula 2023). In areas like Birendranagar, consumer trust in organic labeling and understanding of health benefits significantly shape preferences (Rokaya and Pandey 2023). Many consumers are willing to pay a premium for pesticide-free and organic fruits due to their perceived nutritional and environmental benefits (Ghimire and Khadka 2023; Regmi et al. 2023). This trend highlights the need for improved access and marketing strategies to support organic market expansion in urban areas (Paudel et al. 2023).

6.2 Role of Cooperatives and NGOs

Cooperatives and non-governmental organizations (NGOs) have been instrumental in promoting organic farming in Nepal by providing training, technical support, and market access to smallholder farmers (Sharma 2014). These organizations have enabled farmers in remote areas to adopt organic practices by supplying necessary resources and knowledge. Expanding these initiatives could help overcome barriers to organic fruit production, especially in underserved regions (Chandran et al. 2019).

Additionally, cooperatives can play a crucial role in organizing farmers into larger production units, which enables them to achieve economies of scale and enhance their bargaining power in the market (Sharma 2014). Organic agriculture in Nepal contributes to health and environmental sustainability, with many farmers expressing satisfaction with their income. Family farming is vital for food security, and farmers' cooperatives can address the challenges faced in this sector; therefore, the government needs to promote organic agriculture (Banjara and Poudel 2016). By pooling resources and coordinating production, cooperatives can help farmers access larger markets and negotiate better prices for organic products.

6.3 Policy Recommendations

The government must implement comprehensive policies supporting organic farmers to realize Nepal's full potential in organic fruit production. This includes simplifying the certification process, providing financial incentives, and investing in infrastructure such as cold storage and transportation (Hijazi 2021). Additionally, the government should focus on long-term strategies that promote sustainable farming practices rather than short-term projects that offer limited benefits. While consumer awareness is growing, current government policies hinder progress. Karnali Province strives to achieve full organic status due to constitutional support for organic initiatives and promoting climate adaptation through subsidies (Baral et al. 2020). Increasing consumer awareness through public campaigns, school programs, and media outreach could shift demand toward organic products (Hijazi 2021; Chand et al. 2022). By fostering a more informed consumer base, the government can help create a more substantial domestic market for organic fruits, encouraging more farmers to adopt organic practices.

Conclusion

Organic fruit production in Nepal has significant potential due to the country's diverse agro-climatic conditions and traditional farming practices. However, various challenges hinder the growth of this sector, including inadequate infrastructure, limited market access, high certification costs, and insufficient government support. To overcome these barriers, comprehensive policies are necessary, alongside increased support for smallholder farmers and improved consumer awareness. Strengthening cooperatives, streamlining certification processes, and enhancing market access are essential steps for Nepal to establish itself as a significant player in the global organic market. With the right strategies and support, Nepal can transform its organic fruit production sector, benefiting local farmers and consumers while contributing to environmental sustainability.

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Conflict of Interests

There is no conflict of interest among the authors about the contents of this manuscript.

Ethical Clearance

Not applicable.

References

Acharya, L. N., & Atreya, P. N. (2012). *Fruit development in Nepal: Past efforts, present status, and future needs/way forward*. Proceedings of the Eight National Horticulture Workshop, Nepal. Retrieved from https://horticulturenepal.org/uploads/main_attachment/1643013469_Proceedings%20of%20the%20%20Eight%20%20National%20Horticulture-133-147.pdf. Retrieved September 2024.

Alves, D. S., & Ascari, J. (2019). Use of Natural Chemical Products for Pest Control. In B. Souza, L. Vázquez, & R. Marucci (eds) *Natural Enemies of Insect Pests in Neotropical Agroecosystems* (pp. 479–488). Springer, Cham. https://doi.org/10.1007/978-3-030-24733-1_38

Atreya, K., Subedi, B. P., Ghimire, P. L., Khanal, S. C., & Pandit, S. (2020). A review on history of organic farming in the current changing context in Nepal. *Archives of Agriculture and Environmental Science*, *5*(3), 406-418.

Baig, A., Ali, G., & Raza, H. (2022). *Assessment of the organic potential in Gilgit-Baltistan*. International Centre for Integrated Mountain Development (ICIMOD). DOI: 10.53055/ICIMOD.786

Banjara, R. K. (2016). Opportunity of organic farming to improve the socio-economic life of Nepalese farmers. *European Journal of Agriculture and Forestry Research*, *4*, 22-31.

Banjara, R. K., & Poudel, M. (2016). Sustainable model of organic agriculture: a case study of Nepalese farmers. *Journal of Advanced Academic Research*, *3*(1), 142-163.

Baral, P., Paudel, R., Kharal, S., & Khadka, A. (2020). Organic agriculture in Nepal: Policies and practices. *Journal of the Institute of Agriculture and Animal Science*, *36*, 279-289.

Barański, M., Srednicka-Tober, D., Volakakis, N., Seal, C., Sanderson, R., et al. (2014). Higher antioxidant and lower cadmium concentrations and lower incidence of pesticide residues in organically grown crops: a systematic literature review and meta-analyses. *The British journal of nutrition*, *112*(5), 794–811. https://doi.org/10.1017/S0007114514001366.

Böhme, M. H., & Dimitrov-Skatov, A. G. (2018). Investigations to biological and organic treatments against pests in vegetable cultivation. *IOP Conference Series: Earth and Environmental Science*, *215*, 012012. https://doi.org/10.1088/1755-1315/215/1/012012

Chand, P. B., Joshi, K., Thapa, R., Lamsal, A., & Chauhan, B. (2022). Factors Affecting The Adoption Of Organic Farming In Farwestern Hill, Darchula District Of Nepal. *Tropical Agroecosystems*, *3*(1), 16-22.

Chandran, C. S., Thomas, S., & Unni, M. R. (Eds.). (2019). *Organic farming: New advances towards sustainable agricultural systems*. Springer.

Dahal, S., Dangi, B., Dangi, R. K., Bista, P., Bhandari, A., Adhikari, P., &Bhattarai, M. (2023). Determinants of adoption of multiple sustainable agriculture practices among mandarin producing farmers in Salyan District of Karnali Province, Nepal. *European Journal of Sustainable Development Research*, *7*(4), em0230. https://doi.org/10.29333/ejosdr/13440.

De Wilde, N. A., Gubbels, T. H., Heinen, D., & Arnabal, E. H. (2024). *Professional apiculture in the Netherlands: sector analysis*. Wageningen University & Research.

Demo, A. H., & Bogale, G. A. (2024). Enhancing crop yield and conserving soil moisture through mulching practices in dryland agriculture. *Frontiers in Agronomy*, *6*. https://doi.org/10.3389/fagro.2024.1361697

Food and Agriculture Organization of the United Nations. (2023). *Agricultural production statistics (2000–2022)*. Retrieved from https://www.fao.org/statistics/highlights-archive/highlights-detail/agricultural-production-statistics-(2000-2022)/ November 21, 2024.

Ghimire, D., & Khadka, K. (2023). Consumer Willingness to Pay for Pesticides-Free Fruits and Vegetables (A Case Study of Bhaktapur District). *International Journal of Atharva*, *1*(1), 83–95. https://doi.org/10.3126/ija.v1i1.58846

Granatstein, D., & Kirby, E. (2016). *Status of organic production of temperate fruits in the USA*. Eco fruit. Retrieved from http://www.ecofruit.net/wp-content/uploads/2020/04/17_Granatstein_128bis134.pdf

Granatstein, D., Kirby, E., & Willer, H. (2013). Global area and trends of organic fruit production. *ActaHortic*, *1001*(1001), 383-394.

Gubbuk, H., Polat, E., & Pekmezci, M. (2004). Organic fruit production in Turkey. *Journal of Fruit and Ornamental Plant Research*, (*Special ed*), *12*, 23-29.

Hijazi, Y. (2021). *Converting Avocado Farming in Lebanon from Conventional to Organic: The Economic Perspective*. Master

dissertation. Retrieved from https://api.pageplace.de/preview/DT0400.9783346551924_A42842944/preview-9783346551924_A42842944.pdf

Lamy, F. C., Lamy, F. C., Poinsot, D., Poinsot, D., Cortesero, A. M., Cortesero, A. M., Dugravot, S., & Dugravot, S. (2017). Artificially applied plant volatile organic compounds modify the behavior of a pest with no adverse effect on its natural enemies in the field. *Journal of Pest Science*, *90*(2), 611–621. https://doi.org/10.1007/S10340-016-0792-1

M'sakni, N. H., Alsufyani, T., Alotaibi, N. J., Al-Malki, M. A. S., & Alghamdi, E. M. (2024). *Interspecific Eavesdropping on Chemical Communication in the Field between Taif Pomegranate, Aphis punicae, Enemies, and Protectors*. Preprint available on Research Square https://doi.org/10.21203/rs.3.rs-4008859/v1

Mairata, A., Labarga, D., Puelles, M., Rivacoba, L., Martín, I., Portu, J., & Pou, A. (2024). Impact of organic mulches on grapevine health, growth and grape composition in nutrient-poor vineyard soils. *OENO One*, *58*(4). https://doi.org/10.20870/oeno-one.2024.58.4.8265

Mondal P. (2012). Major problems and constraints for organic farming in India. Retrieved from http://www.yourarticlelibrary.com/essay/major-problems-and-constraints-for-organicfarming-in-india/25013 access on December, 2024.

Muhammad, A., & Umma, D. M. B. B. (2024). *Challenges of Implementing Anchor Borrower Programme for Food Security in Nasarawa State, Nigeria*. *Journal of Business and Innovation in Emerging Economies*, *8*(9), 594–604.

Munna, M. R., Trisa, S. A., Islam, M. M., Islam, M. J., Pal, D. C., & Rahman, M. K. (2021). Commercially available organic manures on growth, yield and nutrient content of Burmese Grapes (*Baccaurea ramiflora*). *Journal of Biodiversity Conservation and Bioresource Management*, *7*(1), 63-72.

Neeson, R. (2008). Organic fruit production. *Orange: New South Wales Department of Primary Industries. Available at http://www. dpi. nsw. gov. au/__data/assets/pdf_file/0020/237800/organic-fruit-production. pdf.*RetrievedSeptember 2024.

Neilsen, G. H., Lowery, D. T., Forge, T. A., & Neilsen, D. (2009). Organic fruit production in British Columbia. *Canadian Journal of Plant Science*, *89*(4), 677-692.

Pathak, R. K., & Ram, R. A. (2004). *Manual on Vedic Krishi*. Central Institute for Subtropical Horticulture.

Peck, G. M., Merwin, I. A., Watkins, C. B., Chapman, K. W., & Padilla-Zakour, O. I. (2009). Maturity and quality of 'Liberty'apple fruit under integrated and organic fruit production systems are similar. *HortScience*, *44*(5), 1382-1389.

Paudel, R., Nepali, P. B., Panta, H. K., & Acharya, G. D. (2023). Transforming Food, Embracing Inclusivity: Nepal's Journey towards Sustainable Food Systems. *Orchid Academia Siraha*, *2*(1), 53–61. https://doi.org/10.3126/oas.v2i1.65603.

Ram, R. A., & Kumar, A. (2019). Growing fruit crops organically: challenges and opportunities. *Current Horticulture*, *7*(1), 3-11.

Regmi, K. R., Pokhrel, A., Regmi, S. D., Kandel, S., & Lamichhane, J. (2023). Assessment on Consumers Willingness to Pay for Organic Products in Rupandehi District, Nepal. *Journal of the Institute of Agriculture and Animal Science*, *37*(1), 19–30. https://doi.org/10.3126/jiaas.v37i1.56970

Renzi, N., Pacetti, T., Lompi, M., Castelli, G., Caporali, E., Setti, A., & Bresci, E. (2024). *Water footprint and water productivity analysis of an alternative organic mulching technology for irrigated agriculture*. EGU General Assembly 2024, Vienna, Austria, 14–19 Apr 2024, EGU24-8048, https://doi.org/10.5194/egusphere-egu24-8048

Rokaya, A. B., & Pandey, A. C. (2023). Analysis of Entrepreneurial Activities for Promoting Organic Products in Karnali Province. *KMC Journal*, *5*(2), 119-138.

Rozpara, E., & Głowacka, A. (2012). The usefulness of stone fruit species and cultivars for organic fruit production in Poland. *In Proceedings of the 15th International Conference on Organic Fruit Growing* (pp. 20-22). Hohenheim, Germany.

Schupp, J., & Moran, R. (2004). Mineral nutrient management for organic fruit production. *Pennsylvania Fruit News*, *84*, 14-19.

Shaltout, F. A. (2024). *Preservatives Added to Food Preservation and Their Impact Against the Bacteria*. Clinical Research and Clinical Reports. Retrieved from https://www.clinicsearchonline.org/uploads/articles/1733887890CRC-24-RA-89-Galley_Proof.pdf accessed on September, 2024

Sharma, S. K. (2014). Family as an autonomous institution for local agricultural resources governance: discourse on organic agriculture development in the mountains of Nepal. *International Journal of Agricultural Resources, Governance and Ecology*, *10*(4), 325-334.

Shashank, D. U., Sujatha, G. S., Sai Teja, K. S., Hema, A., Mishra, R., Abinaya, S., Shewta, K., & Kumar, G. A. (2024). A Comprehensive Review of Advances in Semiochemical Exploitation for Insect Pest Management. *Journal of Advances in Biology & Biotechnology*, *27*(9), 845–863. https://doi.org/10.9734/jabb/2024/v27i91357

Shcherbatyuk, N., Wortman, S. E., McFadden, D., Weiss, B., Weyers, S. L., et al. (2024). Alternative and Emerging Mulch Technologies for Organic and Sustainable Agriculture in the United States: A Review. *Hortscience*, *59*(10), 1524–1533. https://doi.org/10.21273/hortsci18029-24

Shrestha, R. M. (2021). Factors Affecting Consumer Purchase Intentions of Organic Products in Lalitpur District. *Pravaha*, *27*(1), 125-130.

Singh, K., Kumar, S., Pradhan, S., & Patidar, O. P. (2018). Organic Farming for Sustainable and Nutritional Fruit Production in India: A Review. *International Journal of Current Microbiology and Applied Sciences*, *7*(5), 3033-3039.

Tamang, S., Dhital, M., & Acharya, U. (2011). *Status and scope of organic agriculture in Nepal*. Food and Sustainable Agriculture Initiative, Forest Action. Retrieved from https://www.academia.edu/download/31431148/1._2011_Scopes_and_Challanges_of_Organic_Agriculture_in_Nepal_3_June.pdf accessed on 14 October 2024.

Tamm, L., Häseli, A., Fuchs, J. G., Weibel, F. P., & Wyss, E. (2002). Organic fruit production in humid climates of Europe: Bottlenecks and new approaches in disease and pest control. *Acta Horticulturae, 638*, 333-339.

Uma, S., & Kumar, P. S. (2025). *Organic Production of Fruits: Issues, Challenges, and Opportunities*. In H. Pathak, W.S. Lakra, A. Gopalakrishnan, & K.C. Bansal (Eds) *Advances in Agri-Food Systems*. Springer, Singapore. https://doi.org/10.1007/978-981-96-0759-4_9.

Upadhyay, J. P., & Niraula, B. (2023). Consumer Purchase Intention towards Organic Food Products in Nepal. *Pravaha*, *29*(1), 103–110. https://doi.org/10.3126/pravaha.v29i1.71410

Van Zonneveld, M., Volk, G. M., Dulloo, M. E., Kindt, R., Mayes, S., & others. (2023). Safeguarding and using fruit and vegetable biodiversity. In J. von Braun, K. Afsana, L.O. Fresco, & M.H.A. Hassan (Eds.) *Science and Innovations for Food Systems Transformation* (pp. 553–567). Springer, Cham. https://doi.org/10.1007/978-3-031-15703-5_30.

Weibel, F. P., Häseli, A., Schmid, O., & Willer, H. (2002). Present status of organic fruit growing in Europe. *Acta Horticulturae, 638*, 375-385.

Weibel, F. (2002). Organic fruit production in Europe. *The Compact Fruit Tree, 35*(3), 77-82.

Willer, H., Meier, C., Schlatter, B., & Trávníček, J. (2022). *The state and evolution of organic fruit and vegetables: Production and market at a world scale*. In W. W. Ocimati, T. Lecot, & Lehrer, K. (Eds.) XXXI International Horticultural Congress (IHC2022): XII International Symposium on Banana: Celebrating Banana Organic Production. 1367 edition. Acta Hortic. International Society for Horticultural Science, Leuven, pp. 1-12.

Willer, H., Trávníček, J., & Schlatter, S. (2024). *The world of organic agriculture: Statistics and emerging trends 2024*. Research Institute of Organic Agriculture (FiBL) & IFOAM – Organics International. Retrieved from https://www.fibl.org/en/shop-en/1747-organic-world-2024.

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An overview of betulin: botanical source, derivatives and biological potential: Mini Review

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ABSTRACT

This review aims to provide insight into and summarize the potential of betulin and its derivatives as important pharmaceutical molecules, including their underlying mechanisms of action. This investigation compiles comprehensive scientific data regarding betulin as a botanical raw material for industrial and pharmaceutical applications. Betulin, a natural pentacyclic lupane-triterpenoid, exhibits diverse biological activities, addressing metabolic dysfunctions, infectious diseases, cardiovascular disorders, and carcinogenic activity. The extraction of betulin from natural sources, mainly birch bark, is relatively simple and cost-effective, making it an attractive compound for the pharmaceutical and cosmetic industries. This study lists 93 plant sources of betulin and explores its repurposing as an effective therapeutic agent. It highlights its potential as an antiviral, anti-inflammatory, anticancer, and hepatoprotective compound, emphasizing the benefits of derivatizing betulin with various groups or moieties, such as imidazole carboxylic ester, hemisuccinate, hemiphthalate, nicotinate, acetylbetulin-28-o-triphenylphosphonium, succinyl, and 3-substituted glutaryl. The information gathered comes from various sources, including plant databases, Google Scholar, PubMed, ethnobotanical references, and classical texts.

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1 Introduction

Betulin is a natural compound with the systematic name 3β, 28-dihydroxy-20(29)-lupen or lup-20(29)-en-3β, 28-diol. Its molecular formula is $C_{30}H_{50}O_2$, with a molecular weight of 442.728 g/mol (Alakurtti et al. 2006). Betulin is primarily isolated from various species in the families Betulaceae, Platanaceae, Dilleniaceae, Rhamnaceae, Rosaceae, and Fagaceae, particularly from the bark of different species of Betulaceae (Cichewicz and Kouzi 2004; Ghaffari et al. 2012). The structure of betulin is based on a complex polycyclic system characterized by a cyclopentane-perhydrophenanthrene (sterane) core, which comprises four cyclohexane rings and one saturated cyclopentane ring condensed together. The carbon atoms in this structure are designated by the letters A, B, C, D, and E, following the recognized numbering system for carbon skeletons (Takibayeva et al. 2023). Notably, the α-isopropenyl group at the C-19 carbon atom and a five-membered ring E are distinctive features of betulin (Kislitsyn 1994; Krasutsky 2006; Bergelin and Holmbom 2008).

Betulin has a pentacyclic ring structure with hydroxyl groups at positions C_3 and C_{28} (Figure 1). An alkene moiety is also present at carbon 20. The hydroxyl and alkene groups serve as binding sites for simple modifications, while the pentacyclic lupane skeleton contributes to the lipophilicity of betulin, leading to poor water solubility. Betulin is separated from birch bark using sublimation or solvent extraction methods, and its concentration in the extract may vary depending on the birch species and the tree's location. Although betulin can be found in other plant sources, it is abundantly derived from Betula species (Hayek et al. 1989; Tolstikov et al. 2005; Adepoju et al. 2023). The chemical structure and betulin's physical and chemical properties are discussed in detail in several studies (Akihisa et al. 2002; Fulda 2008; Lesellier et al. 2012; Uryash et al. 2014; Amiri et al. 2020). The primary objective of this review is to highlight the potential of betulin and its derivatives as effective therapeutics for various biological activities through the derivatization of groups or moieties at the molecule's C_3 and C_{28} positions.

2 Sources of literature

The information published on Betulin was gathered from various sources, including Google, Web of Science, Elsevier, PubMed, Google Scholar, and Semantic Scholar. The search terms used for this review included "Betulin," "Introduction to Betulin," "structure-activity relationship (SAR) of Betulin," "sources of Betulin," "derivatives of Betulin," and "pharmacological activities of Betulin." The search was conducted in all available languages. The chemical structures of the compounds were drawn using ChemDraw 15.0 software.

3 Botanical sources of betulin

Betulin is extracted from various plant sources. Table 1 lists the different plant sources, their families, the specific plant parts utilized, and their reported percent yields.

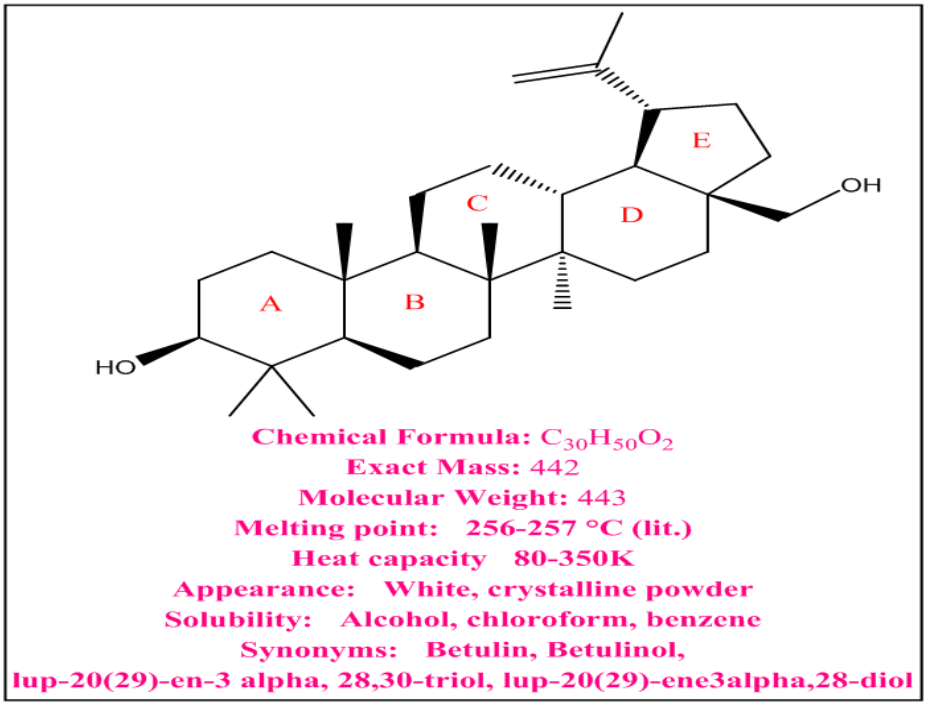


Figure 1 Structure of Betulin with their Chemical and Physical Properties

Table 1 Botanical sources of betulin with its % yield

S.No	Name of Plant	Family	Plant part	% yield	Reference
1	*Betula pumila*	Betulaceae	Bark	18.2% to 22.7%	Rastogi et al. 2015
2	*B. pendula*	Betulaceae	Bark	16-22%	Rastogi et al. 2015
3	*B. jacquemontii*	Betulaceae	Bark	-	Zhang et al. 2023
4	*B. pubescens*	Betulaceae	Bark	95-98%	Abyshev et al. 2007
5	*B. platphylla*	Betulaceae	Bark	51%	Chen et al. 2020
6	*B. papyrifera*	Betulaceae	Bark	28-32%	Blondeau et al. 2020
7	*B. nana*	Betulaceae	Bark	-	Atkinson 1992
8	*B. nigra*	Betulaceae	Bark	20-30%	Alakurtti et al. 2006
9	*B. lente*	Betulaceae	Bark	45%	Holonec et al. 2012
10	*B. alba*	Betulaceae	Bark	95-98%	Abyshev et al. 2007
11	*B. occidentalis*	Betulaceae	Bark	-	White and Bernstein 2003
12	*B. utilis*	Betulaceae	Bark	71%	Siman et al. 2016
13	*Bahuhina recemosa*	Caesalpiniaceae	Root	-	Gupta et al. 2004
14	*Bupleurum flavum*	Apiaceae (Umbelliferae)	Aerial part	-	Tykheev et al. 2020
15	*Buxus microphylla*	Buxaceae	Stem and leaf	-	Fujun et al. 2015
16	*B. papillosa*	Buxaceae	Whole plant	-	Saleem et al. 2020
17	*Byrsonima crassifolia*	Malpighiaceae	Leaf	-	Peraza-Sánchez et al. 2005
18	*B. microphylla*	Malpighiaceae	Wood	0.5% to 2.5%	Aguiar et al. 2005
19	*Cappasis sepiaria*	Capparaceae	Leaf	0.1% - 2%	Mishra et al. 2007
20	*Careya arborea*	Lecythidaceae	Bark	-	Gupta et al. 2012
21	*Carlina corymbosa*	Asteraceae	Bark	-	Oz 2021
22	*Cassia siamea*	Caesalpiniacea	Trunk and Bark	-	Kamagaté et al. 2014
23	*Castanea sativa*	Fagaceae	Leaf	-	Tchatchoua and Aravanopoulos 2015
24	*Celastrus punctalus*	Celastraceae	Stem	2.86%	Huang et al. 2000
25	*Celtis philippinensis*	Capparaceae	Twig	-	Hwang et al. 2003
26	*Ceriops decandra*	Rhizophoraceae	Leaf	-	Hossain et al. 2012
27	*E. divinorum*	Ebenaceae	Root	-	Kaingu et al. 2012
28	*E. kellau*	Ebenaceae	Branch	-	Orzalesi et al. 1970
29	*E. natalensis*	Ebenaceae	Root bark	-	Lall et al. 2006
30	*Euphorbia lathyris*	Euphorbiaceae	Seed oil	-	Ma et al. 2020
31	*Flindersia pimentelliana*	Rutaceae	Bark	0.01 to 0.1 %	Hu et al. 2020
32	*Grevia asiatica*	Malvaceae	Bark and heart wood	-	Zia-Ul-Haq et al. 2013
33	*G. tiliaefolia*	Tiliaceae	Bark	-	Sharma et al 2015
34	*Guazuma tomentosa*	Sterculiaceae	Bark	-	Magid et al. 2008
35	*Guioa villosa*	Sapindaceae	Leaf	-	Magid et al. 2008

S.No	Name of Plant	Family	Plant part	% yield	Reference
36	*Gymnosporia variabilis*	Celastraceae	Aerial part	-	Bhat et al. 2024
37	*Holacantha emoryi*	Simaroubaceae	Seed	-	Stöcklin et al. 1969
38	*Hovenia acerba*	Rhamnaceae	Fruit	-	Zhang et al. 2020
39	*Hypoestes purpurea*	Acanthaceae	Aerial part	45%	Shen et al. 2004
40	*Ilex latifolia*	Aquifoliaceae	Stem bark	-	He et al. 2020
41	*I. macropoela*	Aquifoliaceae	Twig	0.24% to 0.54%	Hu 1949
42	*Ixora chinensis*	Rubiaceae	Leaf	-	Sajini and Chamundeeswari 2023
43	*Jasminum lanceolarium*	Oleaceae	Stem and leaf	-	Ning et al.2013
44	*Juglans regia*	Juglandaceae	Bark	-	Eberle et al. 2023
45	*Lagerstroemia guilinensis*	Lythraceae	Stem	-	Lin et al. 2024
46	*Lasianthus chinensis*	Rubiaceae	Leaf	-	Zhu 2002
47	*Lespedeza bicolor*	Fabaceae	Stem bark	-	Lee et al. 2016
48	*Lithocarpus attenuta*	Fagaceae	Leaf and stem	-	Ye et al. 2024
49	*L. elegans*	Fagaceae	Bark	-	Ye et al. 2024
50	*L. cornea*	Fagaceae	Leaf and stem	-	Ye et al. 2024
51	*L. elizabethae*	Fagaceae	Leaf and stem	-	Ye et al. 2024
52	*L. glabra*	Fagaceae	Leaf and stem	-	Ye et al. 2024
53	*L. haipinii*	Fagaceae	Leaf and stem	-	Ye et al. 2024
54	*L. hancei*	Lauraceae	Leaf and stem	-	Ye et al. 2024
55	*L. harlandii*	Lauraceae	Leaf and stem	-	Ye et al. 2024
56	*L. irwinii*	Lauraceae	Leaf and stem	-	Ye et al. 2024
57	*L. litchioides*	Lauraceae	Leaf and stem	-	Ye et al. 2024
58	*L. polystachyus*	Lauraceae	Leaf and stem	-	Ye et al. 2024
59	*Lonchocarpus laxiflorus*	Lauraceae	Stem	-	Desta et al. 2022
60	*Lophopetalum rigidum*	Celestraceae	Bark	-	Mahajan and Chauhan 2024
61	*L. toxicum*	Celestraceae	Bark	-	Mahajan and Chauhan 2024
62	*Lyonia ovalifolia*	Ericaceae	Shoot	-	Bhandari et al. 2020
63	*Mallotus philippensis*	Euphorbiaceae	Leaf	-	George et al. 2023
64	*Matayba elaeagnoides*	Elaeagnaceae	Bark	-	Rorato et al. 2018
65	*M. chiapensis*	Maytenaceae	Leaf	-	De Figueiredo et al. 2024
66	*M. cuzcoina*	Maytenaceae	Root bark	-	De Figueiredo et al. 2024
67	*M. elacodendroides*	Maytenaceae	Stem bark	-	De Figueiredo et al. 2024
68	*Melaleuca leucadendron*	Myrtaceae	Leaf	-	Melani et al. 2023
69	*Melodinus australis*	Apocynaceae	Aerial part	-	Linde 1965
70	*Valeriana laxiflora*	Valerianaceae	-	88.67	Kaur et al. 2022
71	*Nerium oleander*	Apocynaceae	Leaf	-	Induja et al. 2024

S.No	Name of Plant	Family	Plant part	% yield	Reference
72	*Ocimum basilicum*	Lamiaceae	Root and stem	-	Wirtu et al. 2024
73	*Ormosia emarginata*	Fabaceae	Leaf	-	Tang et al. 2023
74	*Osmanthus cymosus*	Oleaceae	Leaf	-	Kubba et al. 2005
75	*Viscum album*	Loranthaceae	Leaf	27.40	Jäger et al. 2007
76	*Vicia fuba*	Fabaceae	Seed	92.7	Chen et al. 2020
77	*Vaccinium ashei*	Ericaceae	Fruit	27-40	Szakiel et al. 2012
78	*Sophora japonica*	Fabaceae	Flower	0.5-1.5	Guo et al. 2024
79	*Salvia officinalis*	Lamiaceae	Leaf	0.9	Vieira et al. 2020
80	*Phlogacanthus curviflorus*	Acanthaceae	Aerial part	-	Jia et al. 2023
81	*Phyllanthus flexuous*	Phyllanthaceae	Stem bark	-	Guo et al. 2024
82	*Prunus duclis*	Rosaceae	Leaf	-	Vieira et al. 2020
83	*Pterocarpus santalinus*	Fabaceae	Leaf	-	Jia et al. 2023
84	*Pyrostegia venusta*	Apiaceae	Aerial part	-	Pratima et al. 2024
85	*Quercus suber*	Fagaceae	Bark	-	Manzoor et al. 2024
86	*Rhododendron adamsii*	Ericaceae	-	-	Dhadse and Saxena 2024
87	*R. aureum*	Ericaceae	-	-	Dhadse and Saxena 2024
88	*R. dahuricum*	Ericaceae	-	-	Dhadse and Saxena 2024
89	*R. kotschyi*	Ericaceae	-	-	Dhadse and Saxena 2024
90	*R. ledebourii*	Ericaceae	-	-	Dhadse and Saxena 2024
91	*R. luteum*	Ericaceae	-	-	Dhadse and Saxena 2024
92	*R. mucronulatum*	Ericaceae	-	-	Dhadse and Saxena 2024
93	*R. schlippenbachii*	Ericaceae	-	-	Dhadse and Saxena 2024

4 Chemical modifications

Three key locations in betulin allow for simple chemical modification: the primary hydroxyl group at position C-28, the secondary hydroxyl group at position C-3, and the alkene moiety at position C-20. The parent structure of betulin is modified biochemically at positions C-28 to yield betulinic acid (Boryczka et al. 2013; Baratto et al. 2013; Singh et al. 2016; Boparai et al. 2017). The white birch bark can manufacture physiologically active betulin derivatives due to its high betulin concentration (up to 30%)(Boryczka et al. 2013). Chrobak et al. (2021) reported the synthesis of novel, intriguing acetylenic derivatives of betulin by treating a mixture of betulin and pyridine in dry benzene with propargyl chloroformate (a), 2-butyn-1-yl chloroformate (b), 3-butyn-1-yl chloroformate (c), and ethyl chloroformate (d) (Figure 2). The reported betulin derivatives are: Imidazole carboxylic esters, Hemisuccinates, Hemiphthalates, Nicotinates, Acetylbetulin-28-O-hemiphthalate, Monomethacrylate and Dimethacrylate, Acetylenic, Oxime, Olygomeric ester, Phosphate, Triphenylphosphonium, and Succinyl and 3 substituted glutaryl. The biological activities include hepatoprotective, antileishmanial, antiviral, anti-inflammatory, anticancer, and anti-inflammatory (Santos et al. 2010; Veloso et al. 2010; Tang et al. 2014; Bebenek et al. 2015; Wang et al. 2022).

5 Structure-activity relationship of betulin

A therapeutic agent's structure-activity relationship (SAR) is critically determined by its molecular structure and interactions within the body. Betulin, a naturally occurring triterpenoid molecule, exhibits significant interactive potential due to hydroxyl groups at the C_3 and C_{28} positions. Various groups, including nitrile, carbonyl, amino, succinyl, maleic anhydride, oxime, and imidazole, have demonstrated cytotoxic and inhibitory effects against leishmania, inducible nitric oxide synthase (iNOS), tumors, and cancer. Furthermore, the addition of oxime and imidazole moieties enhances the anticancer and antileishmanial activities (Wang et al. 2022; Bebenek et al. 2015). The structure-activity relationship of betulin is illustrated in Figure 3.

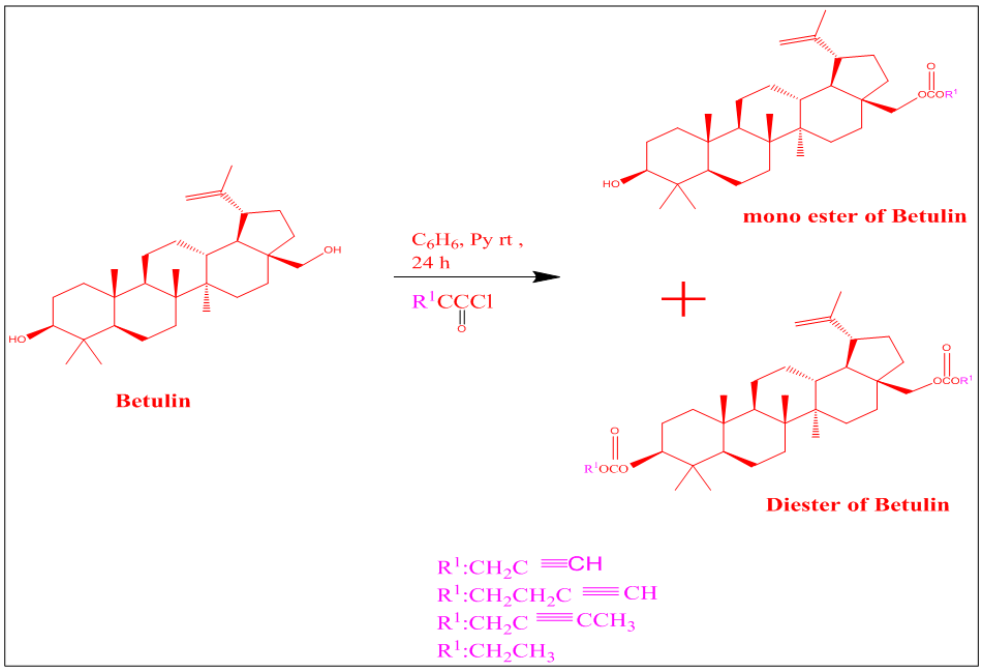


Figure 2 Synthesis of acetylenic derivative of Betulin

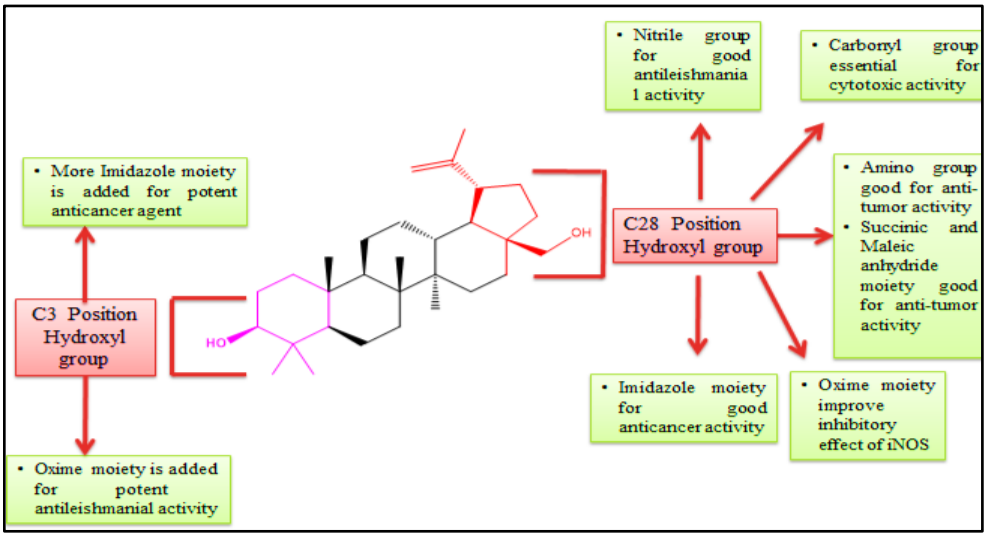


Figure 3 Structure-activity relationship of betulin

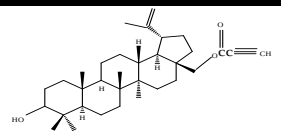
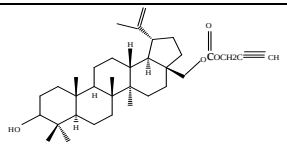
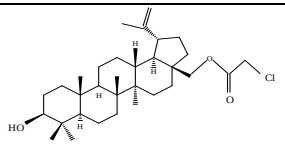
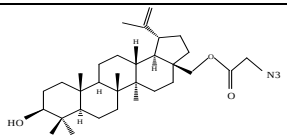
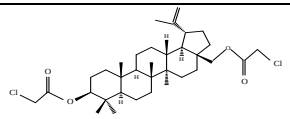
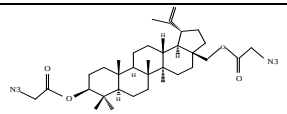
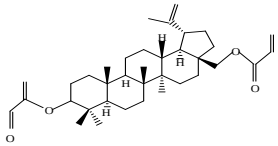
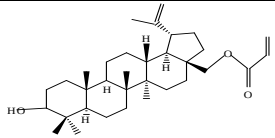
6 Betulin derivatives and its biological activities

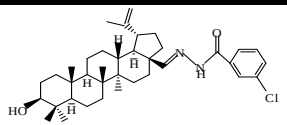
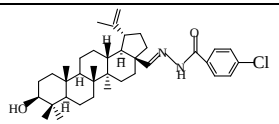
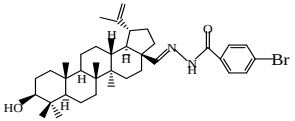
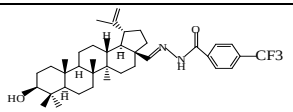
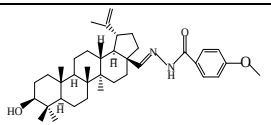
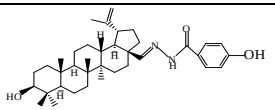
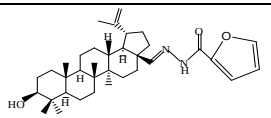
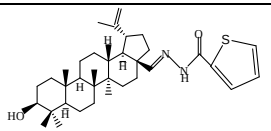
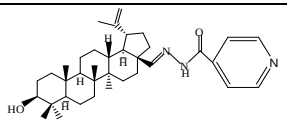
Betulin derivatives exhibit a range of biological activities, including anticancer, antiviral, anti-inflammatory, antioxidant, hepatoprotective, and antidiabetic properties. They present promising opportunities for drug development across various therapeutic areas. Ongoing research aims to create novel compounds demonstrating enhanced potency and selectivity for specific disorders. Tables 2 and 3 detail the biological activities of betulin derivatives, both in vitro and in vivo.

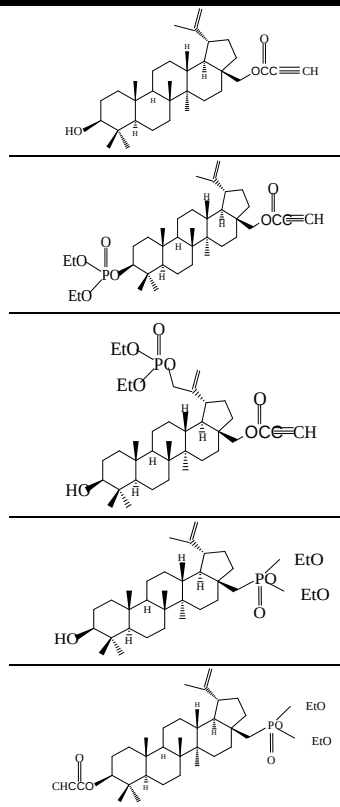
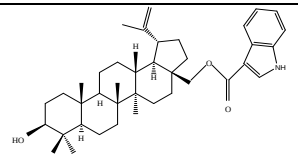
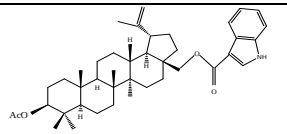
7 Future perspectives

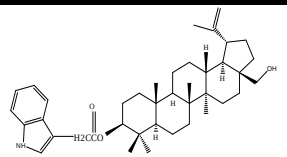
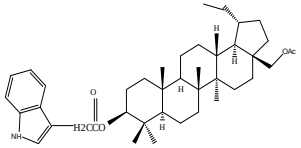
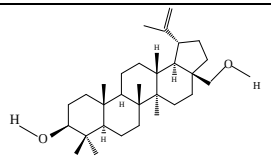
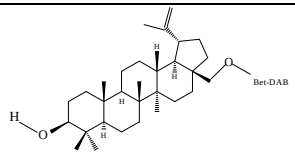
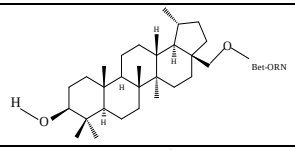
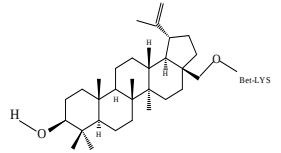
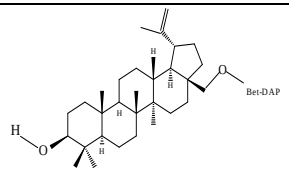
The potential of betulin, particularly in the design and synthesis of derivatives at C_3 and C_{28}, should be explored extensively for therapeutic applications. The study will involve docking studies on the proteins and active sites related to the targeted biological activity. Derivatives such as esters, amino compounds, imidazoles, and chlorinated versions will be commercialized through innovative drug delivery systems. Additionally, preclinical and clinical trials may be conducted to refine these molecules further and develop novel medications to treat various ailments.

Table 2 Betulin derivatives and their biological activities

S. No.	Types of derivatives	Name of the Derivatives	Structure	Biological activities	Mechanism of action	References
1	Acetylenic synthetic derivatives (ASBDs)	28-O-propynoylbetulin		Anticancer	Inhibits tyrosine kinase and C-MET kinase	Mhamdi et al. 2023
		28-O-propargyloxycarbonylbetulin				
2	Glycoconjugation of betulin derivatives	28-O-chloroacetylbetulin		Anticarcinogenic	Caspase cascade activation, modulating signaling pathways (NF-κB and Nrf2), inhibiting angiogenesis, and disrupting mitochondrial membrane	Grymel et al. 2020
		28-O-Azidoacetylbetulin				
		3,28-O,O'-di(chloroacetyl)betulin				
		3,28-O,O'-di(azidoacetyl)betulin				
3	Monomethacrylate- and dimethacrylate-functionalized betulin derivatives	M1Bet		Antibacterial	Disrupting bacterial membrane integrity and causing leakage of intracellular contents	Krol et al. 2020
		M2Bet				

S. No.	Types of derivatives	Name of the Derivatives	Structure	Biological activities	Mechanism of action	References
4	Betulin derivatives containing a hydrazide-hydrazone side-chain	Betulin-28-(3-chloro)benzohydrazide		Anticarcinogenic	Mitochondrial membrane disruption and caspase activation, inhibiting cell proliferation	Zhang et al. 2021
		Betulin-28-(4-chloro) benzohydrazide				
		Betulin-28-(4-bromo) benzohydrazide				
		Betulin-28-(4-trifluoromethyl) benzohydrazide				
		Betulin-28-(4-methoxy) benzohydrazide				
		Betulin-28-(4-hydroxy)benzohydrazide				
		Betulin-28-(2-furan) carbohydrazide				
		Betulin-28-(2-thiophene) carbohydrazide				
		Betulin-28-isonicotinohydrazide				

S. No.	Types of derivatives	Name of the Derivatives	Structure	Biological activities	Mechanism of action	References
5	Acetylenic derivative of betulin phosphate	Betulin derivatives bearing both propynoyloxy and phosphate groups		Breast cancer	Mitochondrial dysfunction, disrupting mitochondrial homeostasis, and activating mitochondrial death pathway	Laiolo et al. 2024
6	Indole betulin derivatives	EB355A		Anticarcinogenic	Mitochondrial membrane disruption and DNA fragmentation, arresting the cell cycle at the G1 phase, and inhibiting tubulin polymerization	Wu et al. 2021
		EB365				

S. No.	Types of derivatives	Name of the Derivatives	Structure	Biological activities	Mechanism of action	References
7	*3-indolyl betulin derivatives*	EB366		Anticancer	Mitochondrial membrane disruption and DNA fragmentation, arresting the cell cycle at the G1 phase to prevent progression to the S phase	Orchel et al. 2021
		EB367				
8	Betulin, and its derivatives with amino acids	1,4-diaminebutane (Dab), 1,3-diaminepropane (Dap), ornithine (Orn), and lysine (Lys)		Reduce inflammation and COX-2 activity in macrophages	Reducing IL-6, reorganizing interferon-gamma receptors (IFNγR), and directly inhibiting COX-2 activity	Rzepka et al. 2022
						
						
						
						

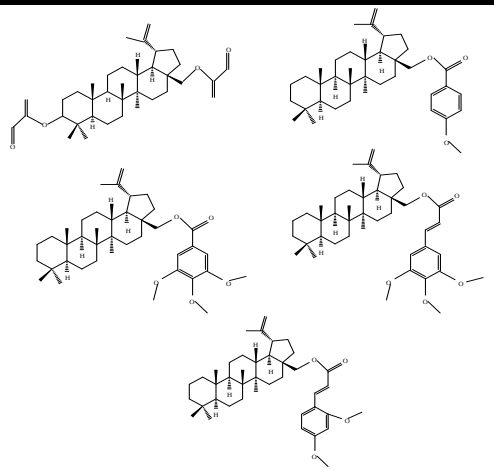
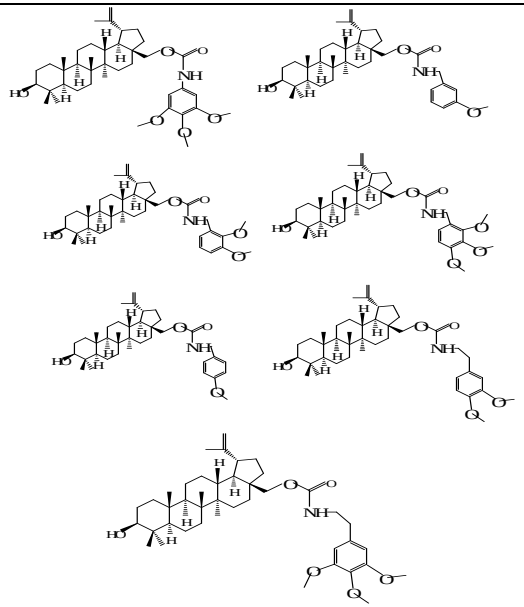
S. No.	Types of derivatives	Name of the Derivatives	Structure	Biological activities	Mechanism of action	References
	Betulin esters					
9	Betulin carbamates	-		Antiproliferative	Reduction of NF-κB, Nrf2, and AMPK, Regulates oxidative stress, inflammation, and energy metabolism	Wu et al. 2021; Bębenek et al. 2022

Table 3 *In-vitro* and *In-vivo* biological effects of betulin and its derivatives

S.No.	Derivatives	*Invitro*	*Invivo*	Activities	Reference
1.	Betulin	HCT116, HT29	-	Anticancer (colon)	Szlasa et al. 2023
2.	Indole	Colo-205, HCT-116	-	Anticancer (colon)	Grymel et al. 2020
3.	Betulin	A549, H1264, Calu-6	Rat	Anticancer (Ling)	Zhou et al. 2018
4.	Triphenylphosphonium	MCF-7, PC-3, MCF-7/Vinb, human skin fibroblast (HSF)	-	Anticancer toward MCF-7/Vinb cells	Khusnutdinova et al. 2018
5.	Ester	Me-45	-	Anticancer (melanoma)	So et al. 2018
6.	Betulin	SGC7901	-	Anticancer (gastric)	Tsepaeva et al. 2017
7.	Azole	A549, Hep-G2, HCT 116, MS, RDTE32	Mice	Anticancer	Drąg-Zalesińska et al. 2017
8.	Hydroxypropargylamines	SW1736, MCF-7, LIPO, DLD-1, A549, A2780, A253, 8505C, 518A2, NiH 3 T3	-	Anticancer	Li et al. 2016
9.	Carbamate and N-acylheterocyclic	Hep-G2, Jurkat, HeLa, HT-29, PC-3	-	Anticancer (carcinoma, leukemia, cervical, colon, prostate)	Grishko et al. 2017
10.	γ-Butyrolactones and butenolides	518A2, A431, A253, FADU, A549, A2780, DLD-1, HCT-8, HCT-116, HT-29, SW480, 8505C, SW1736, MCF-7, Lipo	-	Anticancer	Csuk et al. 2013
11.	α- and β- D-glucopyranose B anomers	8505C, SW1736, A253, FaDu, A431, A2780, DLD-1, HCT-8, HCT-116, HT-29, SW480, MCF-7, 518A2, A549	-	Anticancer (thyroid, head and neck, cervical, ovarian, colon, breast, melanoma, lung)	Santos et al. 2010
12.	Betulin	A431, MCF-7, HeLa	-	Anticancer	Csuk et al. 2010
13.	Betulin	RAW264.7 murine macrophage, Murine J774 macrophages, and RAW 264.7 mouse macrophage	Mice	Antiinflammatory	Kommera et al. 2010; Şoica et al. 2012; Ci et al. 2017
14.	3′-Azido-3′-deoxythy-midine	MT-4	-	AntiHIV-1 maturation	Laavola et al. 2016
15.	Betulin	*Chlamydia pneumoniae*	-	Antimicrobial	Wu et al. 2014
16.	Disuccinyl	*Leishmania donovani*	Mice	Antimicrobial	Xiong et al. 2010
17.	Heterocyclic	*Leishmania donovani*, THP-1	-	Antimicrobial	Salin et al. 2010; Chowdhury et al. 2014
18.	Betulin	CFSC-2G	-	Antifibrotic	Szuster-Ciesielska et al. 2011
19.	Triphenylphosphonium	-	Mice	Antimicrobial (antischistosoma mansoni)	Spivak et al. 2014

Conclusion

Betulin and its derivatives are potent chemotherapeutic agents that warrant further exploration. Betulin can be extracted, purified, and isolated with optimal yield using straightforward methods, such as Soxhlet extraction with green solvents. The C3 and C28 positions are the active sites for derivatization, which can enhance its biological activities.

Acknowledgment

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Conflicts of interest

The authors declare no conflict of interest.

Ethical Approval

Not Applicable for this study.

References

Abyshev, A. Z., Agaev, E. M., & Guseinov, A. B. (2007). Studies of the chemical composition of birch bark extracts (Cortex betula) from the Betulaceae family. *Pharmaceutical Chemistry Journal*, *41*(8), 419-423.

Adepoju, F. O., Duru, K. C., Li, E., Kovaleva, E. G., & Tsurkan, M. V. (2023). Pharmacological potential of betulin as a multitarget compound. *Biomolecules*, *13*(7), 1105.

Aguiar, R. M., David, J. P., & David, J. M. (2005). Unusual naphthoquinones, catechin and triterpene from Byrsonima microphylla. *Phytochemistry*, *66*(19), 2388-2392.

Akihisa, T., Takamine, Y., Yoshizumi, K., Tokuda, H., Kimura, Y., et al. (2002). Microbial transformations of two lupane-type triterpenes and anti-tumor-promoting effects of the transformation products. *Journal of natural products*, *65*(3), 278-282.

Alakurtti, S., Mäkelä, T., Koskimies, S., & Yli-Kauhaluoma, J. (2006). Pharmacological properties of the ubiquitous natural product betulin. *European Journal of Pharmaceutical Sciences*, *29*(1), 1-13.

Amiri, S., Dastghaib, S., Ahmadi, M., Mehrbod, P., Khadem, F., Behrouj, H., & Ghavami, S. (2020). Betulin and its derivatives as novel compounds with different pharmacological effects. *Biotechnology advances*, *38*, 107409.

Atkinson, M. D. (1992). *Betula pendula* Roth (*B. verrucosa* Ehrh.) and *B. pubescens* Ehrh. *Journal of Ecology*, *80*(4), 837-870.

Baratto, L. C., Porsani, M. V., Pimentel, I. C., Netto, A. B. P., Paschke, R., & Oliveira, B. H. (2013). Preparation of betulinic acid derivatives by chemical and biotransformation methods and determination of cytotoxicity against selected cancer cell lines. *European journal of medicinal chemistry*, *68*, 121-131.

Bebenek, E., Chodurek, E., Orchel, A., Dzierzewicz, Z., & Boryczka, S. (2015). Antiproliferative activity of novel acetylenic derivatives of betulin against G-361 human melanoma cells. *Acta Poloniae Pharmaceutica*, *72*(4), 699-703.

Bębenek, E., Chrobak, E., Rzepka, Z., & Wrześniok, D. (2022). New Betulin Derivatives with Nitrogen Heterocyclic Moiety—Synthesis and Anticancer Activity In Vitro. *Biomolecules*, *12*(10), 1540.

Bergelin, E., & Holmbom, B. (2008). Reactions and distribution of birch extractives in kraft pulp oxygen delignification. *Journal of Wood Chemistry and Technology*, *28*(4), 261-269.

Bhandari, P., Shrestha, K. K., Kunwar, R. M., Bussmann, R. W., & Paniagua-Zambrana, N. Y. (2020). *Lyonia ovalifolia* (Wall.) Drude Ericaceae. In: R. Kunwar, H. Sher, R.W. Bussmann (Eds.) *Ethnobotany of the Himalayas. Ethnobotany of Mountain Regions* (pp 1-8). Springer, Cham. https://doi.org/10.1007/978-3-030-45597-2_145-1

Bhat, A. T., Prabhukumar, K. M., & Rana, T. S. (2024). Lectotypification of four names in the genus Gymnosporia (Celastraceae). *Phytotaxa*, *645*(3), 286-293.

Blondeau, D., St-Pierre, A., Bourdeau, N., Bley, J., Lajeunesse, A., & Desgagné-Penix, I. (2020). Antimicrobial activity and chemical composition of white birch (*Betula papyrifera* Marshall) bark extracts. *Microbiologyopen*, *9*(1), e00944.

Boparai, A., Niazi, J., Bajwa, N., & Singh, P. A. (2017). Betulin a pentacyclic tri–terpenoid: an hour to rethink the compound. *Open Access Journal of Translational Medicine & Research*, *1*(2), 53-59.

Boryczka, S., Bębenek, E., Wietrzyk, J., Kempińska, K., Jastrzębska, M., Kusz, J., & Nowak, M. (2013).Synthesis, structure and cytotoxic activity of new acetylenic derivatives of betulin. *Molecules*, *18*(4), 4526-4543.

Chen, H., Xiao, H., & Pang, J. (2020). Parameter optimization and potential bioactivity evaluation of a betulin extract from white birch bark. *Plants*, *9*(3), 392.

Chowdhury, S., Mukherjee, T., Chowdhury, S. R., Sengupta, S., Mukhopadhyay, S., Jaisankar, P., & Majumder, H. K. (2014). Disuccinyl betulin triggers metacaspase-dependent endonuclease G-mediated cell death in unicellular protozoan parasite *Leishmania donovani*. *Antimicrobial agents and chemotherapy*, *58*(4), 2186-2201.

Chrobak, E., Jastrzębska, M., Bębenek, E., Kadela-Tomanek, M., Marciniec, K., et al. (2021). Molecular structure, in vitro anticancer study and molecular docking of new phosphate derivatives of betulin. *Molecules*, *26*(3), 737.

Ci, X., Zhou, J., Lv, H., Yu, Q., Peng, L., & Hua, S. (2017). Betulin exhibits anti-inflammatory activity in LPS-stimulated macrophages and endotoxin-shocked mice through an AMPK/AKT/Nrf2-dependent mechanism. *Cell death & disease*, *8*(5), e2798-e2798.

Cichewicz, R. H., & Kouzi, S. A. (2004).Chemistry, biological activity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Medicinal research reviews*, *24*(1), 90-114.

Csuk, R., Barthel, A., Schwarz, S., Kommera, H., & Paschke, R. (2010). Synthesis and biological evaluation of antitumor-active γ-butyrolactone substituted betulin derivatives. *Bioorganic & medicinal chemistry*, *18*(7), 2549-2558.

Csuk, R., Sczepek, R., Siewert, B., & Nitsche, C. (2013). Cytotoxic betulin-derived hydroxypropargylamines trigger apoptosis. *Bioorganic & medicinal chemistry*, *21*(2), 425-435.

de Figueiredo, P. T. R., Duarte, M. C., Cordeiro, L. V., de Menezes, R. P. B., Lima, E. D. O., et al. (2024). Sesquiterpenes alkaloids from *Maytenus distichophylla* (Celastraceae). *Quimica nova*, *47*(4),e-20230123.

Desta, G. T., Ferede, Y. A., Zewdu, W. S., Adugna, B. Y., Arega, T., & Alemu, M. A. (2022). Validation of Antidiabetic and Antihyperlipidemic Effects of 80% Methanolic Extract of the Lonchocarpus laxiflorusLeaves in Streptozotocin-Induced Diabetic Swiss Albino Mice. *Evidence-Based Complementary and Alternative Medicine*, *2022*(1), 8411851.

Dhadse, P., & Saxena, J. (2024). Formulation and Evaluation of Phytosomes of *Pterocarpus santalinus* Extract. *International Journal for Research in Applied Science and Engineering Technology*, *12*, 450-457.

Drąg-Zalesińska, M., Drąg, M., Poręba, M., Borska, S., Kulbacka, J., & Saczko, J. (2017). Anticancer properties of ester derivatives of betulin in human metastatic melanoma cells (Me-45). *Cancer Cell International*, *17*, 1-7.

Eberle, L. V., Tsisak, A. O., Radaieva, I. M., & Kazantseva, A. S. (2023). Analysis of phenolic compounds in black walnut (*Juglans nigra* L.) fruit extract using high-performance liquid chromatography. *Farmatsevtychnyizhurnal*, (2), 49-57.

Fujun, M. A., Qiujun, W., Junling, D. O. N. G., Yi, Y., Wenjia, W., Defei, Y., & Bainian, S. (2015). *Buxus* leaves from the Oligocene of Guangxi, China and their biogeographical significance. *Acta Geologica Sinica-English Edition*, *89*(5), 1453-1469.

Fulda, S. (2008). Betulinic acid for cancer treatment and prevention. *International journal of molecular sciences*, *9*(6), 1096-1107.

George, S., Duraisamy, M., Venugopalan, R., & Kalaiselvan, D. (2023).Neuroprotective Effect of Mallotusphilippensis Extract in Streptozotocin Induced Diabetic Neuropathy in Rats. *Research Journal of Pharmacy and Technology*, *16*(10), 4649-4657.

Ghaffari Moghaddam, M., Ahmad, B. H., & Samzadeh-Kermani, A. (2012). Biological activity of betulinic acid: a review. *Pharmacology & Pharmacy*, *3*(02), 119-123.

Grishko, V. V., Tolmacheva, I. A., Nebogatikov, V. O., Galaiko, N. V., Nazarov, A. V., Dmitriev, M. V., & Ivshina, I. B. (2017). Preparation of novel ring-A fused azole derivatives of betulin and evaluation of their cytotoxicity. *European Journal of Medicinal Chemistry*, *125*, 629-639.

Grymel, M., Pastuch-Gawołek, G., Lalik, A., Zawojak, M., Boczek, S., Krawczyk, M., & Erfurt, K. (2020). Glycoconjugation of betulin derivatives using copper-catalyzed 1, 3-dipolar azido-alkyne cycloaddition reaction and a preliminary assay of cytotoxicity of the obtained compounds. *Molecules*, *25*(24), 6019.

Guo, N., Chen, Z., Cao, S. Q., & Shang, F. D. (2024). *Sophora japonica* L. bioactives: Chemistry, sources, and processing techniques. *Food Frontiers*, *5*(3), 1166-1187.

Gupta, M., Mazumder, U. K., Siva, K. T., Gomathi, P., & Sambath, K. R. (2004). Antioxidant and hepatoprotective effects of *Bauhinia racemosa* against paracetamol and carbon tetrachloride induced liver damageinrats. *Iranian Journal of Pharmacology and Therapeutics*, *3*, 12-20.

Gupta, P. C., Sharma, N., & Rao, C. V. (2012). Pharmacognostic studies of the leaves and stem of Careyaarborea Roxb. *Asian Pacific journal of tropical biomedicine*, *2*(5), 404-408.

Hayek, E. W., Jordis, U., Moche, W., & Sauter, F. (1989). A bicentennial of betulin. *Phytochemistry*, *28*(9), 2229-2242.

He, P., Hua, H., Tian, W., Zhu, H., Liu, Y., & Xu, X. (2020). Holly (*Ilex latifolia* Thunb.) polyphenols extracts alleviate hepatic damage by regulating ferroptosis following diquat challenge in a piglet model. *Frontiers in Nutrition*, *7*, 604328.

Holonec, L., Ranga, F., Crainic, D., Truta, A., & Socaciu, C. (2012).Evaluation of betulin and betulinic acid content in birch bark from different forestry areas of Western Carpathians. *NotulaeBotanicaeHortiAgrobotanici Cluj-Napoca*, *40*(2), 99-105.

Hossain, M. H., Hassan, M. M., Jahan, I. A., Nimmi, I., & Islam, A. (2012).Antidiarrhoeal activity, nitric oxide scavenging and total

tannin content from the bark of *Ceriops decandra* (griff.) Ding hou. *International Journal of Pharmaceutical Sciences and Research*, *3*, 1306-1311.

Hu, J., Herbohn, J., Chazdon, R. L., Baynes, J., & Vanclay, J. K. (2020). Long-term growth responses of three Flindersia species to different thinning intensities after selective logging of a tropical rainforest. *Forest Ecology and Management*, *476*, 118442.

Hu, S. Y. (1949). The genus Ilex in China. *Journal of the Arnold Arboretum*, *30*(3), 233-344.

Huang, H. C., Shen, C. C., Chen, C. F., Wu, Y. C., & Kuo, Y. H. (2000). A novel agarofuran sesquiterpene, celahin D from Celastrus hindsiiBenth. *Chemical and pharmaceutical bulletin*, *48*(7), 1079-1080.

Hwang, B. Y., Chai, H. B., Kardono, L. B., Riswan, S., Farnsworth, N. R., et al. (2003). Cytotoxic triterpenes from the twigs of *Celtis philippinensis*. *Phytochemistry*, *62*(2), 197-201.

Induja, M. S., Anuradha, A., Mulasseri, S., Jadav, R., Aneaus, S., Karthic, A., &Arjumand, T. (2024). Oleander Leaf: Fatal poisoning in children. *Current Science (00113891)*, *126*(2).

Jäger, S., Winkler, K., Pfüller, U., & Scheffler, A. (2007). Solubility studies of oleanolic acid and betulinic acid in aqueous solutions and plant extracts of *Viscum album* L. *Planta medica*, *73*(02), 157-162.

Jia, L. I., Xiao, M. E. N. G., Chengyue, Y. I. N., Zhang, L., Bin, L. I. N., et al. (2023). Antimalarial and neuroprotectiveent-abietanediterpenoids from the aerial parts of *Phlogacanthus curviflorus*. *Chinese Journal of Natural Medicines*, *21*(8), 619-630.

Kaingu, C. K., Oduma, J. A., & Kanui, T. (2012). Preliminary investigation of contractile activity of *Ricinus communis* and *Euclea divinorum* extracts on isolated rabbit uterine strips. *Journal of ethnopharmacology*, *142*(2), 496-502.

Kamagaté, M., Koffi, C., Kouamé, N. M., Akoubet, A., Alain, N., Yao, R., & Die, H. (2014). Ethnobotany, phytochemistry, pharmacology and toxicology profiles of *Cassia siamea* Lam. *The Journal of Phytopharmacology*, *3*(1), 57-76.

Kaur, P., Arora, S., & Singh, R. (2022). Isolation, characterization and biological activities of betulin from Acacia nilotica bark. *Scientific Reports*, *12*(1), 9370.

Khusnutdinova, E. F., Petrova, A. V., Apryshko, G. N., Kukovinets, O. S., & Kazakova, O. B. (2018). Synthesis and cytotoxicity of indole derivatives of betulin, erythrodiol, and uvaol. *Russian Journal of Bioorganic Chemistry*, *44*, 322-329.

Kislitsyn, A. N. (1994). Ekstraktivnyeveshchestvaberesty: vydelenie, sostav, svoistva, primenenie [Birch bark extractives: isolation, composition, properties, application]. *Khimiiadrevesiny—Wood chemistry*, *3*, 3-28.

Kommera, H., Kaluđerović, G. N., Bette, M., Kalbitz, J., Fuchs, P., et al. (2010). In vitro anticancer studies of α-and β-d-glucopyranose betulin anomers. *Chemico-biological interactions*, *185*(2), 128-136.

Krasutsky, P. A. (2006). Birch bark research and development. *Natural Product Reports*, *23*(6), 919-942.

Krol, S. K., Bębenek, E., Sławińska-Brych, A., Dmoszyńska-Graniczka, M., Boryczka, S., & Stepulak, A. (2020). Synthetic betulin derivatives inhibit growth of glioma cells in vitro. *Anticancer Research*, *40*(11), 6151-6158.

Kubba, A., Tillequin, F., Koch, M., Litaudon, M., & Deguin, B. (2005). Iridoids, lignan, and triterpenes from Osmanthuscymosus. *Biochemical systematics and ecology*, *33*(3), 305-307.

Laavola, M., Haavikko, R., Hämäläinen, M., Leppänen, T., Nieminen, R., et al. (2016). Betulin derivatives effectively suppress inflammation in vitro and in vivo. *Journal of natural products*, *79*(2), 274-280.

Laiolo, J., Graikioti, D. G., Barbieri, C. L., Joray, M. B., Antoniou, A. I., et al. (2024). Novel betulin derivatives as multidrug reversal agents targeting P-glycoprotein. *Scientific Reports*, *14*(1), 70.

Lall, N., Weiganand, O., Hussein, A. A., & Meyer, J. J. M. (2006). Antifungal activity of naphthoquinones and triterpenes isolated from the root bark of Eucleanatalensis. *South African Journal of Botany*, *72*(4), 579-583.

Lee, S. J., Hossaine, M. A., & Park, S. C. (2016). A potential anti-inflammation activity and depigmentation effect of *Lespedeza bicolor* extract and its fractions. *Saudi Journal of Biological Sciences*, *23*(1), 9-14.

Lesellier, E., Destandau, E., Grigoras, C., Fougère, L., & Elfakir, C. (2012). Fast separation of triterpenoids by supercritical fluid chromatography/evaporative light scattering detector. *Journal of Chromatography A*, *1268*, 157-165.

Li, Y., Liu, X., Jiang, D., Lin, Y., Wang, Y., et al. (2016). Betulin induces reactive oxygen species-dependent apoptosis in human gastric cancer SGC7901 cells. *Archives of pharmacal research*, *39*, 1257-1265.

Lin, H., Jiang, X., Qian, C., Zhang, Y., Meng, X., et al. (2024). Genome-Wide Identification, Characterization, and Expression

Analysis of the HD-Zip Gene Family in Lagerstroemia for Regulating Plant Height. *Genes*, *15*(4), 428.

Linde, H. H. (1965). Die alkaloide aus *Melodinus australis* (F. MUELLER) PIERRE (Apocynaceae). *Helvetica Chimica Acta*, *48*(8), 1822-1842.

Ma, W., Lv, C., Jiang, D., Kang, C., & Zhao, D. (2020). The complete chloroplast genome sequence of *Euphorbia lathyris* L.(Euphorbiaceae). *Mitochondrial DNA Part B*, *5*(3), 3660-3662.

Magid, A.A., Voutquenne-Nazabadioko, L., Bontemps, G., Litaudon, M., Lavaud, C. (2008). Tyrosinase inhibitors and sesquiterpene diglycosides from *Guioa villosa*. *Planta Medica*, *74*(1), 55-60.

Mahajan, G., & Chauhan, B. S. (2024).Novel use pattern of pendimethalin for annual ryegrass (*Lolium rigidum* Gaudin) control in wheat and barley. *Frontiers in Agronomy*, *5*, 1341880.

Manzoor, M. A., Xu, Y., Lv, Z., Xu, J., Wang, Y., et al. (2024). Comparative genomics of N-acetyl-5-methoxytryptamine members in four *Prunus* species with insights into bud dormancy and abiotic stress responses in *Prunus avium*. *Plant Cell Reports*, *43*(4), 89.

Melani, I. R., Wafi, M. F., Riandinata, M. R., Halim, P. A. R., Muti'ah, R., & Santosaningsih, D. (2023). Potential Inhibition of *Melaleuca leucadendron* L. Compounds Against the NSP5 SARS CoV-2 Protein. *Indonesian Journal of Cancer Chemoprevention*, *13*(3), 195-206.

Mhamdi, S., Yahia, K.B., Montpied, P., Brendel, O., & Dreyer, E. (2023).Inter-specific differences in architectural and morphological traits on seedlings of cork oak (*Quercus suber* L.), zeen oak (*Quercus canariensis* Willd.) and their hybrid Afares oak (*Quercus afares* Pomel) growing in the greenhouse. *IOBC-WPRS Bulletin*, *168*, 49-54.

Mishra, S. N., Tomar, P. C., & Lakra, N. (2007).Medicinal and food value of Capparis—a harsh terrain plant. *The Indian Journal of Traditional Knowledge*, *6*(1), 230-238.

Ning, K. Q., Meng, D. L., Lou, L. L., & Li, X. (2013). Chemical constituents from the *Jasminum lanceolarium* Roxb. *Biochemical Systematics and Ecology*, *51*, 297-300.

Orchel, A., Chodurek, E., Jaworska-Kik, M., Paduszyński, P., Kaps, A., et al. (2021). Anticancer activity of the acetylenic derivative of betulin phosphate involves induction of necrotic-like death in breast cancer cells in vitro. *Molecules*, *26*(3), 615.

Orzalesi, G., Mezzetti, T., Rossi, C., & Bellavita, V. (1970). Pentacyclic triterpenoids from Euclea kellau. *Plantamedica*, *18*(05), 30-36.

Öz, U. (2021). Anatomy of *Carlina corymbosa* L., *Carthamus dentatus* Vahl. And *Picnomon acarna* (L.) Cass.(Asteraceae). *Celal Bayar University Journal of Science*, *17*(2), 215-222.

Peraza-Sánchez, S. R., Poot-Kantún, S., Toores-Tapia, L. W., May-Pat, F., Simá-Polanco, P., & Cedillo-Rivera, R. (2005). Screening of Native Plants from Yucatan for Anti–Giardia lamblia Activity. *Pharmaceutical biology*, *43*(7), 594-598.

Pratima, H., Shiraguppi, A., Joojagar, P., Shah, K., Cheeraladinni, S. S., et al. (2024). Phytochemical profile and hepatoprotective potentiality of *Phyllanthus* genus: a review. *Journal of Pharmacy and Pharmacology*, *77*(2):189-205.

Rastogi, S., Pandey, M. M., & Rawat, A. K. S. (2015). Medicinal plants of the genus Betula—Traditional uses and a phytochemical–pharmacological review. *Journal of ethnopharmacology*, *159*, 62-83.

Rorato, D., Araujo, M., Dutra, A., Turchetto, F., Tabaldi, L., & Mieth, P. (2018).Seed analysis and seedling production of *Matayba elaeagnoides* Radlk. *Floresta e Ambiente*, *25*, e20160043.

Rzepka, Z., Bębenek, E., Chrobak, E., & Wrześniok, D. (2022).Synthesis and anticancer activity of indole-functionalized derivatives of betulin. *Pharmaceutics*, *14*(11), 2372.

Sajini, R. J., & Chamundeeswari, D. (2023).Ixoraspecies: A Review on chemical constituents and Pharmacological activities. *Latin American Journal of Pharmacy: A Life Science Journal*, *42*(3), 1000-1024.

Saleem, H., Htar, T. T., Naidu, R., Zengin, G., Locatelli, M., Tartaglia, A., & Ahemad, N. (2020). Phytochemical composition and enzyme inhibition studies of *Buxus papillosa* CK Schneid. *Processes*, *8*(7), 757.

Salin, O., Alakurtti, S., Pohjala, L., Siiskonen, A., Maass, V., Maass, M., &Vuorela, P. (2010). Inhibitory effect of the natural product betulin and its derivatives against the intracellular bacterium *Chlamydia pneumoniae*. *Biochemical pharmacology*, *80*(8), 1141-1151.

Santos, R. C., Salvador, J. A., Marín, S., Cascante, M., Moreira, J. N., & Dinis, T. C. (2010). Synthesis and structure–activity relationship study of novel cytotoxic carbamate and N-acylheterocyclic bearing derivatives of betulin and betulinic acid. *Bioorganic & medicinal chemistry*, *18*(12), 4385-4396.

Sharma, M., Chopra, S., & Prasad, S. B. (2015). *Guazuma tomentosa*: A valuable medicinal plant. *International Journal of Pharmacognosy and Phytochemical Research*, *7*(1), 197-200.

Shen, C. C., Ni, C. L., Huang, Y. L., Huang, R. L., & Chen, C. C. (2004). Furanolabdane Diterpenes from *Hypoestes purpurea*. *Journal of natural products*, *67*(11), 1947-1949.

Šiman, P., Filipová, A., Tichá, A., Niang, M., Bezrouk, A., & Havelek, R. (2016). Effective method of purification of betulin from birch bark: The importance of its purity for scientific and medicinal use. *PloS one*, *11*(5), e0154933.

Singh, P. A., Brindavanam, N. B., Kimothi, G. P., Verma, R., & Aeri, V. (2016).A validated HPLC method for the determination of betulin in the stem bark of *Tectona grandis* Linn. *International Journal of Pharmaceutical Sciences and Research*, *7*(2), 719.

So, H. M., Eom, H. J., Lee, D., Kim, S., Kang, K. S., et al. (2018). Bioactivity evaluations of betulin identified from the bark of *Betula platyphylla* var. japonica for cancer therapy. *Archives of pharmacal research*, *41*, 815-822.

Şoica, C. M., Dehelean, C. A., Peev, C., Aluas, M., Zupkó, I., KásaJr, P., &Alexa, E. (2012). Physico-chemical comparison of betulinic acid, betulin and birch bark extract and in vitro investigation of their cytotoxic effects towards skin epidermoid carcinoma (A431), breast carcinoma (MCF7) and cervix adenocarcinoma (HeLa) cell lines. *Natural product research*, *26*(10), 968-974.

Spivak, A. Y., Keiser, J., Vargas, M., Gubaidullin, R. R., Nedopekina, D. A., Shakurova, E. R., & Odinokov, V. N. (2014). Synthesis and activity of new triphenylphosphonium derivatives of betulin and betulinic acid against *Schistosoma mansoni* in vitro and in vivo. *Bioorganic & medicinal chemistry*, *22*(21), 6297-6304.

Stöcklin, W., De Silva, L.B., Geissman, T.A.(1969). Constituents of Holacanthaemoryi. *Phytochemistry*, *8*(8),1565-9.

Szakiel, A., Paczkowski, C., & Huttunen, S. (2012).Triterpenoid content of berries and leaves of bilberry *Vaccinium myrtillus* from Finland and Poland. *Journal of Agricultural and Food Chemistry*, *60*(48),11839-49.

Szlasa, W., Ślusarczyk, S., Nawrot-Hadzik, I., Abel, R., Zalesińska, A., et al. (2023). Betulin and its derivatives reduce inflammation and COX-2 activity in macrophages. *Inflammation*, *46*(2), 573-583.

Szuster-Ciesielska, A., Plewka, K., Daniluk, J., & Kandefer-Szerszeń, M. (2011). Betulin and betulinic acid attenuate ethanol-induced liver stellate cell activation by inhibiting reactive oxygen species (ROS), cytokine (TNF-α, TGF-β) production and by influencing intracellular signaling. *Toxicology*, *280*(3), 152-163.

Takibayeva, A. T., Zhumabayeva, G. K., Bakibaev, A. A., Demets, O. V., Lyapunova, M. V., et al. (2023). Methods of analysis and identification of betulin and its derivatives. *Molecules*, *28*(16), 5946.

Tang, J., Jones, S. A., Jeffery, J. L., Miranda, S. R., Galardi, C. M., et al. (2014). Synthesis and biological evaluation of macrocyclized betulin derivatives as a novel class of anti-HIV-1 maturation inhibitors. *The Open Medicinal Chemistry Journal*, *8*, 23.

Tang, J., Zou, R., Wei, X., & Li, D. (2023). Complete chloroplast genome sequences of five Ormosia Species: Molecular structure, comparative analysis, and phylogenetic analysis. *Horticulturae*, *9*(7), 796.

Tchatchoua, D. T., & Aravanopoulos, F. A. (2015). Selection strategy for Chestnut (*Castanea sativa* Mill.) families originating from contrasting European populations. *Open Journal of Forestry*, *5*(05), 489.

Tolstikov, G. A., Flekhter, O. B., Shultz, E. E., Baltina, L. A., & Tolstikov, A. G. (2005). Betulin and its derivatives: Chemistry and biological activity. *Chemistry for sustainable development*, *13*(1), 1-29.

Tsepaeva, O. V., Nemtarev, A. V., Abdullin, T. I., Grigor'eva, L. R., Kuznetsova, E. V., et al. (2017). Design, synthesis, and cancer cell growth inhibitory activity of triphenylphosphonium derivatives of the triterpenoid betulin. *Journal of natural products*, *80*(8), 2232-2239.

Tykheev, Z. A., Anenkhonov, O. A., Zhigzhitzhapova, S. V., Taraskin, V. V., Radnaeva, L. D., & Zhang, F. (2020). Do Compositions of Lipid Fraction Correspond to Species Differentiation in Bupleurum L.(Apiaceae)?. *Plants*, *9*(11), 1407.

Ur'yash, V. F., Kokurina, N. Y., Larina, V. N., Kashtanov, E. A., & Gruzdeva, A. E. (2014). Physicochemical properties of Betulin and CO 2 extract from birch bark. *Russian Journal of Physical Chemistry B*, *8*, 1100-1109.

Veloso, C. C., Bitencourt, A. D., Cabral, L. D., Franqui, L. S., Dias, D. F., et al. (2010). Pyrostegia venusta attenuate the sickness behavior induced by lipopolysaccharide in mice. *Journal of Ethnopharmacology*, *132*(1), 355-358.

Vieira, S. F., Ferreira, H., & Neves, N. M. (2020). Antioxidant and anti-inflammatory activities of cytocompatible *Salvia officinalis* extracts: A comparison between traditional and Soxhlet extraction. *Antioxidants*, *9*(11), 1157.

Wang, J., Wu, J., Han, Y., Zhang, J., Lin, Y., et al. (2022). Synthesis and biological evaluation of novel betulin derivatives with aromatic hydrazone side chain as potential anticancer agents. *Journal of the Brazilian Chemical Society*, *33*(03), 227-237.

White, J. F., & Bernstein, D. I. (2003). Key pollen allergens in North America. *Annals of Allergy, Asthma & Immunology*, *91*(5), 425-435.

Wirtu, S. F., Mishra, A. K., Jule, L. T., & Ramaswamy, K. (2024). *Ocimum basilicum* and *Ocimum americanum*: A Systematic Literature Review on Chemical Compositions and Antimicrobial Properties. *Natural Product Communications*, *19*(4), 1934578X241247640.

Wu, J., Wang, J., Han, Y., Lin, Y., Wang, J., & Bu, M. (2021).Synthesis and cytotoxic activity of novel betulin derivatives containing hydrazide-hydrazone moieties. *Natural Product Communications*, *16*(10), 1934578X211055345.

Wu, Q., Li, H., Qiu, J., & Feng, H. (2014). Betulin protects mice from bacterial pneumonia and acute lung injury. *Microbial pathogenesis*, *75*, 21-28.

Xiong, J., Kashiwada, Y., Chen, C. H., Qian, K., Morris-Natschke, S. L., Lee, K. H., &Takaishi, Y. (2010). Conjugates of betulin derivatives with AZT as potent anti-HIV agents. *Bioorganic & medicinal chemistry*, *18*(17), 6451-6469.

Ye, Z., Zhang, H., Lin, X., Huang, S., Zou, S., & Zou, X. (2024). Effect of Biochar Using N, P, and K Fertilisers on Growth and Quality of *Lithocarpus litseifolius*. *Agronomy*, *14*(4), 728.

Zhang, H., Ding, J., Holstein, N., & Wang, N. (2023). Betula mcallisteri sp. nov.(sect. Acuminatae, Betulaceae), a new diploid species overlooked in the wild and in cultivation, and its relation to the widespread B. luminifera. *Frontiers in Plant Science*, *14*, 1113274.

Zhang, L., Ma, Z., Wang, R., & Zhu, M. (2021).Synthesis and characterization of methacrylate-functionalized betulin derivatives as antibacterial comonomer for dental restorative resins. *ACS Biomaterials Science & Engineering*, *7*(7), 3132-3140.

Zhang, L., Mao, R., Bi, H., Shen, J., Wang, Y., & Li, M. (2020).Characterization of the complete chloroplast genome of Hoveniaacerba (Rhamnaceae). *Mitochondrial DNA Part B*, *5*(1), 934-935.

Zhou, Z., Zhu, C., Cai, Z., He, L., Zhao, F., Lou, X., & Qi, X. (2018). Betulin induces cytochrome c release and apoptosis in colon cancer cells via NOXA. *Oncology letters*, *15*(5), 7319-7327.

Zhu, H. (2002). A revision of the genus Lasianthus (Rubiaceae) from China. *Systematics and Geography of Plants*, *72*(2)63-110.

Zia-Ul-Haq, M., Stanković, M. S., Rizwan, K., & De Feo, V. (2013).Grewiaasiatica L., a food plant with multiple uses. *Molecules*, *18*(3), 2663-2682.

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Agrobiodiversity integration in farming systems for income generation and livelihood options of smallholder farmers in Nepal: A case study of Bhimphedi Municipality

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ABSTRACT

Food and nutrition security are critical global concerns, particularly for smallholder farmers in Nepal who face challenges with resources and inputs. This study explores how the Chabeli Farmers Group in Bhimphedi Municipality can utilize agrobiodiversity to enhance their farming systems and income. To achieve this, a survey was conducted involving 20 farmers (10 male and 10 female), supplemented by focus group discussions and field observations. The study identified 60 species across cereals, vegetables, fruits, forage, and fodder crops, representing 99 genotypes and 10 types of livestock. Farmers preserve and manage these species through traditional practices, such as storing seeds in Bhakari containers made of bamboo and mud. The findings of this study highlight the importance of passing knowledge from one generation to the next and the role of traditional methods in protecting biodiversity. Additionally, a Participatory Guarantee System (PGS) facilitates the sale of organic cash crops. The study demonstrates that effective biodiversity management enhances resilience, diversifies income, and improves market access. In conclusion, integrating agrobiodiversity with community-driven systems improves food security, protects biocultural heritage, and offers scalable solutions for long-term sustainable farming.

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1 Introduction

Agrobiodiversity is a complex and multidimensional concept used to assess biodiversity in agricultural lands. It encompasses the biological diversity within land use and food systems, as well as social-ecological activities. including sociocultural skills, economic influences, and political interactions. Agrobiodiversity includes genetic, species, and ecosystem levels essential for sustaining key functions (Parris 2001; Zimmerer and Vaca 2016; Matthies et al. 2023). There are several ways to develop indicators for agrobiodiversity. The Convention on Biological Diversity defines three indicators, each focusing on one of the levels: genetic diversity, species diversity, and ecosystem diversity (Gugerli et al. 2008). Furthermore, agrobiodiversity is considered a crucial adaptation strategy for mitigating risks associated with climate change. It enhances food security, promotes sustainable land management, supports biodiversity, improves soil quality, and fosters ecosystem services (Chaudhary et al. 2020; Cordoba Vargas et al. 2020; Rist et al. 2020; Agnoletti and Santoro 2022; Liu et al. 2022).

Agrobiodiversity is seen as particularly significant for farmers who cultivate relatively small areas due to its relevance for environmental sustainability, food security, and climate adaptation (Altieri et al. 2012; Williams 2017; Zimmerer et al. 2018; Bukchin-Peles and Fishman 2021). The terms "family farmers" and "smallholders" are often used interchangeably (Garner and de la O Campos 2014), with small farms or smallholders typically classified as family farms with less than two hectares of land (Lowder et al. 2021). In addition to contributing to 70-80% of food production, smallholder farming methods provide around 60% of household income and are essential for boosting food security. These farming practices also serve as vital pathways for preserving agrobiodiversity (Forsythe 2017; Fan and Rue 2020; Gautam et al. 2020; Guarin et al. 2020; Lowder et al. 2021; Dagunga et al. 2023).

Macqueen (2024) highlights the various benefits of agrobiodiversity, which include food security, livelihood resilience (Zimmerer and de Haan 2020; Kerr et al. 2021), nutritional and health benefits (Fanzo et al. 2013; Remans et al. 2014; Harris et al. 2022; Zaccari et al. 2023), provision of biomass energy and household materials (Immerzeel et al. 2013; Subedi et al. 2020), preservation of biocultural heritage (Agnoletti and Santoro 2022; Swiderska et al. 2022), and ecosystem services, including climate change mitigation (Altieri 1999; Gerits et al. 2021; Drucker et al. 2022).

Eighty percent of the world's population lives in impoverished rural areas (FAO 2019), where agriculture, often practiced in smallholdings, is the dominant activity. Significant changes are needed in the global agriculture system to meet the future food demands of a growing, increasingly wealthy, and urbanized population. In this context, smallholder farmers in developing countries are crucial in ensuring food security. Over 80% of the world's farms operate on less than 2 hectares of land. Though these smallholder farms account for only 12% of the world's farmland, they produce 80% of the food in Asia and sub-Saharan Africa (Lowder et al. 2014).

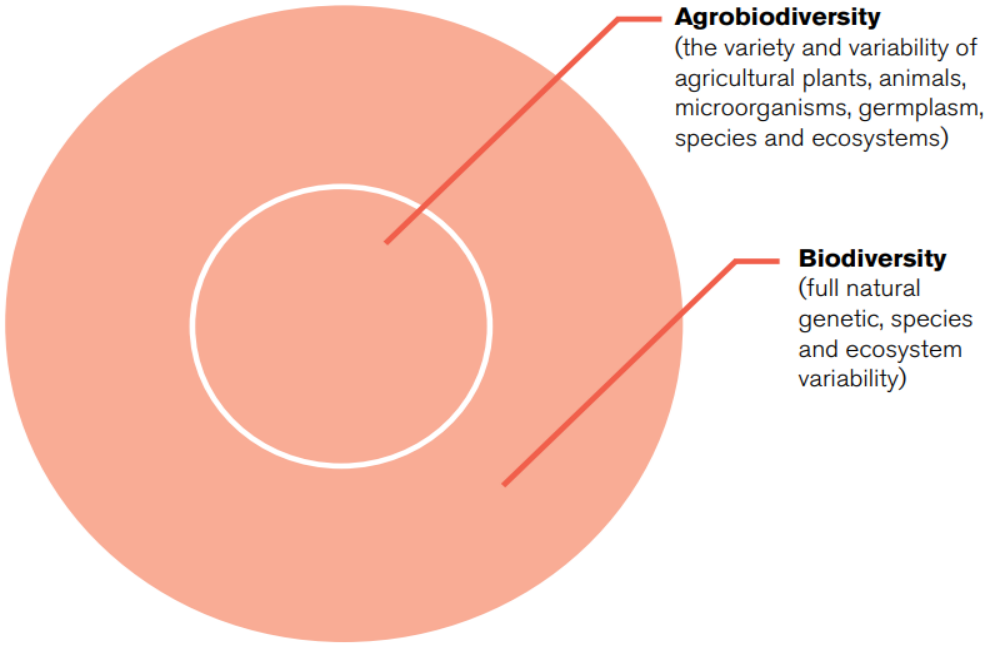

Figure 1 Agrobiodiversity as a subset of biodiversity (adopted from Macqueen 2024)

Smallholder farms face both naturally occurring challenges and man-made obstacles that impact their ability to increase production and transition to more commercial and profit-driven farming systems. In many developing countries, women play a vital role in agriculture, making up 43% of the workforce on small farms. However, they face numerous barriers, including limited access to essential resources such as land, livestock, agricultural inputs, technology, markets, and financial or extension services. These challenges hinder their ability to improve their livelihoods and contribute fully to the agricultural economy (FAO 2011). Smallholder farmers must either intensify their farming practices or shift from agriculture to overcome these constraints. Promoting land rights and efficient land markets, enhancing risk management and adaptation strategies, supporting inclusive food value chains, closing gender gaps, fostering young farmers, and scaling up productive cross-sector social safety nets are essential steps in this process. The potential for positive change is within reach, offering hope and driving necessary actions (Fan and Rue 2020).

The concept of smallholders' livelihoods is closely tied to sustainability. According to the UNDP (2010), sustainable livelihoods enable smallholder farmers to cope with and recover from shocks and stressors, paving the way for a brighter future for them and their future generations. This sustainable approach, which does not exploit natural resources, offers long-term benefits. Livelihoods encompass the activities, assets, and access that collectively determine the living conditions of individuals or households (Ellis 1998). According to Nzima et al. (2024), household food security and income are vital for sustainable global, national, and local development. Additionally, the factors influencing farmers' adoption of income-generating activities and adaptive strategies in vulnerable contexts have been widely discussed worldwide (Wang et al. 2023). Bravo-Pena and Yoder (2024) assert that agrobiodiversity is often regarded as a crucial adaptation strategy for mitigating the risks associated with climate change, enhancing the system's resilience and responsiveness to external shocks.

In Nepal, agrobiodiversity is a critical component of biodiversity, encompassing six significant elements: crops, forages, livestock, aquatic species, insects, and microorganisms. It also includes four sub-components: domesticated species, semi-domesticated varieties, wild relatives, and wild edibles (Joshi et al. 2020). Many farming areas in Nepal are environmentally marginal and increasingly at risk of land degradation and biodiversity loss due to climate change. Agrobiodiversity's ongoing availability and use, especially among smallholder farmers, are vital for adapting to these climate challenges (Regmi and Paudyal 2009). Agriculture remains a cornerstone of Nepal's economy, contributing 27.6% to the GDP and involving 66% of smallholder farmers engaged in agricultural activities to ensure national food security (Karki et al. 2020). Smallholders farming less than 0.5 hectares per household account for over 50% of Nepalese farmers (Central Bureau of Statistics 2011). Research has shown that agricultural diversification positively impacts dietary diversity and enhances the livelihoods of smallholder farmers. A varied farming system increases the availability of healthy foods and promotes environmental stability and resilience (Wilson 2010). A recent study by Nepali et al. (2024) demonstrated a positive relationship between agricultural diversification and dietary diversity. This study focuses on the Chabeli Farmer Group and provides valuable insights into how incorporating agrobiodiversity into farming practices can strengthen livelihoods and enhance stability through innovative farming methods. It highlights the significant contributions of smallholder and marginalized farmer groups in managing agrobiodiversity. Their efforts in integrating diverse crop varieties, livestock species, and wild varieties support agrobiodiversity and honor traditional and Indigenous knowledge systems. The study aims to explore how integrating agrobiodiversity into smallholder farming systems in Bhimphedi Municipality, Nepal, can enhance income generation, improve livelihoods, and strengthen community resilience. It seeks to document traditional knowledge, biodiversity management practices, and the role of community-driven initiatives like the Chabeli Farmers Group in fostering sustainable agriculture through diverse crop and livestock systems. Additionally, the study aims to assess the impact of these practices on food security, market access, and climate adaptation strategies.

2 Methodology

2.1 Description of the case study area and Chabeli Farmer Group

This case study was conducted in the Makwanpur District of Bagmati Province, Nepal. It is located 23 km from Hetauda Bazar, the headquarters of Bagmati Province, and 53 km from Kathmandu, the capital city of Nepal. The district has an altitude ranging from 300 to 1800 meters and features subtropical to temperate climatic conditions. Geographically, it lies at approximately 27.4217°N latitude and 85.0301°E longitude. The annual rainfall in the study area is 1700 mm, primarily occurring during the monsoon season from June to September. The soil in the area predominantly consists of loamy types.

The region is home to subtropical broadleaf forests at lower altitudes, characterized by a diverse range of tree species, including Sal (*Shorea robusta*), Indian rosewood (*Dalbergia sissoo*), khayar (*Acacia catechu*), and chir pine (*Pinus roxburghii*). Subtropical pine forests, which are represented by chir pine, can be found in drier regions. In contrast, oak forests dominate at higher altitudes and are characterized by temperate broadleaf and mixed forests (Nepal and Koirala 2023).

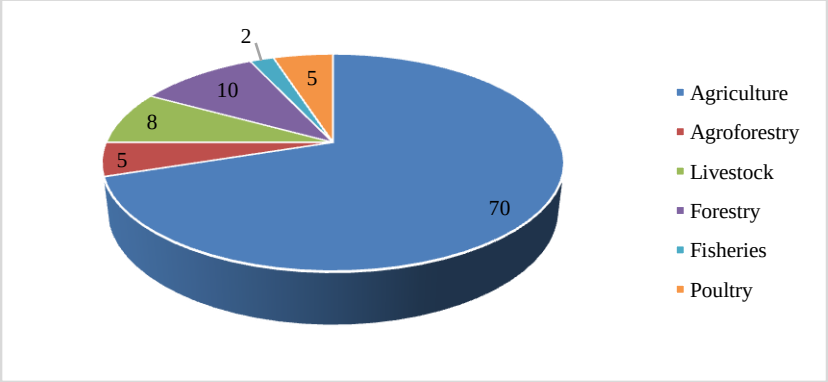

Figure 2 Use of formal agricultural land by land-use categories share (in % age)

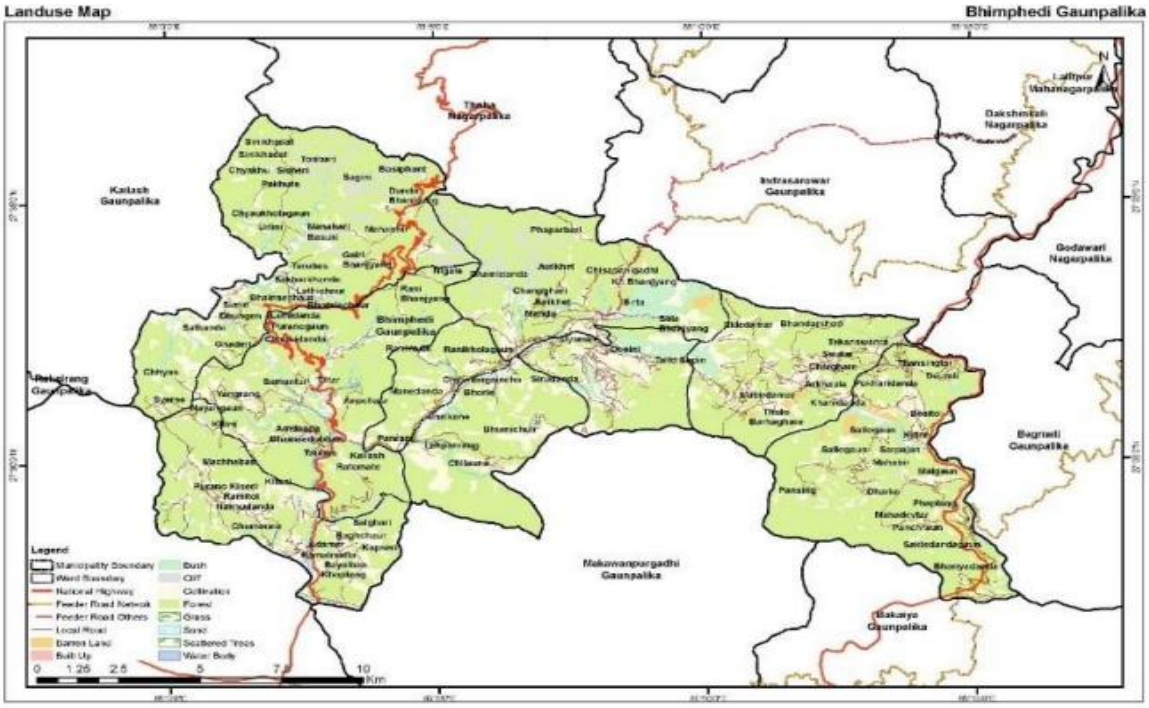

Figure 3 Land-use map of Bhimphedi Municipality (Source: Department of Forest Research and Survey 2017)

The Chabeli Farmers Group was established in 2012 AD (2069 BS) and comprises 40 smallholder farmers from various ethnic and caste groups: 8 members from the Brahman and Chhetri communities, 22 members from Indigenous communities, 7 members from Dalit communities, and 3 members from other castes and ethnicities. The members of the Chabeli Farmers Group are categorized into two groups: marginal farmers, who have landholdings of 0.01 to 0.5 hectares, and smallholder farmers, who have landholdings ranging from 0.5 to 8.0 hectares. The income sources of the group members are diverse, with 11% generated from agroforestry activities, 78% from agriculture (both subsistence and commercial), and 11% from livestock production and poultry management (Nepal and Koirala 2023; Macqueen 2024).

Bhimphedi Municipality consists of rural and hilly areas, with 95% of the land comprising hilly terraces and 5% flat slopes. Of the municipality's total land area, 48% is covered by forest. Agriculture occupies 70% of the land used, while the remaining 30% includes other income-generating activities such as agroforestry, livestock, forestry, fisheries, and poultry (Figures 2 and 3).

2.2 Arrangement of the field study: Questionnaire development and arrangement of the field study

To gather primary data and gain a better understanding of the agrobiodiversity practices of the Chabeli Farmer Group, a questionnaire was developed to collect information on the following areas: (i) Geographical Structure: Climate conditions

and weather patterns, (ii) Demographic Information: Age, gender, household composition, and landholding sizes, (iii) Composition of the Farmer Group: Male, female, and youth representation, (iv) Land Use Patterns: Agroforestry, types of agriculture, types of forests present, and forest-based resources, (v) Agricultural Practices: Gender-specific practices regarding crop production and sales, wild harvesting, livestock preferences, neighborhood transfer, in-house transfer, and the types of commercial and subsistence crops grown, along with the crop varieties used, (vi) Bio-Cultural Heritage: Selection of seeds for crops grown for nutritional security and the influence of cultural and traditional practices based on market goals and farm functions, (vii) Knowledge Sources and Transfer: Identification of traditional knowledge, training received at home, sources of agricultural information, neighborhood knowledge sources, knowledge transfer within households, and community knowledge networks, and (viii) Market and Enterprises: Strategies for enterprises and challenges faced in accessing markets for their products. Based on the prepared questionnaire, the field study was conducted with assistance from lead farmers and volunteers, supported by local leaders and the farmer group to ensure community participation.

2.3 Field study and data (primary) collection and analysis of the study

A series of orientations were conducted with volunteer farmers to assess the feasibility of data collection. The primary data gathered included information on crop diversity, gender roles in managing agrobiodiversity, the use of cultural and medicinal plants, challenges encountered in farming, and sources of knowledge. This data was collected through surveys, interviews, and focus group discussions. In addition, a Focused Group Discussion (FGD) was held to identify the necessary information and sources. Interviews were conducted with 10 male and 10 female group members, concentrating on the commercial and subsistence crops grown and identifying the agrobiodiversity managed by different genders, including youth members. The volunteer farmers were categorized into three distinct respondent groups based on age and gender: women aged 30–65, men aged 30–70, and youth aged 16–35.

The survey revealed that the Chabeli Farmers Group utilizes a variety of crops and plants for multiple purposes, such as food and livestock production, as well as medicinal, cultural, and religious uses. Members of the farmer group manage 56 different plant species, including a range of varieties. Data collection involved both quantitative and qualitative methods. The quantitative approach was used to analyze numerical data gathered from questionnaires. In contrast, the qualitative approach included FGDs and open-ended responses to identify challenges, knowledge sources, and agrobiodiversity management. The findings assessed the effectiveness of Farmer-Focused Producer Organizations (FFPOs) and apex organizations like the Chabeli Farmers Group in promoting and preserving agrobiodiversity. The results also emphasized how integrating traditional knowledge with modern practices, including climate-smart techniques, has enhanced resilience against climate change impacts and improved the socioeconomic patterns of local development through diversified farming systems. This methodology provided a comprehensive understanding of the Chabeli Farmers Group's operations. It is a model for other farmer groups seeking to enhance their agricultural practices through community collaboration and knowledge sharing.

2.4 Secondary data collection

The secondary data was extracted and reviewed from a previous report, case studies, and a midterm evaluation of the Forest and Farm Facility (FFF) project conducted by FAO in 2021. This project aims to support forest and farm producers in showcasing climate-resilient landscapes and improving livelihoods. The evaluation highlighted the purpose and priorities of Forest and Farm Producer Organizations (FFPOs) concerning national and sub-national policies that address governance, advocacy, issue-based discussions, entrepreneurship, and sustainable forest management. The project's progress has demonstrated improvements in service delivery, responsible governance, green skill development, and the protection and promotion of indigenous knowledge. The report indicates that sustaining the project requires responsible governance and entrepreneurship while recognizing that external factors such as market dynamics and land tenure present additional challenges. Similarly, a case study reviewed a document published by IIED titled "*Selling Stories that Conserve Bio-Cultural Diversity and Promote Resilience*". This document describes how FFPOs operate Nepal's Participatory Guarantee System (PGS). The source focuses on agroecological practices and a certification system that targets smallholder and marginalized farmers, aligning with community-driven approaches. The study reveals that the National Farmer's Group Federation (NFGF) and its apex organizations, such as the Chabeli Farmer Group, promote organic farming and participatory grant systems, enabling smallholder farmers to connect with better markets and improve product quality. However, challenges remain in accessing finance, local government support, policy endorsement, and skills training. Additional publications, such as "*Food Security in Nepal*" and "*Agriculture Model Learning Facilitation Resource Book*," highlight various case studies and frameworks about agricultural development and learning models adopted in the country. Another article discussing policies and perspectives on food health and safety in Nepal provides an academic viewpoint on food safety challenges and readiness (Koirala 2023). A report overviewing agricultural biodiversity contributes to a global perspective on sustaining agricultural functions (FAO 1999). These sources were selected based on their relevance, research focus, credibility, and level of detail. The data was chosen to cover topics like PGSs,

agricultural governance, apex organizations, and community-driven agricultural practices. This ensured a comprehensive understanding of the implications for Nepalese smallholder farmers and food sovereignty. The selected topics also consider the socioeconomic status of smallholder farmers and the influence of top organizations, farmer groups, and local chains in strengthening the community. The sources, articles, and publications from National Farmers Group Federation Nepal (NFGF) aided in contextualizing the overall process and findings.

3 Results

3.1 Status of agrobiodiversity utilization

3.1.1 Gender-wise cultivation of commercial crops

The study examined gender participation in the commercial cultivation of various agricultural products, including cereal crops, grain legumes (pulses), horticultural fruits and vegetables, oilseed crops, livestock, poultry, wild harvests, and medicinal herbs (Table 1). The assessment revealed that men and women play significant agricultural roles and actively contribute to agrobiodiversity management. However, men are more involved in commercial cultivation, with 80% of men reported to be engaged in the commercial production of these crops (Lima and Cunha 2024). Additionally, the findings indicated that women primarily focus on family care and prioritize maintaining household nutritional values. They tend to sell larger quantities of products specifically grown for market purposes. For instance, 70% of women reported growing vegetable crops for commercial sale. In livestock production, women typically concentrate on small animals, such as goats and chickens, which they raise for subsistence and market needs. Women generally sell these animals within their communities and prefer to sell dairy products, as they can generate quick cash income.

Table 1 Gender participation in crop production and estimates of Sales (in % age)

Gender Participation in Commercial and Subsistence Crop Production								
S.N	Crops	Participation in Commercial crop Production		Involvement of Males and Females in Sales of commercial crops (%)		Participation in Subsistence crop production		Crops classification
		Male	Female	Male	Female	Male	Female	
1	Maize	Yes	Yes	90.0	90.0	Yes	Yes	Cereals
2	Wheat	Yes	Yes	90.0	90.0	Yes	Yes	Cereals
3	Buckwheat	Yes	Yes	80.0	80.0	Yes	Yes	Cereals
4	Paddy	Yes	Yes	80.0	80.0	Yes	Yes	Cereals
5	Finger Millet	Yes	Yes	90.0	90.0	Yes	Yes	Cereals
6	Barley	Yes	Yes	90.0	80.0	Yes	Yes	Cereals
7	Pigeon Pea	Yes	Yes	90.0	80.0	Yes	Yes	Legumes /Pulses
8	Chick Pea	Yes	Yes	90.0	90.0	Yes	Yes	Legumes /Pulses
9	Green Pea	Yes	Yes	95.0	90.0	Yes	Yes	Legumes /Pulses
10	Yellow Pea	Yes	Yes	95.0	90.0	Yes	Yes	Legumes /Pulses
11	Kidney Beans	Yes	Yes	95.0	80.0	Yes	Yes	Legumes /Pulses
12	Butter Beans	Yes	Yes	95.0	80.0	Yes	Yes	Legumes /Pulses
13	Fawa Beans	Yes	Yes	95.0	80.0	Yes	Yes	Legumes /Pulses
14	Soybeans	Yes	Yes	95.0	90.0	Yes	Yes	Legumes /Pulses
15	Black Grams	Yes	Yes	95.0	80.0	Yes	Yes	Legumes /Pulses
16	Green Gram	Yes	Yes	95.0	90.0	Yes	Yes	Legumes /Pulses
17	Red Lentil	Yes	Yes	90.0	80.0	Yes	Yes	Legumes /Pulses
18	Banana	Yes	Yes	90.0	75.0	Yes	Yes	Fruits
19	Jackfruit	Yes	Yes	90.0	75.0	Yes	Yes	Fruits
20	Peach	Yes	Yes	90.0	80.0	Yes	Yes	Fruits

S.N	Crops	Gender Participation in Commercial and Subsistence Crop Production						Crops classification
		Participation in Commercial crop Production		Involvement of Males and Females in Sales of commercial crops (%)		Participation in Subsistence crop production		
		Male	Female	Male	Female	Male	Female	
21	Persimmons	Yes	Yes	90.0	80.0	Yes	Yes	Fruits
22	Raspberries	Yes	Yes	95.0	80.0	Yes	Yes	Fruits
23	Strawberries	Yes	Yes	95.0	80.0	Yes	Yes	Fruits
24	Pears	Yes	Yes	90.0	90.0	Yes	Yes	Fruits
25	Plums	Yes	Yes	90.0	90.0	Yes	Yes	Fruits
26	Kiwi	Yes	Yes	95.0	95.0	Yes	Yes	Fruits
27	Avocado	Yes	Yes	95.0	95.0	Yes	Yes	Fruits
28	Potato	Yes	Yes	85.0	80.0	Yes	Yes	Vegetables
29	Cabbage	Yes	Yes	85.0	80.0	Yes	Yes	Vegetables
30	Raddish	Yes	Yes	85.0	80.0	Yes	Yes	Vegetables
31	Carrot	Yes	Yes	85.0	80.0	Yes	Yes	Vegetables
32	Turnip	Yes	Yes	85.0	85.0	Yes	Yes	Vegetables
33	Tomatoes	Yes	Yes	90.0	80.0	Yes	Yes	Vegetables
34	Chilly	Yes	Yes	90.0	75.0	Yes	Yes	Vegetables
35	Cucumber	Yes	Yes	90.0	90.0	Yes	Yes	Vegetables
36	Pumpkin	Yes	Yes	90.0	90.0	Yes	Yes	Vegetables
37	Bottle gourd	Yes	Yes	90.0	90.0	Yes	Yes	Vegetables
38	Okra	Yes	Yes	90.0	90.0	Yes	Yes	Vegetables
39	Chayote	Yes	Yes	100.0	80.0	Yes	Yes	Vegetables
40	Cauliflower	Yes	Yes	90.0	80.0	Yes	Yes	Vegetables
41	Bamboo Shoots	Yes	Yes	95.0	85.0	Yes	Yes	Vegetables
42	Mushroom	Yes	Yes	95.0	90.0	Yes	Yes	Vegetables
43	Black Mustard	Yes	Yes	95.0	80.0	Yes	Yes	Oilseeds
44	Sesame	Yes	Yes	95.0	80.0	Yes	Yes	Oilseeds
45	Linseed/Flaxseed	Yes	Yes	95.0	80.0	Yes	Yes	Oilseeds
46	Soybean	Yes	Yes	95.0	80.0	Yes	Yes	Oilseeds
47	Cows	Yes	Yes	40.0	30.0	Yes	Yes	Livestock
48	Buffaloes	Yes	Yes	30.0	25.0	Yes	Yes	Livestock
49	Goats	Yes	Yes	70.0	85.0	Yes	Yes	Livestock
50	Pigs	Yes	Yes	60.0	55.0	Yes	Yes	Livestock
51	Ducks, Geese, Quail, Pigeons	No	No	-	-	Yes	Yes	Poultry Birds
52	Indian gooseberry	Yes	Yes	95.0	90.0	Yes	Yes	Wild Harvests
53	Malabar nut	Yes	Yes	90.0	95.0	Yes	Yes	Wild Harvests

S.N	Crops	Gender Participation in Commercial and Subsistence Crop Production						Crops classification
		Participation in Commercial crop Production		Involvement of Males and Females in Sales of commercial crops (%)		Participation in Subsistence crop production		
		Male	Female	Male	Female	Male	Female	
54	Red Silk Cotton	Yes	Yes	100.0	100.0	No	No	Wild Harvests
55	Stinging Nettle Leaf	No	Yes	0	95.0	Yes	Yes	Wild Harvests
56	Fiddle Head Wild Fern	No	Yes	0	90.0	Yes	Yes	Wild Harvests
57	Ginger	Yes	Yes	95.0	80.0	Yes	Yes	Medicinal herbs
58	Garlic	Yes	Yes	95.0	80.0	Yes	Yes	Medicinal herbs
59	Holy Basils	Yes	Yes	95.0	95.0	Yes	Yes	Medicinal herbs
60	Chirata	Yes	Yes	95.0	95.0	Yes	Yes	Medicinal herbs

3.1.2 Gender-wise raising of subsistence crops and Livestock

The study revealed that male farmers are involved periodically and seasonally in growing crops for household purposes, especially when cultivating labor-intensive cereal crops like maize and wheat. To maximize land use, these crops are often intercropped (Rusinamhodzi et al., 2012). Men tend to be less involved in kitchen and home garden management, which includes producing various vegetables, spices, and fruits, as their primary responsibilities focus on land preparation and fertilizer management (Abebe and Mulu 2017). Male respondents noted that when production is geared toward subsistence farming, they share the workload of livestock management since it is very time-consuming to rear animals. Interviews with farmers indicated that women are responsible for intercultural operations and other tasks related to the cultivation of subsistence crops. Similarly, women raise livestock, such as cows and buffaloes, for dairy products, goats, and chickens for sale in the neighborhood. They are also responsible for related tasks, including preparing biogas and managing livestock manure to enhance soil fertility (Binge et al. 2023). Additionally, women handle crop harvesting and post-harvest management, selecting and storing seeds for the next season.

3.1.3 Gender-wise harvesting of wild crops "Non-timber forest crops" (NTFPs)

Various wild plants, mentioned below, have been gathered and utilized to prepare organic manure. Men are primarily involved in the collection of these wild plants, which include several commonly collected resources (i) Malabar nut and mulberry are valued for their medicinal properties and serve as fodder and forage (Gupta et al. 2021), (ii) Stinging nettle leaves are used for both food and medicine (Ghasemi et al. 2024), and (iii) Chinaberry is collected explicitly for manure preparation (Ahmed et al. 2023).

On the other hand, women harvest various wild berries, including raspberries, blueberries, and blackberries, which are particularly beneficial as a fruit source for children. Wild ferns are gathered for household consumption and sold in local markets. These nutrient-rich ferns are often found in forests near canal sources (Rahmawati et al. 2017).

Other commonly collected resources include: (i) Stinging nettle leaves (used as food and medicine) (Ghasemi et al. 2024), (ii) Mushrooms, which serve as a dietary staple, (iii) Bamboo shoots (locally known as *Tama*), consumed as a food source, and (iv) Wild ferns, which are used as leafy greens and a source of nutrition (Rahmawati et al. 2017).

3.1.4 Existing agrobiodiversity of the area

The main crop varieties cultivated by the members of the Chabeli Farmers Group include additional valuable species, such as crops, oilseed crops, fodder and forage crops, species used for plant protection, and medicinal herbs, totaling 99 plant varieties (Table 2).

3.2 Knowledge transfer system for crops cultivation and animal management

Centuries-old traditional practices play a significant role in maintaining and protecting agro-biodiversity, as highlighted by Leoni (2024). Farmers in the local community engage in various methods of food cultivation, production, and value addition. These practices are influenced by cultural traditions associated with caste and religion. Their ancestors' Critical knowledge, incorporating local cultural and religious practices, has been passed down. For instance, the Janjati communities possess extensive knowledge of wild edibles and depend on forests for these forest foods, which are integral to their traditional culture. They rely on various resources, including wild mushrooms, bamboo shoots, wild ferns, berries, and medicinal herbs. These plants serve multiple purposes: food, medicine, fodder,

Table 2 Existing agrobiodiversity amongst the Chabeli groups

English/Local Name	Scientific Name	Characteristics of genotype	Genotype
A. Cereal Crops			
Maize (Makai)	*Zea mays*	Hybrids	Arun-2
			Manakamana-4
			Rampur Composite
			Sikhar-1
Wheat (Gahu)	*Triticum aestivum*	Improved/Semi-dwarf Varieties/High Yield	Gautam(improved)
			NL-971(semi-dwarf)
			WK 1204(semi-dwarft)
			NL 1110(high-yield)
Buckwheat (Fapar)	*Fagopyrum esculentum*	Early grown/Cold resistance	Jyoti
			Manang Local
			Rangli
			Madhupati
Paddy (Dhan)	*Oryza sativa*	Resistance varieties of Paddy	Dhan
			Khumal-4
			Radha-4
			Mansuli
Finger Millet(Kodo)	*Setaria Millet*	Foxtail Millet	Kaguno
	Pennisetum glaucum	Pearl Millet	MotiKodo
	Panicum miliaceum	Proso Millet	Chino
Barley (jau)	*Hordeum vulgare*	Improved /Local	Himalayan-4(improved)
			Goutum(local)
			Harsha(local)
Sorghum	*Sorghum bicolor*	Improved varieties	Lalit
B. Grain Legumes and Pulses			
Pigeon pea (Rahar Dal)	*Cajanus arietinum*	Local Varieties	Dhan Maya
			Madhukanya
Chickpea (Chana)	*Cicer arietinum*	High Yield	ICCV-10
			V-4
Yellow Pea (Pahelo Matar)	*Pisum sativum*	High yield/Disease resistance	Green Arrow
			Lincoln
Cow Pea (Bodhi)	*Vigna unguiculata*	High Yield	Arkel
Kidney Bean (Rajma)	*Phaseolus vulgaris*	High Yield	Red Kidney
			White Kidney
Butter Beans (Ghiu Simi)	*Phaseolus lunatus*	High Yield	Fordhook 242
Fawa Beans (Bacula)	*Vicia faba*	High Yield	Windsor

English/Local Name	Scientific Name	Characteristics of genotype	Genotype
Soyabeans (Bhatmas)	*Glycine max*		
Black Gram (Kalo Mas)	*Vigna mungo*	High Yield	T9
Green Gram (Hariyo Mas)	*Vigna radiata*	Early Maturity	NM-92
Red Lentil (Masoor)	*Lens culinaris*	High Yield Potential	Dhaulagiri
C. Horticulture (Fruit crops)			
Banana (Kera)	*Musa sapientum*	Large (size)	Rajapuri
		Medium(size)	Basrai
		Small(size)	Rasthali
Jackfruit (Katahar)	*Artocarpus heterophyllus*	Large (size)	Katahar
Peach (Aru)	*Prunus persica*	Prunuspersica	Elberta
Apricot (Khubani)	*Prunus armeniaca*	Taste(Sweet)	Goldcot
Persimmons (Lapsi)	*Diospyros kaki*	Astringent	Hachiya
Berries	*Rubus idaeus*	Taste	Raspberries(Aiselu)
	Rubus fruitcosus		Blackberries(KaloAiselu)
	Fragaria ananassa		Strawberries(Chino)
Pears (Naspati)	*Pyrus communis*	Popular Variety	Barlett
Plums (Aloo Bukhara)	*Prunus domestica*	Popular Variety	Methley
D. Horticulture (Vegetable crops)			
Potato (Aalu)	*Solanum tuberosum*	Popular Variety	KhumalRato
Cabbage (Banda)	*Brassica oleracea*	Popular Variety	Green Cabbage
Raddish (Mula)	*Raphanus sativus*	Popular Variety	Red Radish
		Popular Variety	White Radish
Carrot (Gajar)	*Daucus carota*	Hybrid	Nepa-Dhim
Turnip (Salgum)	*Brassica rapasubsp*	Size /Popular Variety	Purple Top Turnip
			White Globe Turnip
			Golden Ball Turnip
Tomato (Tamatar)	*Solanum lycopersicum*	Popular Variety	Cherry Tomato
			Surakhsya
Chilli (Khursani)	*Capsicum annuum*	Popular Variety	Chilli Pepper
	Capsicum chinense Habanero	Popular Variety	Habanero Chilli
Cucumber (Kakro)	*Cucumissativus*	Popular Variety	Dynasty
Pumpkin (Farsi)	*Cucurbita moschata*	Popular Variety	Chhanga
Bottle gourd (Lauka)	*Lagenaria siceraria*	Popular Variety	Pandhera
Okra (Bhindi)	*Abelmoschus esculentus*	Popular Variety	DharaneBhindi
Chayote (Iskush)	*Sechium edule*	Popular Variety	Iskus
Cauliflower (Kauli)	*Brassica oleracea var. botrytis*	Popular Variety	Phoolkobi

English/Local Name	Scientific Name	Characteristics of genotype	Genotype
Bamboo Shoot (Tama)	*Bambusa vulagaris*(sp.)	Popular Variety	Bans
Mushroom (Chiyau)	*Pleurotus ostreatus*	Popular Variety	Oyster Mushroom
Stinging Nettle Leaf (Sisno)	*Urticodiocia*	Wild Variety	
Fiddle Head Wild Fern (Ningro)	*Matteuccia struthiopteris*	Wild Variety	
E. Oil Seeds			
Black Mustard (KaloToori)	*Brassica nigra*	Popular Variety	Varuna
Sesame (Teel)	*Sesamum indicum*	Hybrid	-
Linseed/Flaxseed	*Linum usitatissimum*	Popular Variety	-
Soybean	*Glycine max*	Popular Variety	-
F. Fodder and Forage			
Berseem	*Trifolium alexandrinum*	Popular Variety	-
Malabar nut	*Justicia adhatoda*	Wild Variety	-
Mulberry	*Morus nigra*	Wild Variety	-
Stinging nettle leaf	*Urtica dioica*	Wild Variety	-
G. Materials used for Plant Protection			
Red Silk Cotton	*Bombax ceiba*	Popular Variety	-
Rhododendron	*Rhododendron arboretum*	Wild	-
Malabur nut	*Justicia adhatoda*	Wild	-
Neem	*Azadirachta indica*	Popular Variety	-
China Berry	*Melia azedarach*	Wild	-
Indian gooseberry	*Phyllanthus embica*	Wild	-
Holy Basil	*Ocimum tenuiflorum*	Wild	-
Gokshura	*Tribulus terrestris*	Wild	-
Sarpagandha	*Rauvolfia serpentina*	Wild	-
Livestock Dung	-	-	-
Livestock urine	-	-	-
H. Medicinals Herbs			
Ginger	*Zingiber officinale*	Popular Variety	-
Garlic	*Allium sativum*	Popular Variety	-
Satavari	*Asparagus racemosus*	Wild	-
Spikenard	*Nardostachys jatamansi*	Wild	-
Holy Basils	*Ocimum tenuiflorum*	Wild	-
Chirata	*Swertia chirayita*	Wild	-
I. Livestock and Poutry			
Cow (jersey)	*Bos taurus*	Popular	-
Buffalo	*Bubalus bubalis*	Popular	-

English/Local Name	Scientific Name	Characteristics of genotype	Genotype
Goat	*Capra aegagrushircus*	Popular	-
Pig	*Sus scrofa domesticus*	Popular	-
Ox	*Bos taurus*	Popular	-
Chicken	*Gallus domesticus*	Popular	-
Duck	*Anas platyrhynchos domesticus*	Popular	-
Pigeon	*Columba livia*	Popular	-
Geese	*Anser anser domesticus*	Popular	-
Quails	*Coturnix japonica*	Popular	-

and insect and pest repellents. Similarly, Newari communities have unique cultural and religious practices tied to the cropping cycle and food that correspond with the changing seasons. They celebrate various rituals and festivals aligned with agricultural production practices. Notably, they are famous for producing a popular local wine called Chyang, which is made from cereals such as rice, millet, and sorghum.

3.2.1 Medicine and Pesticide Preparation Using Plants

There are longstanding practices for preparing medicine and pesticides from wild plants, including: (a) The creation of liquid manure using Asuro and livestock excreta and urine to control pests and manage diseases (Tiwari et al. 2023), (b) The use of cow urine and Mugwort (*Artemisia vulgaris*) for pest management (Baruah et al. 2024), (c) The application of medicinal herbs such as Bozo (*Acorus calamus*) for sore throats, Holy Basil for coughs and fevers, and ginger for inflammation (Nair and Groot 2021), (d) Various wild (such as Tanki "*Bauhinia purpurea"*) and cultivated legumes are utilized to maintain soil fertility and provide fodder, and (e) The incorporation of wild edible herbs and fruits, including cardamom, chiraito, bamboo shoots, berries, chili, and turmeric, which are commonly used spices that enhance flavor in cooking and meet dietary needs (Thapa et al. 2018).

3.2.2 Bio-cultural heritage

Farmers primarily grow staple crops to ensure food security and nutrition for their families, making staple crop production a key priority for farming households (Andriessen et al. 2025). They actively preserve agrobiodiversity by cultivating traditional crop varieties with cultural and nutritional significance. These farmer-selected varieties, including rice, maize, millet, beans, vegetables, fruits, and livestock breeds, have evolved through local farmers' selection, adaptation, and improvement processes over generations. Approximately 80% of staple crops such as rice, maize, millet, and barley are sold in the market, while the remaining 20% is stored for household consumption or livestock feed. However, the growing emphasis on commercial farming and adopting high-yielding crop varieties has led farmers to explore alternative production systems, such as vegetable farming (Nguyen and Singh 2024). Most vegetables cultivated are modern varieties designed to meet market demand and secure higher prices. For instance, farmers in the Chabeli group incorporate various fodder and forage species to enhance dairy production.

Additionally, farmers grow neglected and underutilized crops, such as *Amaranthus spp*., naked barley, and sesame, to provide essential micronutrients (Akram et al. 2023). Staple crops like wheat, rice, maize, and millet are primarily grown as daily sources of carbohydrates, with only surplus production being sold. Vegetables and fruits, including root crops like potatoes and yams, and others like tomatoes, onions, chilies, cauliflower, and cabbages are cultivated for their vitamins, minerals, and dietary fiber (Górska-Warsewicz et al. 2021). Certain crops are cultivated due to their cultural and religious significance:

1. Rice (*Oryza sativa*): Rice is considered sacred and is used in various ceremonies, such as weddings, death rituals, and the traditional rice-feeding ceremony for infants (Malla 2018).

2. Wheat (*Triticum aestivum*): Wheat symbolizes the sun's power and is integral to rituals associated with the sun.

3. Holy Basil (*Ocimum sanctum*): A sacred plant in Hinduism, holy basil is used in religious ceremonies and is known for its medicinal properties, including reducing inflammation and aiding digestion.

4. Asparagus (*Asparagus racemosus*): This plant is used in Ayurvedic medicine to address digestion, respiration, and reproductive health issues.

5. Amla (*Emblica officinalis*): Amla is known for boosting immunity and improving digestion. It has both medicinal and religious significance.

6. Ginger (*Zingiber officinale*): Valued for its medicinal properties, ginger also carries spiritual significance in traditional Hindu practices.

Farmers are increasingly attracted to cultivating high-value horticultural crops such as kiwi, avocado, and strawberry as cash crops (Rai and Rai 2024a; Rai and Rai 2024b). These fruits are gaining popularity in Bhimphedi Municipality, where agricultural officers and local governments provide training and support in organic farming, organic manure preparation, and mushroom cultivation to enhance commercial viability. Such initiatives also aim to engage youth and reduce migration rates. Additionally, common spices like ginger, garlic, turmeric, and chilies are widely grown and processed by microenterprises for sale.

Farmers raise chickens, goats, and pigs for meat and eggs, while cows and buffaloes are raised for dairy products like milk, butter, and ghee. Although livestock production traditionally focuses on meeting household nutritional needs, it is becoming increasingly market-oriented (Yitayih et al. 2024). Farmers also use local plants for various farming functions, such as planting fodder along traditional canals to reduce seepage. Fodder and forage species are grown on terrace risers to stabilize the terraces and prevent soil erosion, reducing dependence on forest resources.

3.2.3 Crop Management Practices

Farmers in this region integrate traditional cultural practices into their planting, harvesting, and seed-sowing activities. These practices continually evolve as they incorporate scientific techniques to adapt to changing environmental and agricultural conditions (Ismail et al. 2025). Smallholder farmers play an active role in conserving agro-biodiversity through these varied approaches. Many farmers practice crop rotation to improve soil fertility, control pests and diseases, and prevent soil erosion (Akbar et al. 2024). The commonly used crop rotation patterns identified in the study include: (i) Maize-Paddy-Wheat-Finger Millet: This traditional rotation involves growing maize in the summer, paddy during the rainy season, and wheat and finger millet in the winter, (ii) Maize-Beans/Vegetables: Maize is grown in the summer, while beans are cultivated in the winter. This combination helps maintain soil fertility and reduces labor costs, (iii) Paddy-Wheat: This rotation is favored due to the high demand for rice and wheat. Paddy is grown during the rainy season, followed by wheat in the winter. In spring, cold-resistant paddy varieties such as Sukha-4, Chandanath-1, and Khumal-4 are planted, which require less water, and (iv) Root Crops Followed by Fruits: Farmers grow root crops like ginger and carrots alongside fruit crops. They believe this mixture enhances productivity, as root crops deplete nitrogen from the soil, while fruit and legume crops replenish it.

3.2.3.1 Integrated Systems: Mixed Cropping/Intercropping

This practice involves planting multiple crops to enhance biodiversity, reduce soil erosion, and improve soil fertility (Li et al. 2024). Examples include: (i) Maize and beans: These complementary crops grow well together, (ii) Cucumbers and pumpkins intercropped with maize or millet: Vegetables are grown alongside cereal crops to optimize space and time, benefiting household and commercial production.

3.2.3.2 Cover Cropping

Cover crops are cultivated not for harvest but to enhance soil health and decrease reliance on synthetic fertilizers (Priya and Ramesh Kumar 2025). Some effective combinations of cover crops include: (i) Alfalfa with Maize or Potatoes: This combination helps reduce weed growth, prevents soil erosion, and increases the organic matter in the soil, (ii) Legumes (Peas, Beans, Lentils): These crops improve soil fertility and provide a food source for farmers and livestock.

3.2.3.3 Traditional Seed Storage and Mulching

Farmers utilize techniques like Mór storage, which is particularly effective for storing potatoes. Additionally, leaves from local trees, such as maple and banana, are commonly used for mulching to conserve soil moisture and suppress weeds.

3.2.3.4 Biocontrol Agents and Biopesticides

Farmers protect their crops using traditional biocontrol methods and plant-based biopesticides made from local materials. These practices promote sustainable pest and disease management while minimizing the use of chemical inputs. By integrating traditional knowledge with modern techniques, farmers create sustainable ecosystems, maintain soil health, and protect biodiversity while adapting to contemporary agriculture's challenges.

3.2.3.5 Traditional method of nursery preparation

Farmers have reported that the traditional method of nursery preparation involves planting seedlings using wild tree leaves. This approach helps keep the nursery bed free of weeds. Additionally, these practices are cost-effective as they use locally available materials to create a healthy nursery environment. This method is particularly effective for preparing nurseries for climbing and creeping plants.

3.3 Knowledge transfer system

3.3.1 Generation to generational knowledge transfer (within the household)

Farmers play a crucial role in preserving various plant species on their land and nearby forests to meet the needs of their households and farming communities (Czembor et al. 2024). These needs include food, fodder, medicinal plants, and spices. Knowledge and practices related to plant conservation have been passed down through generations and are deeply rooted in local and traditional

wisdom. This intergenerational knowledge transfer occurs during daily farm work and interactions, ensuring that sociocultural beliefs and traditional practices are maintained while managing the farms.

3.3.2 Knowledge transfer (Neighbor)

Farmers can share knowledge with their neighbors through informal interactions (Wei et al. 2025). More structured opportunities arise during local events, fairs, and festivals. Additionally, the establishment of farmer groups, such as the Chabeli Farmers Group and the Mai Farmers Group, has further improved the exchange of information. These groups allow lead farmers to demonstrate best practices, fostering learning among their fellow farmers. Such gatherings also provide valuable platforms for sharing experiences related to modern farming techniques and introducing new crop varieties and livestock breeds. Events like seed fairs, farmer field schools, and integrated pest management (IPM) initiatives, organized by various supporting institutions collaborating with farmers' groups, offer opportunities to exchange seeds, plant cuttings, and other planting materials.

3.3.3 Group (Chabeli) Knowledge Network

The Chabeli Farmers Group actively promotes knowledge sharing among its members. For instance, the group demonstrates biofertilizer production techniques at designated demonstration sites supported by the NFGF. Farmers quickly recognized that biofertilizer production builds on their traditional knowledge while adapting it to modern practices, and they soon began replicating these techniques on their farms. With guidance, the group also adopted advanced composting methods, such as mixing *Trichoderma* with animal dung to produce vermicompost. This method has gained significant popularity among farmers in Bhimphedi Municipality for organic vegetable cultivation. Furthermore, the group has encouraged adopting climate-resilient practices, including rehabilitating traditional reservoir ponds, harvesting rainwater, and collecting wastewater. Wastewater from households and farms is stored in ponds to cultivate algae, an effective biofertilizer. Additionally, tree species are integrated into degraded or abandoned agricultural land, transforming into productive agroforestry systems. Farmers have highlighted the benefits of domesticating and sustainably managing wild tree species, which enhance dietary diversity and provide wood for various farm needs. Some tree species are also valued for improving livestock feed, medicinal uses, and plant protection measures, reflecting a holistic approach to sustainable farming. The Chabeli Farmers Group has established a demonstration garden to showcase agro-biodiversity organic farming practices. The garden features a mix of local and improved crop and vegetable varieties. Group members are encouraged to learn from this site and implement the practices on their farms.

3.3.4 Forest and Farm Producer Organizations (FFPOs) Knowledge Network

As part of the NFGF, the group benefits from a knowledge-sharing network that provides advanced insights into farm management and production, including (i) Technical expertise in organic farming, updated soil fertility management practices, establishing and managing a participatory guarantee system (PGS), and strategies for building resilient production systems, and (ii) Market-oriented farming knowledge is disseminated through farmer field schools, exchange visits, and targeted coaching sessions on specific agricultural topics.

Additionally, the group engages with specialized knowledge and technologies through various initiatives, such as (a) Participatory action planning to promote the adoption of resilient and proven techniques that combine traditional and modern practices, (b) Onsite pest and disease management training to enhance farmers' skills and capacities, (c) Access to diverse information sources, including publications, agri-bulletins, radio programs from the National Agricultural Research Council (NARC), and materials shared by local governments and civil society organizations, (d) Market access facilitation supported by programs like the Forest and Farm Facility (FFF), (e) Agroforestry improvements that upgrade traditional practices for sustainable land use, (f) Capacity development for PGS implementation to enhance production standards and certification, and (g) Adopt climate-smart water management practices emphasizing efficient water use and conservation techniques.

Table 3 Lists of Traditional Biopesticides used by Chabeli

S. N.	Name of Bio pesticides (Traditionally Used)	Common name of Bio pesticides	Applications
1	Neem Oil,Titepati Extract	Neem Oil	Neem is a natural pesticide extracted from the seeds of the neem tree. It is effective against many pests and is commonly used in Nepal to control aphids, thrips, whiteflies, and caterpillars.
2	Garlic-based Biopesticides	Lasunkojhol	Garlic is known for its antifungal and antibacterial properties, which make it an effective natural pesticide. Garlic extract is used in Nepal to control fungal diseases in crops such as potatoes and tomatoes.
3	Liquid Manure	Jholmal	Biological control liquid manure against insect pests such as aphids, whiteflies, and mealybugs. It effectively controls pests in various crops, including vegetables, fruits, and ornamental plants.

These initiatives strengthen the group's ability to integrate sustainable, innovative, and market-responsive farming methods while preserving and enhancing traditional agricultural practices.

3.4 Resource management system (Crop seeds for cultivation and Livestock raising)

3.4.1 Self-provisioning and multiplication

Farmers rely on traditional methods for managing seeds for personal use and selling at local markets. These practices include: (i) Seed collection and storage: Farmers gather seeds from locally adapted plants, including traditional and heirloom varieties. They carefully dry and store these seeds in optimal, dry conditions to ensure seed viability (Davidson et al. 2024), (ii) Labeling and organization: Each seed packet includes the plant name, variety, and relevant details about its characteristics or growing requirements. Farmers organize their seed collections systematically to allow for easy access and retrieval, (iii) Record keeping: Detailed logs are maintained, noting the collection date, location, and other plant observations. These records help track seed performance and identify potential issues, (iv) Marketing: Farmers sell their collected seeds at local markets while sourcing seeds from other producers, fostering a dynamic exchange of planting materials.

These practices help ensure seed quality, promote agrobiodiversity, and support local agricultural economies.

Grains are stored in a Bhakari, a traditional container made from bamboo and mud. Typically, Bhakaris have a single chamber, although some variants have two chambers. The double-chambered Bhakari is especially useful in regions prone to flooding, as it allows farmers to use the grains from the lower chamber first, reserving the contents of the upper chamber for the monsoon season. These containers can hold up to two tons of grains.

Another storage method involves using Ghaita, clay pots for holding beans and mustard seeds. These pots are filled with seeds and sealed with clay lids. To help retain moisture, a layer of straw may be placed inside the Ghaita. Additionally, the Chaaita technique uses bamboo to store smaller quantities of seed grain. This method involves covering the Chaaita with mud to protect it from pests and moths. Such practices are commonly observed among Janajati or Indigenous communities.

3.4.2 Neighbor barter or purchase

As the leading federation of Forest and farm producer organizations (FFPO), the NFGF organizes regular fairs and exchanges to facilitate seed sharing among farming communities. Its goal is to promote agrobiodiversity and enhance local seed stocks. Community members actively participate in seed fairs hosted by producers in the Bhimphedi municipality. These fairs showcase unique local seed varieties well-adapted to the region's soil, climate, and environmental conditions, making them invaluable for farmers and breeders. Such events also strengthen connections between farmers and market actors, supporting seed swaps and encouraging collaboration.

The Chabeli Farmers Group contributes by hosting a demonstration stand at nutrition fairs organized by schools and local governments. These fairs highlight the group's diverse produce and aim to inspire young people to engage in agriculture while emphasizing the importance of nutritional values.

The NFGF has also introduced mobile applications, including the Krishipath and Krishi Guru apps. These platforms provide farmers with a wealth of resources, such as information on crops and varieties, seed availability, market prices, production guidelines, crop insurance, and government schemes. The Chabeli Farmers Group utilizes these apps to connect with fellow farmers and agricultural experts, facilitating experience sharing and mutual support. These tools empower farmers with the knowledge and resources to identify high-quality seeds and improve their agricultural practices.

3.4.3 Formal system of a community seed bank

Smallholder farmers actively participate in on-farm conservation practices, focusing on preserving crop landrace seeds for both household use and market sales. These farmers rely heavily on traditional and local knowledge to carefully select and store viable seeds for the next planting cycle. This approach ensures farmers have easy and reliable access to seeds and planting materials for various crops. The apex producer organization supports and advocates for these practices, emphasizing the importance of farmers' rights and food sovereignty. Local seed preservation efforts typically concentrate on maintaining native varieties of key crops, including maize, millet, spices, and horticultural products, thus safeguarding their agricultural heritage and enhancing resilience.

3.5 Enterprise strategies and agrobiodiversity

The Chabeli Farmers Group, in partnership with the local government, operates an integrated pest management (IPM) learning center. This initiative has allowed the group to adopt more environmentally friendly agricultural practices, enhancing market opportunities. The center has also trained five additional farmer groups from neighboring communities and four community-led farmers from each group. The IPM center promotes organic and climate-resilient farming through improved soil and pest management techniques.

The Chabeli Farmers Group is dedicated to producing various products using organic methods, mainly focusing on local varieties

of legumes and vegetables. They prioritize the conservation of these traditional varieties and cultivate them using organic practices. Their strategy includes selling vegetables in commercial markets and targeting niche markets with diverse local products.

NFGF is implementing a Participatory Guarantee System (PGS) to improve its services and add value. This system enables farmer groups within the same area to agree on organic standards and ensure compliance through peer audits. These groups receive support from NFGF, the local government, and the FFF program to develop enterprise plans that establish a clear vision and actionable steps for their agribusiness ventures.

3.5.1 Cash crops

The primary commodity the farmer group sells is organically produced vegetables, although they also cultivate local varieties of cereals and legumes. The commercially grown crops include leafy greens, tomatoes, cereal grains, and legumes. However, the shift toward commercial organic vegetable production in Bhimphedi may lead to a reduction in on-farm agro-biodiversity as new and improved vegetable varieties such as black tomatoes and fruit species like strawberries, kiwis, and avocados replace areas previously dedicated to local crops. Despite this shift, farmers continue to rely on traditional knowledge and agro-biodiversity to maintain organic production, utilizing wild plant materials for pest management and soil fertility.

Farmers are encouraged to incorporate sustainable traditional practices, such as crop rotation, minimum tillage, using natural mulching sources, and integrating beneficial fodder, forage, and horticultural species. They are also urged to protect and manage beneficial plants from forests, maintaining integrated forest-farm systems. The rise in commercial production has raised environmental concerns, such as the increased use of polybags, plastics, and machinery, which contribute to climate pollution and a decline in traditional practices like intercropping, crop rotations, and zero tillage. The impact of these changes on soil health and microbial organisms remains unclear. Furthermore, introducing improved varieties may lead to replacing traditional local crops, and some improved varieties may be more susceptible to pests and diseases, potentially reducing the resilience of the agricultural system to climate change.

Despite these challenges, farmers, the local government, and the National Farmers' Group Federation (NFGF) are collaborating to align commercial production more closely with traditional methods. They also advocate for environmentally friendly practices that are resilient to climate change and focus on protecting natural plants, animals, and crop varieties. Additionally, adopting value-added agroforestry is encouraged, allowing marginalized farmers to grow marketable fodder plants, like Malabar nuts, alongside horticultural crops such as fruits and vegetables.

3.5.2 Changing pattern of cash crops cultivation

Farmers initially produced local varieties of cereals and legumes, taking advantage of their unique characteristics and flavors. These commodities were sold in local markets and nearby cities. There was also a focus on seasonal vegetable production in Bhimphedi municipality, including the Chabeli farmer groups. However, due to the growing demand for their vegetables, these farmers are now transitioning to commercial production, including seasonal and off-season crops.

3.5.3 The role of the Chabeli Farmers Group in Shaping sale and income generation

The Chabeli Farmers Group is increasingly becoming a recognized brand for safe and organic vegetable production while strengthening its relationships with various market stakeholders. To maintain consistent quality and scalability, the group has developed a crop calendar that guides the cultivation of their crops. While commercial production has expanded the variety of crops and animal species being raised, there are concerns that this dominance might threaten agro-biodiversity.

Additionally, the group has received training in mushroom production from local municipality officials and has adopted new methods for growing mushrooms, including systematically preparing dark houses. These mushrooms are now packaged and sold at local markets in Bhimphedi. Furthermore, the group is promoting the development of value-added enterprises, such as crop processing and packaging, which are expected to enhance the value of their products and provide additional sources of income.

3.5.4 Future Enterprise Plan

The Chabeli farmer group aims to transform their area into an organic production hub and enhance their capacity to produce local products, thereby preserving local agro-biodiversity. They have begun promoting their organic products in local and provincial markets and have developed a marketing strategy that emphasizes the benefits of organic farming, particularly its positive effects on biodiversity and climate change. The group encourages farmers to participate in local farmers' markets and connect with online platforms, establishing a direct link to consumers.

This approach has been identified as a crucial strategy for building resilience against the impacts of climate change. By implementing these plans, they can contribute to biodiversity conservation, mitigate the effects of climate change, and promote healthy, sustainable food production. Smallholder farmers are prepared to engage with microfinancing options to scale up the production and

distribution of commercial products. Additionally, they have partnered with the local government to initiate a Participatory Guarantee System (PGS) to recognize and brand their local and organic products.

A case study by Karki et al. (2020) on climate change adaptation among subsistence and smallholder farmers revealed both climatic and non-climatic factors that influence adaptation practices. Moreover, diversifying income sources is one of the primary adaptation strategies. Climatic factors beyond human control pose significant threats to the livelihoods of rural smallholders, whose income relies heavily on natural resources.

The food system will substantially change to address nutrition, food security, and sustainability challenges. These changes will enhance resilience, democracy, and sustainability to support the ever-growing population while mitigating the adverse environmental impacts on agriculture (Chaudhary et al. 2023). Furthermore, in the context of Nepal, research on farming systems plays a vital role in improving agricultural productivity, sustainability, and the livelihoods of smallholder farmers (Shrestha et al. 2024). In contrast to conventional agricultural practices, which contribute to resource degradation and biodiversity loss, sustainable agricultural methods, mainly organic bio-intensive farming and agroecological practices, have proven effective in conserving natural resources, improving rural livelihoods, diversifying farm income, and stabilizing the agricultural sector (Rai et al. 2024).

Conclusion

In summary, smallholder farmers are crucial in preserving and enhancing agrobiodiversity through their traditional, regionally adapted agricultural methods. Despite the challenges posed by modern technologies and hybrid varieties, these farmers maintain a rich knowledge repository that has been developed over generations. Their reliance on local practices promotes sustainable and environmentally friendly farming while revitalizing Nepal's unique agricultural and culinary heritage. The business-oriented actions of agricultural producer groups have facilitated the rise of organic farming and the establishment of local certification systems, making it easier for farmers to access profitable markets.

Furthermore, the shift towards ecological production has empowered farmers to transform traditional farming systems into resilient and sustainable practices that benefit plant and animal health. This approach reduces dependency on external inputs and better equips farmers to manage challenges posed by climate change and other external pressures. Farmers enhance their self-sufficiency by adopting agroecology-based value chains and actively contributing to local biodiversity conservation. Combining advanced technologies with indigenous knowledge continues to reshape local agricultural practices, ensuring long-term sustainability and prosperity of smallholder farming communities.

Recommendations

The Chabeli farmer group is actively engaged in their community and deserves recognition for their efforts. Documenting their traditional knowledge and practices is essential. Mechanisms like seed banks should be promoted to manage farmer landraces sustainably while safeguarding their rights to natural resources. Seed banks can help smallholder farmer groups, such as Chabeli, conserve and sustainably utilize seed resources, thereby supporting the maintenance of agrobiodiversity.

Additionally, the group should analyze market trends and develop a specific strategy for local niche products to ensure sustainable production and distribution of their goods. Gaining product recognition is vital, so they need to enhance their branding efforts and systematically label their products.

In partnership with farmers, the local government should prioritize the documentation and registration of agrobiodiversity and traditional knowledge, as local cultivars and native plants are gradually disappearing. The local government must identify and protect the agrobiodiversity that local communities have conserved and utilized. This collaboration is vital to ensure a resilient supply of seeds and promote agrobiodiversity conservation. Additionally, it is essential to recognize the value of agrobiodiversity. There is a need to develop mechanisms that compensate farmers for their efforts in using and conserving biodiversity, and these mechanisms should be established and formalized.

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Conflict of Interest

The authors declare that they have no prevailing conflict of interest in terms of financial, academic, commercial, political, or personal regarding the conduct of case studies and the content and authors' position in the manuscript.

Ethical Clearance

Not Applicable.

References

Abebe, T., & Mulu, D. (2017). The role of women in the management and utilization of home garden: The case of Dale

District in Southern Ethiopia. *Asian Journal of Plant Science and Research, 7*(4), 41-54.

Agnoletti, M., & Santoro, A. (2022). Agricultural heritage systems and agrobiodiversity. *Biodiversity and Conservation, 31*(10), 2231–2241. https://doi.org/10.1007/s10531-022-02460-3

Ahmed, M. S., Yasin, M., Zia, M. U., & Nadeem, M. A. (2023). Composted *Melia azedarach* L. (Chinaberry tree) sawdust mixtures regulate the sprouting and growth of single bud node seedlings of sugarcane. *Journal of Soil Science and Plant Nutrition, 23*, 2465-2475.

Akbar, A., Arshad, K., Shehnaz, S., Ahmad, W., Ullah, A., Sattar, A., & Ali, A. (2024). The role of sustainable farming practices in enhancing soil fertility and crop yield. *Dialogue Social Science Review (DSSR), 2*(5), 137–147.

Akram, I., Layla, A., & Ismail, T. (2023). Exploring neglected and underutilized plant foods to fight malnutrition and hunger in South Asia. In T. Ismail, S. Akhtar, C.E. Lazarte, (Eds), *Neglected plant foods of South Asia: Exploring and valorizing nature to feed hunger* (pp. 51–71). Springer, Cham. https://doi.org/10.1007/978-3-031-37077-9_3.

Altieri, M. A. (1999). Applying agroecology to enhance the productivity of peasant farming systems in Latin America. *Environment, Development and Sustainability, 1*, 197–217.

Altieri, M. A., Funes-Monzote, F. R., & Petersen, P. (2012). Agroecological efficient agricultural systems for smallholder farmers: Contributions to food sovereignty. *Agronomy for Sustainable Development, 32*(1), 1–13. https://doi.org/10.1007/s13593-011-0065-6

Andriessen, T., van der Horst, H., & Morrow, O. (2025). Forms of autonomy and dependence in food aid: Unravelling how they are related and perceived by recipients. *Agriculture and Human Values*, 1–15. https://doi.org/10.1007/s10460-025-10715-2

Baruah, K., Rana, M., & Sharma, L. (2024). Ethnobotanical insights into the medicinal, agricultural, and veterinary applications of *Artemisia vulgaris* in the Sikkim region. *Ecology Environment and Conservation, 30*(4), 1780-1784. DOI:10.53550/EEC.2024.v30i04.052.

Binge, B., Jalango, D., & Tesfaye, L. (2023). Post-harvest losses management through climate-smart innovations: A collaborative approach among value chain actors. Retrieved from https://www.cgiar.org/research/publication/post-harvest-losses-management-through-climate-smart-innovations-collaborative-approach-among-value-chain-actors/ accessed on 15th January 2025.

Bravo-Peña, F., & Yoder, L. (2024). Agrobiodiversity and smallholder resilience: A scoping review. *Journal of Environmental Management, 351*, 119882.

Bukchin-Peles, S., & Fishman, R. (2021). Should long-term climate change adaptation be focused on smallholders? *Environmental Research Letters, 16*(11), 114011.

Central Bureau of Statistics. (2011). *National living standard survey, 2010/2011*. (Commission, N. P. ed.). Kathmandu, Nepal: Government of Nepal.

Chaudhary, P., Upadhya, D., Dhakal, B., Dhakal, R., & Gauchan, D. (2020). Generation, gender, and knowledge gap in agrobiodiversity among smallholders in Nepal. *Journal of Agricultural Science, 12*(9), 62-73.

Chaudhary, S., Shrestha, A. K., Rai, S., Acharya, D. K., Subedi, S., & Rai, R. (2023). Agroecology integrates science, practice, movement, and future food systems. *Journal of Multidisciplinary Sciences, 5*(2), 39–60.

Córdoba Vargas, C. A., Hortúa Romero, S., & León-Sicard, T. (2020). Resilience to climate variability: The role of perceptions and traditional knowledge in the Colombian Andes. *Agroecology and Sustainable Food Systems, 44*(4), 419–445. https://doi.org/10.1080/21683565.2019.1649782

Czembor, E., Tratwal, A., Pukacki, J., Krystek, M., &Czembor, J. H. (2024). Managing fungal pathogens of field crops in sustainable agriculture and AgroVariety internet application as a case study. *Journal of Plant Protection Research*. Retrieved from https://www.plantprotection.pl/Managing-fungal-pathogens-of-field-crops-in-sustainable-agriculture-and-AgroVariety,192507,0,2.html.

Dagunga, G., Ayamga, M., Laube, W., Ansah, I. G. K., Kornher, L., & Kotu, B. H. (2023). Agroecology and resilience of smallholder food security: A systematic review. *Frontiers in Sustainable Food Systems, 7*, 1267630.

Davidson, T., DeSa, M., & Pérez, H. (2024). A beginner's guide to producing and saving open-pollinated seeds for North Florida farmers and gardeners: ENH1383/EP647, 12/2024. *EDIS, 2024*(6). DOI:10.32473/edis-ep647-2024.

Department of Forest Research and Survey (2017) CFUG database record available in MIS. Community Forestry Division, Department of Forest and Survey, Kathmandu.

Drucker, A. G., Pradel, W., Scott, C., Elmes, S., Arpazi Valero, K. G., & Zander, K. K. (2022). Are public good ecosystem services generated by on-farm conservation of crop diversity valued by the

general public? Preprint available at *Research Square*. https://bit.ly/3N5tqeu

Ellis, F. (1998). Household strategies and rural livelihood diversification. *The Journal of Development Studies*, *35*(1), 1–38. https://doi.org/10.1080/00220389808422553.

Fan, S., & Rue, C. (2020). The role of smallholder farms in a changing world. In *S. Fan & C. Rue (Eds.), The role of smallholder farms in food and nutrition security* (pp. 13–28). Springer. https://doi.org/10.1007/978-3-030-42148-9_2

Fanzo, J., Hunter, D., Borelli, T., & Mattei, M. (2013). *Diversifying food and diets: Using agricultural biodiversity to improve nutrition and health.* Routledge Taylor & Francis.

FAO. (2019). *FAO framework on rural extreme poverty: Towards reaching target 1.1 of the sustainable development goals.* Retrieved from http://www.fao.org/3/ca4811en/ca4811en.pdf

Food and Agriculture Organization of the United Nations (FAO). (1999). *Agricultural biodiversity, multifunctional character of agriculture and land conference.* Background Paper 1. Maastricht, Netherlands.

Food and Agriculture Organization of the United Nations (FAO). (2011). *Save and grow A—Policymaker's guide to the sustainable intensification of smallholder crop production.* Rome.

Forsythe, L. (2017). *The impact of staple crop value chain participation on the livelihoods of smallholder farmers in Nigeria and Malawi: Changes in poverty, gender relations, and food security*. Doctoral dissertation submitted to the University of Greenwich, London.

Garner, E., & de la O Campos, A. (2014). Identifying the "family farm": An informal discussion of the concepts and definitions. *ESA Working Paper 14-10.* Food and Agriculture Organization of the United Nations (FAO).

Gautam, A., Bishwakarma, P., &Paudel, I. P. (2020). *Scaling up climate-resilient agriculture for sustainable livelihood of smallholder farmers in Nepal.* Local Initiatives for Biodiversity, Research and Development (LI-BIRD), Nepal. Retrieved from https://libird.org/wp-content/uploads/2022/04/CRA_Baseline_Report_Final.pdf.

Gerits, F., Messely, L., Reubens, B., &Verheyen, K. (2021). A social–ecological framework and toolbox to help strengthen functional agrobiodiversity-supported ecosystem services at the landscape scale. *Ambio, 50*(2), 360–374. https://bit.ly/47Y0JYQ

Ghasemi, P., Behbahani, B. A., & Noshad, M. (2024). Assessment of the Antioxidant Effects of *Urtica dioica* Aqueous Extract on Free Radical Scavenging, Determination of Total Phenol and Flavonoid Content, and the Bioactive Compound Groups. *Journal of Food Science & Technology*, *21* (154), 163-173. DOI: 10.22034/FSCT.21.154.163.

Górska-Warsewicz, H., Rejman, K., Kaczorowska, J., & Laskowski, W. (2021). Vegetables, potatoes, and their products as sources of energy and nutrients to the average diet in Poland. *International Journal of Environmental Research and Public Health*, *18*(6), 3217.

Guarín, A., Rivera, M., Pinto-Correia, T., Guiomar, N., Šūmane, S., & Moreno-Pérez, O. M. (2020). A new typology of small farms in Europe. *Global Food Security*, *26*, 100389. https://doi.org/10.1016/j.gfs.2020.100389

Gugerli, F., Englisch, T., Niklfeld, H., Tribsch, A., Mirek, Z., et al. (2008). Relationships among levels of biodiversity and the relevance of intraspecific diversity in conservation – A project synopsis. *Perspectives in Plant Ecology, Evolution and Systematics, 10*(4), 259–281. https://doi.org/10.1016/j.ppees.2008.07.001

Gupta, S., Verma, D., Tufchi, N., Kamboj, A., Bachheti, A., Bachheti, R.K., & Husen, A. (2021). Food, fodder, and fuelwoods from forests. In A. Husen, R.K. Bachheti, & A. Bachheti (Eds) *Non-Timber Forest Products* (pp.383-425). Springer, Cham. https://doi.org/10.1007/978-3-030-73077-2_17.

Harris, J., van Zonneveld, M., Achigan-Dako, E. G., Bajwa, B., Brouwer, I. D., et al. (2022). Fruit and vegetable biodiversity for nutritionally diverse diets: Challenges, opportunities, and knowledge gaps. *Global Food Security, 33*, 100618. https://bit.ly/3uBVH6b

Immerzeel, D. J., Verweij, P. A., van der Hilst, F., & Faaij, A. P. C. (2013). Biodiversity impacts of bioenergy crop production: A state-of-the-art review. *Bioenergy*, *6*(3), 183–209. https://bit.ly/3N6287I

Ismail, K. A., Lino, F. A., Teggar, M., Laouer, A., Henriquez, J. R., & Rocha, T. T. (2025). Potential applications of phase change materials in the food industry: A review. *Journal of Energy Storage*, *115*, 115946.

Joshi, B. K., Gorkhali, N. A., Pradhan, N., Ghimire, K. H., Gotame, T. P., et al. (2020). Agrobiodiversity and its conservation in Nepal. *Journal of Nepal Agricultural Research Council, 6*, 14–33. https://doi.org/10.3126/jnarc.v6i0.28111

Karki, S., Burton, P., & Mackey, B. (2020). Climate change adaptation by subsistence and smallholder farmers: Insights from three agroecological regions of Nepal. *Cogent Social Sciences*, *6*(1), 1720555.

Kerr, R. B., Madsen, S., Stüber, M., Liebert, J., Enloe, S., et al. (2021). Can agroecology improve food security and nutrition? A review. *Global Food Security, 29*, 100540. https://bit.ly/46G7Sfr

Koirala, N. (2023). Policies and perspectives of food health and safety in Nepal: Is Nepal ready? *EFood, 4*(1), e56. https://doi.org/10.1002/efd2.56.

Leoni, V. (2024). Characterization of agro-biodiversity products for the enhancement of marginal and mountain environments.

Li, Z., Li, Y., Yao, B., & Carmona, C. (2024). Optimising management practices to secure grassland agroecosystems' sustainability. *Frontiers in Sustainable Food Systems, 8*, 1507692.

Lima, H. S. M., & Cunha, H. F. A. (2024). The role of women and the obstacles to biodiversity conservation in developed and developing countries. *Environment, Development and Sustainability*. https://doi.org/10.1007/s10668-024-05407-6

Liu, Q., Sun, X., Wu, W., Liu, Z., Fang, G., & Yang, P. (2022). Agroecosystem services: A review of concepts, indicators, assessment methods, and future research perspectives. *Ecological Indicators, 142*, 109218.

Lowder, S. K., Sanchez, M. V., &Bertini, R. (2021). Which farms feed the world and has farmland become more concentrated? *World Development, 142*, 105455. https://doi.org/10.1016/j.worlddev.2021.105455

Lowder, S., Skoet, J., & Singh, S. (2014). What do we really know about the number and distribution of farms and family farms in the world? *ESA Working Paper 14–02*. Food and Agriculture Organization of the United Nations (FAO), Agricultural Development Economics Division.

Macqueen, D. (2024). *Advancing agrobiodiversity: Why organizations of smallholders and Indigenous Peoples are vital.* IIED London. Available at https://www.iied.org/22251iied.

Malla, M. (2018). Newajewellery–New insights on the jewellery of Nepal. *SAARC Culture, 122.*

Matthies, A. E., Fayet, C. M., O'Connor, L. M., &Verburg, P. H. (2023). Mapping agrobiodiversity in Europe: Different indicators, different priority areas. *Ecological Indicators, 154*, 110744. https://doi.org/10.1016/j.ecolind.2023.110744

Nair, M. B., & Groot, M. J. (2021). *Medicinal plants for home herbal gardens, institutional gardens, and animal health.* Natural Livestock Farming India.

Nepal, A., & Koirala, K. (2023). *Integrating agrobiodiversity for livelihood options in Bhimphedi municipality, Nepal.* National Farmers Group Federation (NFGF) and IIED. Available at https://www.iied.org/21496g

Nepali, B., Jackson, T., & Johns, C. (2024). Exploring the potential of agriculture diversification as a pathway to an improved smallholder livelihood dietary system in Nepal. *Sustainability, 16*, 9384. https://doi.org/10.3390/su16219384

Nguyen, G., Singh, Z., eds. (2024). Recent advances in research and development for vegetable crops under protected cultivation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-5261-2

Nzima, W. M., Ip, R. H., Bhatti, M. A., Godfrey, S. S., Eik, L. O., Gondwe, S. R., & Divon, S. A. (2024). Diversity and heterogeneity of smallholder vegetable farming systems and their impact on food security and income in Malawi. *Frontiers in Sustainable Food Systems, 8*, 1387912.

Parris, K. (2001). *OECD agri-biodiversity indicators: Background paper*. Paper presented at the OECD Expert Meeting on Agri-biodiversity Indicators, 5–8 November 2001, Zurich. Available at https://bit.ly/3N60lzJ

Priya, K., & Ramesh Kumar, S. (2025). Cultivating under-utilized vegetables using organic resources: Unlocking nutritional security and sustainable agriculture. *International Journal of Vegetable Science, 1-23.* https://doi.org/10.1080/19315260.2025.2466493

Rahmawati, D., Hashidoko, Y., & Djajakirana, G. (2017). Concentration of some trace elements in two wild edible ferns, *Diplazium esculentum* and *Stenochlaena palustris*, inhabiting tropical peatlands under different environmental conditions. *Eurasian Journal of Forest Research, 20*(2), 43–56.

Rai S. & Rai R. (2024b). Advancements and practices in budding techniques for kiwifruit propagation. *Journal of Multidisciplinary Sciences*, 6(2), 11- 16.DOI:https://doi.org/10.33888/jms.2024.622

Rai, R., Shrestha, A., Rai, S., Chaudhary, S., Acharya, D. K., & Subedi, S. (2024). Conversion of farming systems into organic bio intensive farming systems and the transition to sustainability in agro-ecology: Pathways towards sustainable agriculture and food systems. *Journal of Multidisciplinary Sciences, 6*(2), 25–34.

Rai, S., & Rai, R. (2024a). Advancement of kiwifruit cultivation in Nepal: Top working techniques. *Journal of Multidisciplinary Sciences, 6*(1), 11–16.

Regmi, B., Paudyal, A., & Bordoni, P. (2009). *Climate change and agrobiodiversity in Nepal: Opportunities to include agrobiodiversity maintenance to support Nepal's National Adaptation Programme of Action (NAPA).* Retrieved from https://openknowledge.fao.org/items/cedc110f-1e6d-4538-a14c-f84c310e6873.

Remans, R., Wood, S. A., Saha, N., Anderman, T. L., & DeFries, R. S. (2014). Measuring nutritional diversity of national food supplies. *Global Food Security*, *3*(3–4), 174–182.

Rist, S., Bonanomi, E. B., Giger, M., Hett, C., Scharrer, B., Jacobi, J., & Lannen, A. (2020). Variety is the source of life: Agrobiodiversity benefits, challenges, and needs. *Zenodo*. https://doi.org/10.5281/ZENODO.3568133

Rusinamhodzi, L., Corbeels, M., & Nyamangara, J. (2012). Maize–grain legume intercropping as an option for ecological intensification that reduces climatic risk for smallholder farmers in central Mozambique. *Field Crops Research*, *136*, 12–22.

Shrestha, A. K., Chaudhary, S., Subedi, S., Rai, S., Acharya, D. K., & Rai, R. (2024). Farming systems research in Nepal: Concepts, design, and methodology for enhancing agricultural productivity and sustainability. *Journal of Multidisciplinary Sciences*, *6*(1), 17–25.

Subedi, R., Karki, M., & Panday, D. (2020). Food system and water–energy–biodiversity nexus in Nepal: A review. *Agronomy*, *10*(8), 1129. Available at https://doi.org/10.3390/agronomy10081129.

Swiderska, K., Argumedo, A., Wekesa, C., Ndalilo, L., Song, Y., Rastogi, A., & Ryan, P. (2022). Indigenous Peoples' food systems and biocultural heritage: Addressing Indigenous priorities using decolonial and interdisciplinary research approaches. *Sustainability*, *14*(18), 11311. https://doi.org/10.3390/su141811311

Thapa, S., Poudel, S., Aryal, K., Kandel, P., Uddin, K., & Karki, S. (2018). A multidimensional assessment of ecosystems and ecosystem services in Taplejung, Nepal. *ICIMOD Working Paper 2018/6. DOI:10.53055/ICIMOD.705*

Tiwari, S., Rai, S., Adhikari, J., & Bista, S. (2023). Organic farming: A reliable strategy for sustainable agriculture in Nepal. *Science Heritage Journal*, *7*(2), 91–103.

United Nations Development Program (UNDP). (2010). *Guidance note on recovery: Livelihood.* https://doi.org/10.1017/CBO9781107415324.004

Wang, J., Jiang, H., & He, Y. (2023). Determinants of smallholder farmers' income-generating activities in rubber monoculture dominated region based on sustainable livelihood framework. *Land*, *12*, 281. https://doi.org/10.3390/land12020281

Wei, B., Chen, H., Shao, L., Huo, X., & Xia, X. (2025). Roles of strong and weak social ties in collaborative network formation for building livelihood resilience of herders: A case study on the Qinghai-Tibetan Plateau. *Rangeland Ecology & Management*, *100*, 14–26.

Williams, D.E. (2017) Agricultural biodiversity and the Columbian Exchange. In: D. Hunter, L. Guarino, C. Spillane, & P.C. McKeown (Eds). *Routledge handbook of agricultural biodiversity* (pp. 192-212). Routledge, London.

Wilson, G. (2010). Multifunctional 'quality' and rural community resilience. *Transactions of the Institute of British Geographers*, *35*(3), 364–381.

Yitayih, M., Goromela, E. H., Gemiyo, D., Ouko, O., Phem, M., Tum, S., & Dessie, T. (2024). An inclusive scaling business model for transforming traditional small-scale poultry production to market-oriented production. *TPGS Experience.* Available at https://hdl.handle.net/10568/169006.

Zaccari, C., De Vivo, R., Pawera, L., Termote, C., Hunter, D., Borelli, T., & Gee, E. (2023). Lessons learned from the Second International Agrobiodiversity Congress: Adopting agricultural biodiversity as a catalyst for transformative global food systems. *Current Opinion in Environmental Science & Health*, *31*, 100411.

Zimmerer, K. S., & de Haan, S. (2020). Informal food chains and agrobiodiversity need strengthening—not weakening—to address food security amidst the COVID-19 crisis in South America. *Food Security*, *12*, 891–894.

Zimmerer, K. S., & Vaca, H. L. R. (2016). Fine-grain spatial patterning and dynamics of land use and agrobiodiversity amid global changes in the Bolivian Andes. *Regional Environmental Change*, *16*, 2199–2214. https://doi.org/10.1007/s10113-015-0897-8.

Zimmerer, K. S., Lambin, E. F., &Vanek, S. J. (2018). Smallholder telecoupling and potential sustainability. *Ecology and Society*, *23*(1), 30. . https://doi.org/10.5751/ES-09935-230130.

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Impact of Storage Duration and Container Materials on Hydroxy Methyl Furfural Levels in Indonesian Trigona Honey

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ABSTRACT

Hydroxymethyl Furfural (HMF) is a six-carbon heterocyclic organic compound containing aldehyde and alcohol functional groups. It is formed from reducing sugars when heated through the Maillard reaction. HMF is widely recognized as an indicator of honey quality, reflecting the time and type of storage container used. In this study, we analyzed HMF content to investigate the effects of different storage container types and durations on HMF levels in honey. The analysis was conducted using High-Performance Liquid Chromatography (HPLC) with the following parameters: a mobile phase of acetonitrile:water in a 10:90 ratio, a stationary phase of octadecylsilane (C18), a flow rate of 1.0 mL/min, and a UV detector set to 280 nm. The results showed an increase in HMF content during the storage process, with variations depending on the container type and the storage duration. The highest HMF level recorded was 47.7931 µg/g in honey stored in transparent glass bottles for 8 months. These findings indicate that both the container type and the storage duration significantly influence HMF accumulation in honey, making it an important parameter for evaluating honey quality.

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1 Introduction

In recent years, there has been increased public concern regarding health issues related to food storage materials. Among dietary choices, unprocessed options like honey, a natural sweetener with a long history, have gained popularity. Honey is preferred because it undergoes minimal processing, helping to preserve its natural properties and qualities (Obiedzińska et al. 2018). Composed primarily of fructose and glucose, honey also contains various proteins, minerals, enzymes, and other substances. Its composition can vary based on the flowers, geographical location, and the insects responsible for its production. Environmental factors that change with seasons, processing techniques, and storage conditions significantly influence honey's composition (Shapla et al. 2018). The main component of honey is sugar, which plays a crucial role in its crystallization. Importantly, crystallization does not detract from honey's quality. However, many consumers prefer honey with a relatively liquid texture and may perceive crystallized honey as inferior quality. This consumer preference influences beekeepers and manufacturers to adopt technological processes, such as carefully timed heating and storage for decrystallization. If not executed properly, these decrystallization attempts can lead to changes in honey's composition (Obiedzińska et al. 2018). One commonly used quality criterion for assessing honey's ripening time and location is the hydroxymethylfurfural (HMF) content. HMF is a byproduct of sugar decomposition, formed during heating and storage (Suhaela et al. 2016). Both temperature and storage duration significantly impact the increase in HMF levels. Quantitative analyses of HMF in honey using spectrophotometry indicate that honey stored at high temperatures tends to have higher HMF levels, influenced by the duration of heating and storage (Shapla et al. 2018). Furthermore, prolonged storage of honey can lead to elevated HMF concentrations. High levels of HMF in honey are associated with various chemical properties, such as water content, pH, free acid concentrations, reduced sugar levels, and enzymatic activity (Suhaela et al. 2016).

The Indonesian National Standards (SNI) regulate honey quality by establishing a maximum limit of 40 mg/kg for HMF content. This regulation aims to help ensure that honey retains its natural properties and is safe for consumption (Badan Standarisasi Nasional 2018). Many researchers are exploring the impact of HMF content on human health regarding honey quality, as this component is linked to honey's quality and chemcial composition. (Fallico et al. 2004). Additionally, previous studies suggest that HMF and its compounds may offer positive health effects, although the concentration of HMF can lead to various consequences, its effects depend on exposure levels. Alongside potential benefits, HMF and its derivatives can have detrimental effects on human health, including genotoxic, mutagenic, carcinogenic, organotoxic, enzym-inhibitory, and DNA-damaging effects. (Suri and Chhabra 2020).

The primary metabolic pathway for HMF involves its oxidation to form 5-hydroxymethyl-2-furanoic acid (HMFA), followed by glycine conjugation to create N-(5-hydroxymethyl-2-furoyl) glycine (HMFG), the main metabolite which is excreted through urine. Additionally, under in vivo conditions, HMF can be converted into 5-sulfoxymethylfurfural (SMF) through the sulfonation of its allylic hydroxyl group, facilitated by sulfotransferases (SULTs) and the sulfate donor, known as 3-phosphoadenosine-5-phosphosulfate (PAPS) (Farag et al. 2020). This highlights the need for further laboratory research to examine the HMF content of honey stored in various containers and over different time intervals and to explore the synthesis and metabolism of HMF concerning human health. One recommended method for measuring HMF levels, suggested by the International Honey Commission (IHC), is High-Performance Liquid Chromatography (HPLC) (Suri and Chhabra 2020). System suitability tests on HMF solutions confirm that the HPLC method is effective for analyzing HMF, offering the advantages of short analysis times and strong separation capabilities, which support its selection among various analytical methods (Dimyati and Marzuki 2023). The current study has evaluated the effect of various storage conditions on the quality of Hydroxy Methyl Furfural content in Indonesian Trigona honey.

2 Materials and Methods

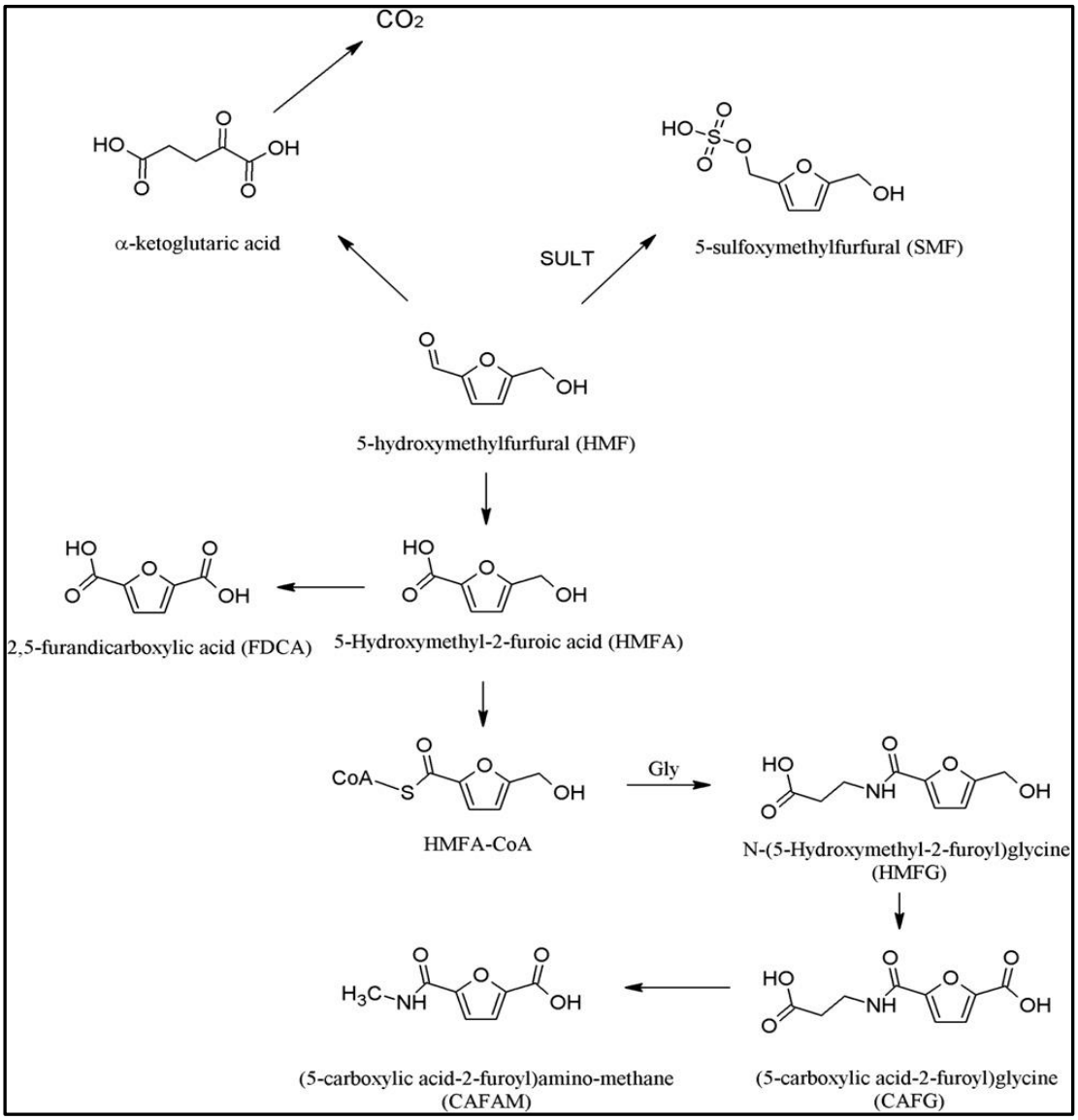
2.1 Protein Identification

Protein identification was conducted by adding biuret reagent to a test tube containing Trigona honey as the sample, chicken egg yolk as the positive control, and distilled water as the negative control. The color changes were then evaluated.

2.2 HMF Content Determination

2.2.1 Honey Sample Preparation

The water content of Trigona honey was measured monthly at room temperature throughout the storage period using a honey refractometer. At the same time, the concentration of hydroxymethylfurfural (HMF) was analyzed using High-Performance Liquid Chromatography (HPLC). For the HMF analysis, 5 grams of honey were dissolved in 25 mL of demineralized water, and then 0.5 mL each of Carrez I and II solutions were added to precipitate proteins. The solution was diluted with demineralized water to reach the required volume, filtered through a 0.45 µm nylon filter, and injected into the HPLC system. The chromatographic conditions were set to use a mobile phase consisting of demineralized water and acetonitrile in a 90:10 ratio, with a C18 column, a flow rate of 1.0 mL/min, and a UV detector set to 280 nm. This method allowed for precise and periodic monitoring of both the water content and HMF levels in Trigona honey, providing insights into the effects of storage duration and container material on honey quality.



2.2.2 Chromatographic Condition

Mobile phase: Deionized water:acetonitrile (90:10); Column: C18 (4.6 mm x 150 mm, particle size 5 µm); flow rate: 1.0 ml/min; injection volume: 20 µl; Detector: UV 280 nm.

2.2.3 Method Validation

Method validation was conducted by evaluating different parameters: linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). Analyte standards were spiked into seven sample replications to assess accuracy until a final concentration of 10 ppm was achieved. These samples were then injected into an HPLC system. The accuracy was determined by calculating the percentage recovery (%recovery). Precision testing involved seven honey samples, each spiked with standard analytes at a concentration of 10 ppm. A volume of 20 µL from each sample was analyzed using HPLC. The peak area obtained from the chromatogram was used to calculate the standard deviation (SD) and the relative standard deviation (RSD). The linearity test was performed by preparing a standard series of six concentrations ranging from 5 to 50 ppm at a wavelength of 280 nm. Linearity was assessed by measuring the slope, intercept values, and correlation coefficient. Determination of detection limits was performed using statistical methods based on a linear regression line derived from the calibration curve. The measurement value corresponds to the intercept (b) in the linear regression equation $y = a + bx$, while the blank standard deviation equates to the residual standard deviation (Sy/x).

3 Results and Discussion

3.1 Protein Identification

The Carrez solutions I and II are used to precipitate proteins found in honey analyzing hydroxymethylfurfural (HMF) content. Proteins are complex organic compounds with high molecular weights, so the Carrez solutions are essential for extraction. This facilitates HMF analysis using High-Performance Liquid Chromatography (HPLC). We conducted the Biuret test to determine the presence of proteins in Trigona honey samples. These findings confirmed the need for Carrez solution in the HMF analysis (Wahdania et al. 2022). We performed tests by adding Biuret reagent was added to three test tubes containing chicken egg yolk (positive control), Trigona honey (sample), and distilled water (negative control). The results of the Biuret test indicated that the Trigona honey sample contained proteins, as the color shifted from yellow to dark yellow or yellowish-purple. While the color change observed did not align precisely with the Biuret test guidelines, where a purple or blue color indicates a positive protein result (Jain et al., 2021), the change in the honey samples still occurred, suggesting the presence of a small percentage of protein. The positive protein result in the Trigona honey samples allows us to use the Carrez solution to analyze HMF content. This is important for precipitating proteins to ensure the analysis yields accurate results for HMF compounds, free from interference by impurities such as proteins (Kurtagić 2021). The Carrez solutions are necessary due to the high molecular weight of proteins, enhancing the effectiveness of HMF analysis using HPLC.

3.2 Suitability System Testing

To achieve reliable results, instrument parameters must be adjusted when analyzing with HPLC instruments. Key factors that can be modified include the flow rate, mobile phase composition, and column type. The chosen system must be appropriate and tailored to optimum conditions to ensure that the results are accurate and valid (Kumar et al. 2023).

The retention time refers to the duration it takes for compounds in a sample to reach the detector. Ideally, the desired retention time (Rt) should not be too short, as this may hinder effective separation, nor should it be too long, as that could impact processing efficiency. For the compound HMF, a retention time of approximately 4 minutes is considered adequate, indicating that the detection time for this compound is efficient. This finding aligns with previous research (Lamerkabel 2011). The results demonstrate that both the Rt and Area Under the Curve (AUC) data for evaluating the suitability of the HMF system using HPLC meet the necessary criteria, with a %RSD value of ≤2.0%.

3.3 Method Validation

3.3.1 Accuracy and Precision

The accuracy test was conducted using an analytical standard (Hydroxymethyl Furfural) spiked method on seven samples, with a reference material concentration spiked at 10 ppm. The results showed a recovery percentage of 106.7% for HMF in honey samples. This recovery value meets and aligns with the established requirement within the 90-107% range. This alignment instills confidence in the accuracy of our method. Since the accuracy test

Table 1 Biuret Test for Protein Identification

Sample	Colour	Results
Trigona honey solution	Yellow-dish purple	Positive
Aquadest as a negative control	No colour	Negative
Egg yolk as positive control	Purple	Positive

Tabel 2 Results of Suitability System

Replication	Rt	AUC
1	4.313	1065.397
2	4.295	1061.774
3	4.268	1065.407
4	4.275	1061.014
5	4.335	1069.757
Average	4.297	1064.669
SD	0.0275	3.4893
%RSD	0.6403	0.3227

Table 3 Results of Accuracy and Precision Test

No.	Replication	Experimental concentration (ppm)	AUC (mAU)	% Recovery
1.	Sample 1	11.15	1065.40	106.50
2.	Sample 2	11.12	1061.77	106.20
3.	Sample 3	11.15	1065.41	106.50
4.	Sample 4	11.11	1061.01	106.10
5.	Sample 5	11.20	1069.76	107.00
6.	Sample 6	11.28	1077.88	107.80
7.	Sample 7	11.16	1066.27	106.60
		Σ		78.17
		Average		11.1671
		Standard Deviation		0.0576
		%Relative Standard Deviation		0.5162
		% Recovery		106.6714
		CV Horwitz		1.3909
		2/3 CV Horwitz		0.9319

yielded recoveries that align with these standards, the tested HPLC method can be considered accurate and capable of providing reliable analysis results.

The precision tests, a key aspect of our evaluation, were performed on the seven honey samples, each representing a separate replication spiked with standard analytes at a concentration of 10 ppm. A volume of 20 µL from each sample was analyzed using HPLC. After measuring the peak area on the chromatogram, we determined the standard deviation (SD) and relative standard deviation (RSD). The % RSD values, less than 2/3 of the CV Horwitz, demonstrate high precision in the testing system, equipment, and analysis method. This HPLC method's precision ensures the validity of the measurement results, which meets the necessary precision criteria.

3.3.2 Linearity, Limit of Detection (LOD), and Limit of Quantification (LOQ)

A linearity graph was constructed using external standardization with six HMF standard solutions at 5 to 50 ppm concentrations. The linear equation obtained from HMF testing on honey is expressed as y = 45.054x + 1.1434, with a correlation coefficient 1. This high correlation coefficient value, which meets the specified requirement of being greater than 0.9997, is a strong indicator of

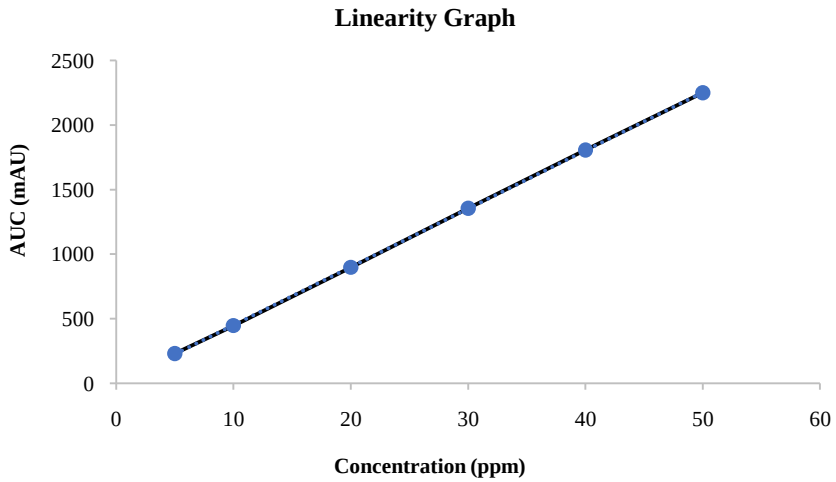


Figure 2 Linearity Graph for HMF standardization

the reliability of the results. This indicates that the method for determining HMF in honey using HPLC exhibits good linearity, as it fulfills the criteria for an acceptable correlation coefficient (r). The limit of detection (LOD) value obtained for HMF was 0.0590, and the limit of quantification (LOQ) was 0.1968. These values demonstrate that the HPLC method for measuring HMF in honey samples is sensitive and can accurately detect HMF concentration levels.

3.4 Evaluation of Water Content and HMF Concentration in Several Period Storage

The water content in Trigona honey samples was determined by measuring the refractive index of honey at room temperature, a crucial step conducted periodically once a month during storage. The results of the analysis showing the relationship between water content and HMF (hydroxy methyl furfural) compound content in Trigona honey, based on variations in container material and storage time, are presented in Table 4. These findings are significant as they shed light on the impact of storage conditions on the quality of Trigona honey. On the day of harvest, the water content of Trigona honey stored in a white plastic container was recorded at 26.0%, with an HMF content of 6.8936 µg/g.

Based on Table 4, it can be observed that there is a decrease in water content accompanied by an increase in HMF (hydroxyl methyl furfural) levels with each increase in honey storage time (Suhaela et al. 2016). However, the decrease in water content across different container types did not significantly correlate with the rise in HMF content. The analysis of container materials and storage duration revealed that the highest water content was found in dark glass containers after 8 months of storage, with a moisture level of 25.5%, while the lowest water content was recorded in dark plastic containers at 24.0%. Conversely, the highest HMF levels were detected in Trigona honey samples stored in transparent glass containers for the same duration, with a concentration of 47.7931 µg/g. In comparison, the lowest HMF levels were found in samples stored in white plastic containers, which exhibited a concentration of 6.8936 µg/g.

The changes in water content during the Trigona honey samples' storage period were insignificant; therefore, no meaningful conclusion can be drawn regarding the relationship between water content and HMF levels. However, it is important to note that the Maillard reaction, a complex chemical process that occurs during prolonged honey storage, alongside the degradation of reducing sugars, coincides with the decrease in water content (Capuano and Fogliano 2011; Hustiany 2016; Shapla et al. 2018; Farag et al. 2020). The increased HMF levels observed were insignificant because no heat treatment was applied to the Trigona honey samples. This underscores the role of the Maillard reaction in HMF formation, a fascinating aspect of honey production that professionals in the field will find intriguing.

3.5 Evaluation of HMF Content in Storage Variation

Hydroxymethyl Furfural (HMF) levels in honey were determined using validated High-Performance Liquid Chromatography (HPLC). The study analyzed HMF compound levels in Trigona honey samples that were treated and stored in four containers for eight months.

The hydroxy methyl furfural (HMF) levels in honey samples were measured in triplicate on the first day of storage, yielding 6.8936,

Table 4 Water Content and HMF Concentration in Several Months of Storage

Storage Container	Monthly storage	Water content (%b/b)	HMF concentration (µg/g)
Transparent Plastic	6	25.75	9.4841
Dark Plastic		24.5	11.5254
Transparent glass		25.5	11.0133
Dark glass		26.0	12.0204
Transparent Plastic	7	25.5	11.3612
Dark Plastic		24.0	14.7059
Transparent glass		25.5	11.9216
Dark glass		26.0	13.9661
Transparent Plastic	8	25.0	31.9106
Dark Plastic		24.0	41.3719
Transparent glass		25.0	47.7931
Dark glass		25.5	42.8390

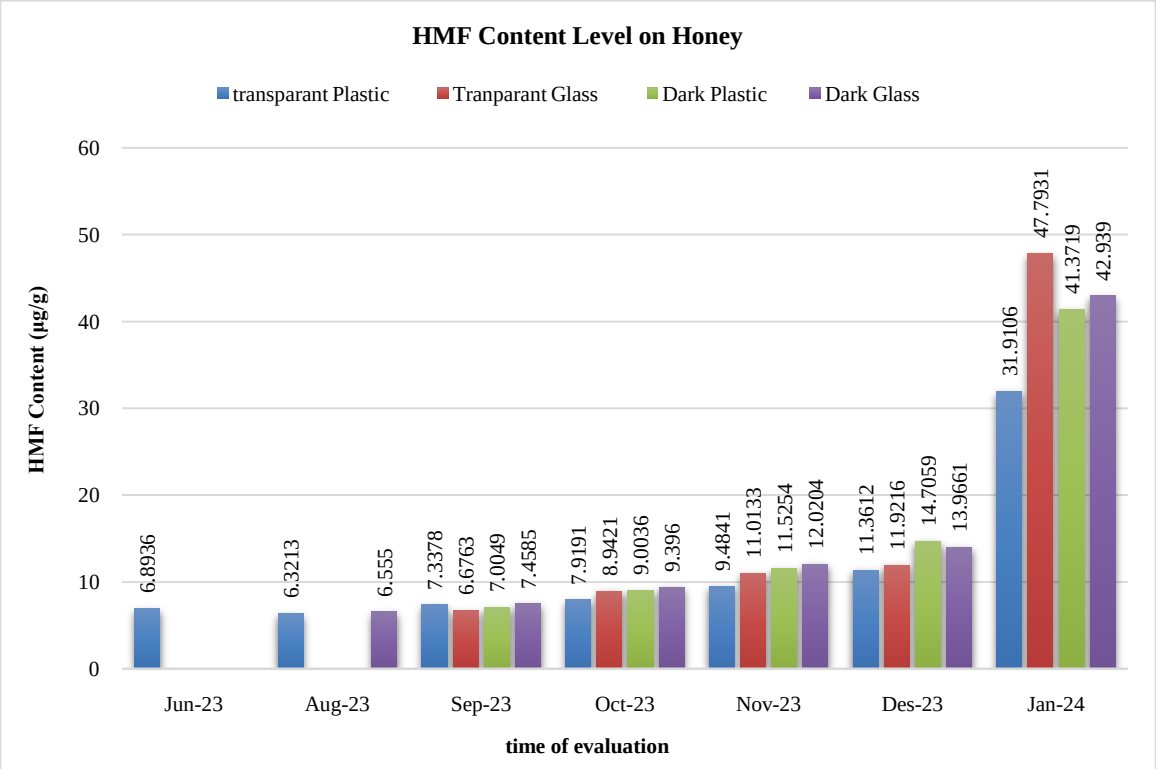


Figure 3 Graph of HMF Content Determination in Honey

6.2884, and 6.5638 ppm, respectively. These measurements establish an initial baseline for evaluating HMF content. Monitoring these levels over time will help assess the impact of storage conditions on honey quality. The resulting HMF levels indicate that fresh honey samples contain only small amounts of HMF. In an experiment to determine HMF levels in honey stored in containers made of four different materials (dark glass, dark plastic, white plastic, and white glass), the highest HMF result was observed in the transparent glass bottle, with levels reaching 47.7931 µg/g after 8 months. This finding is consistent with research indicating that HMF is sensitive to light (Kukurová et al. 2006; Capuano and Fogliano 2011; Suhaela et al. 2016; Ariandi and Khaerati 2017; Obiedzińska et al. 2018; Shapla et al. 2018; Suri and Chhabra 2020; Kurtagić 2021; Petrarca et al. 2020; Pujiarti et al. 2021; Dimyati and Marzuki 2023). The formation of HMF in the transparent glass bottle is more pronounced than in the other three bottles because the transparent nature of the glass allows more light to enter.

Conversely, the transparent plastic bottle had the lowest HMF levels, measuring 31.9106 µg/g. The results indicate lower HMF levels in plastic containers than in glass ones, likely due to the superior insulating properties of plastic. Studies suggest that the insulating ability of a material largely depends on its thickness and thermal conductivity. For optimal insulation, a greater thickness and lower thermal conductivity are necessary. Plastic insulates 5 to 10 times better than glass, with a significantly lower thermal conductivity (Alhamidi et al. 2022). This allows heat to transfer more rapidly in glass containers than in plastic.

Regarding storage duration, the highest HMF levels after 8 months were found as follows: 31.9106 µg/g in transparent plastic bottles, 41.3719 µg/g in dark glass bottles, 47.7931 µg/g in transparent glass bottles, and 42.839 µg/g in dark glass bottles. HMF levels during this 8-month storage period were significantly higher than those measured in fresh honey immediately after harvest, which were 6.8936, 6.2884, and 6.5638 µg/g when stored in a transparent plastic bottle. This increase can be attributed to the absence of significant driving factors influencing large-scale HMF formation during the initial storage period. However, the HMF levels in fresh honey can result from various environmental factors, including the conditions of the bees, the environment where the honey is produced, and the bees' diet during honey production. This complexity in HMF formation underscores the intricate nature of honey production. Additionally, the increase in HMF levels in honey generally coincides with a reduction in water content, although this decrease is relatively stable and not substantial. Water content plays a role in HMF formation through the Maillard reaction, which involves the degradation of reducing sugars.

According to the study's findings, the HMF levels in honey stored in dark plastic bottles, white glass bottles, and dark glass bottles

after 8 months exceeded the maximum allowable limit set by the Indonesian National Standard (SNI) of 40 mg/kg (or µg/g) for HMF content in honey (Badan Standarisasi Nasional 2018; Hidayatullah et al. 2022). This indicates that both the type of storage container and the duration of storage significantly influence the HMF content in honey. This information underscores the responsibility of both producers and consumers to consider storage times for optimal honey quality and safety and to take proactive measures to ensure these standards are met.

Conclusion

Storage time and the type of container significantly impact the levels of Hydroxymethyl Furfural (HMF) in Trigona honey. After eight months, the highest concentration of HMF, measuring 47.7931 µg/g, was found in transparent glass bottles, likely due to exposure to light. In contrast, lower HMF levels were observed in plastic containers, which were better insulated against heat transfer. As storage duration increased, HMF levels rose and were positively correlated with a reduction in water content, which was linked to the Maillard reaction. Notably, the choice of container played a crucial role in reducing this increase in HMF. These findings underscore the potential impact of our research on your work, as they highlight the importance of selecting appropriate storage materials and durations. This is crucial to maintaining honey quality in compliance with regulatory standards, ensuring safety, and satisfying consumers.

Acknowledgment

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Conflict of Interest

The author declares that there are no conflicts of interest.

Ethical Clearance

No animal model was used in this study; therefore, ethical clearance is not required.

References

Alhamidi, A., Anis, A., Al-Zahrani, S. M., Bashir, Z., & Alrashed, M. M. (2022). Conductive Plastics from Al Platelets in a PBT-PET Polyester Blend Having Co-Continuous Morphology. *Polymers*, *14*(6). https://doi.org/10.3390/polym14061092.

Ariandi, & Khaerati (2017). Uji Aktivitas Enzim Diastase, Hidroksmetilfurfural (HMF), Kadar Gula pereduksi, dan Kadar Air Pada Madu Hutan Battan. In *Prosiding Seminar Hasil Penelitian (SNP2M), 1-4*.

Badan Standarisasi Nasional. (2018). *SNI 8664:2018 Madu*. Retrived from www.bsn.go.id.

Capuano, E., & Fogliano, V. (2011). Acrylamide and 5-hydroxymethylfurfural (HMF): A Review on Metabolism, Toxicity, Occurrence In food and Mitigation Strategies. *Food Science and Technology Journal*, *44*(4), 793–810. https://doi.org/10.1016/j.lwt.2010.11.002.

Dimyati, E., & Marzuki, H. (2023). Penetapan Kadar 5-Hydroxymethyl Furfural Dalam Menggunakan Kromatografi Cair Kinerja Tinggi. *Jurnal Multidisipliner Bharasumba*, *2*(1), 672–684.

Fallico, B., Zappalà, M., Arena, E., & Verzera, A. (2004). Effects of conditioning on HMF content in unifloral honeys. *Food Chemistry*, *85*(2), 305–313. https://doi.org/10.1016/j.foodchem.2003.07.010.

Farag, M. R., Alagawany, M., Bin-Jumah, M., Othman, S. I., Khafaga, A. F., et al. (2020). The Toxicological Aspects of the Heat-Borne Toxicant 5-Hydroxymethylfurfural in animals: A Review. *Molecules*, 25 (8). https://doi.org/10.3390/molecules25081941.

Hidayatullah, M., Handoko, C., & Maring, A. (2022). SNI Madu dan Manfaat Madu Untuk Kesehatan. *Standarisasi LHK*, *1*(6), 23–26.

Hustiany, R. (2016). *Reaksi Maillard Pembentuk Citarasa dan Warna Pada Produk Pangan*. PhD Thesis, Lambung Mangkurat University, Indonesia. https://repo-dosen.ulm.ac.id//handle/123456789/17832.

Jain, B. P., Goswami, S. K., & Pandey, S. (2021). *Protein*. In B. P. Jain, S. K. Goswami, & S. Pandey (Eds.), Protocols in Biochemistry and Clinical Biochemistry (pp. 31-48). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-822007-8.00008-8.

Kukurová, K., Karovičová, J., Greif, G., Kohajdová, Z., & Lehkoživová, J. (2006). Determination of 5-Hydroxymethylfurfural After Winkler and By The HPLC Method For Authentication of Honey. *Chemical Papers*, *60*(3), 186–191. https://doi.org/10.2478/s11696-006-0034-8.

Kumar, G. T. J., Andrews, B. S. A., & Abbaraju, V. D. N. K. (2023). The Significance of System Suitability in High-Performance Liquid Chromatography (HPLC) Analysis: Ensuring Accurate and Reliable Results. *International Journal for Research in Applied Science and Engineering Technology*, *11*(10), 29–35. https://doi.org/10.22214/ijraset.2023.56435.

Kurtagić, H. (2021). Determination of Hydroxymethylfurfural Content (HMF) in Fresh Bee Honey Produced in Bosnia and Herzegovina (B&H) by HPLC DAD Method. *International*

Journal of Environmental Sciences & Natural Resources, *26*(5), 556199. https://doi.org/10.19080/ijesnr.2021.26.556199.

Lamerkabel, J. (2011). Mengenal Jenis-Jenis Lebah Madu, Produk-Produk Dan Cara Budidayanya. *Jurnal Ilmu Pengetahuan Dan Teknologi* , *9*(1), 70-78.

Obiedzińska, A., Biller, E., Obiedziński, M. W., & Tyszka, M. (2018). Determination of Hydroxymethylfurfural Content In Natural Honeys In Poland. *Polish Journal of Applied Sciences*, *4*, 60–64. https://doi.org/10.34668/PJAS.2018.4.2.04.

Petrarca, M. H., Meinhart, A. D., & Godoy, H. T. (2020). Dilute-and-Shoot Liquid Chromatography Approach for Simple and High-throughput Analysis of 5-Hydroxymethylfurfural in Fruit-based Baby Foods. *Food Analytical Methods*, *13*(4), 942–951. https://doi.org/10.1007/s12161-020-01713-7.

Pujiarti, R., Amin, A., Ngadianto, A., Septiana, R. M., Purba, B. A. V., & Permadi, D. B. (2021). Quality of Three Forest Honey Types from Baduy Tribe, Lebak District, Banten Province. *Jurnal Ilmu Kehutanan*, *15*(2), 123–136. https://doi.org/10.22146/jik.v15i2.1529.

Shapla, U. M., Solayman, M., Alam, N., Khalil, M. I., & Gan, S. H. (2018). 5-Hydroxymethylfurfural (HMF) Levels In Honey and Other Food Products: Effects On Bees and Human Health. *Chemistry Central Journal*, *12*, 35. https://doi.org/10.1186/s13065-018-0408-3.

Suhaela, Noor, A., & Ahmad, A. (2016). Effect of Heating and Storage Time Levels 5-(Hydroxy Methyl) furan-2-Karbaldehida (HMF) In Honey Origin Mallawa. *International Journal*, *17*(2), 24–31.

Suri, P.S., & Chhabra, P. (2020). A Review Presence of 5-Hydroxymethylfurfural (HMF) in Food Products: Positive and Negative Impacts on Human Health. *International Journal of Forensic Sciences*, *5*(2), 1–10. https://doi.org/10.23880/ijfsc-16000194.

Wahdania, Porodjia, F., & Nurkamiden, S. (2022). Protein Test With Chemical Solution in Identifying Nutrient Content in Food Material. *Journal of Health, Technology and Science*, *2*(4), 41–49. https://doi.org/10.47918/jhts.v2i4.252.

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Molecular Interactions of Mycobacterial Transporter for Novel Antimicrobial Strategies

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Efflux Transporter

Protein-Ligand Interactions

MATE Transporter

Drug repurposing

ABSTRACT

Efflux mechanisms for extruding antimicrobials, mediated by multidrug transporters, are key contributors to multidrug resistance in mycobacteria. The current study focused on molecular interaction analysis of *Mycobacterium tuberculosis* multidrug transporter implicated in multidrug and antimicrobial resistance. We screened a library of efflux transporter inhibitors against the protein structure to identify a lead compound that can potentially inhibit the transporter significantly. The efflux transporter sequence was modeled based on crystallized templates using protein structure prediction and molecular docking. The analysis deduced molecular interactions and critical binding residues that can be targeted as novel biotherapeutics strategies against multidrug transporters of mycobacteria. This study paves the way for targeting multidrug and antimicrobial resistance in the mycobacteria, offering hope for developing effective treatments.

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1 Introduction

In 2023, the World Health Organization (WHO) reported approximately 1.25 million deaths from tuberculosis (TB), making it the leading infectious disease killer, surpassing COVID-19 (Goletti et al. 2025; WHO 2024). The increase in TB cases, along with the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB), highlights the urgent need for innovative therapeutic strategies, especially in high-burden countries such as India, China, Indonesia, Philippines, and Pakistan (Monedero-Recuero et al. 2021; Wilczek et al. 2023).

Mycobacterium tuberculosis (Mtb) employs various mechanisms to evade and tolerate antimicrobial agents (Andre et al. 2017; Srivastav et al. 2014). Its waxy, impermeable cell wall limits drug penetration (Yang et al. 2023), and the upregulation of efflux transporters actively expels drugs from the cell (Campolattano et al. 2023). Genetic mutations, such as those in the rpoB gene, confer resistance to rifampicin (Traoré et al. 2023; Andre et al. 2017), while dormancy decreases antibiotic susceptibility (Shan Chang and Guan 2021; Day et al. 2024). Within the host, Mtb evades immune defenses by inhibiting the fusion of phagosomes and lysosomes (Maphasa et al. 2021) and impairing cytokine signaling and granuloma formation (Peddireddy et al. 2017). Efflux transporters significantly contribute to multidrug tolerance by reducing effective intracellular drug concentrations (Tyagi et al. 2022; Ghajavand et al. 2019). MDR strains resist first-line drugs like isoniazid and rifampicin, while XDR strains also resist second-line agents such as fluoroquinolones and aminoglycosides ("WHO Results Report 2020-2021," n.d.; Seung et al. 2015). Additionally, various uncharacterized efflux transporters in Mtb may be associated with antimicrobial resistance (Huang et al. 2022; Zgurskaya 2021). Therefore, targeting these transporters presents a promising therapeutic strategy against mycobacterial infections (Klukovits and Krajcsi 2015; Rodrigues et al. 2020).

Efflux transporters are classified into major protein families based on their distinct structural and functional characteristics, as illustrated in Figure 1. The Mtb genome encodes at least 65 putative drug efflux pumps from various families, significantly contributing to antimicrobial resistance (Mishra and Daniels 2013; Black et al. 2014). Among these, four families, i.e., ABC, MFS, SMR, and MATE, have demonstrated efflux activities in Mtb, actively removing first- and second-line TB drugs, thereby diminishing their effectiveness (Long et al. 2024). Mycobacterial efflux transporters abundance and broad substrate specificity contribute to high intrinsic drug tolerance (Poulton and Rock 2022; Sharma et al. 2023).

MATE transporters are known for their poly specificity, which allows them to extrude various drugs and contributes to the antimicrobial resistance observed in mycobacterial species, including *M. tuberculosis* (Mishra and Daniels 2013). MATE is classified as a multidrug transporter. The Rv2836c protein, identified as a potential member of the MATE family of efflux transporters, may play a role in mycobacterial drug resistance. Although its structure has not been determined experimentally, it is predicted to have MATE transporters typical 12-transmembrane domain characteristic (Roberts 2022). Stress factors, including exposure to antimicrobials, may induce the expression of this multidrug transporter. However, the regulatory mechanisms governing this expression are not yet fully understood. Targeting

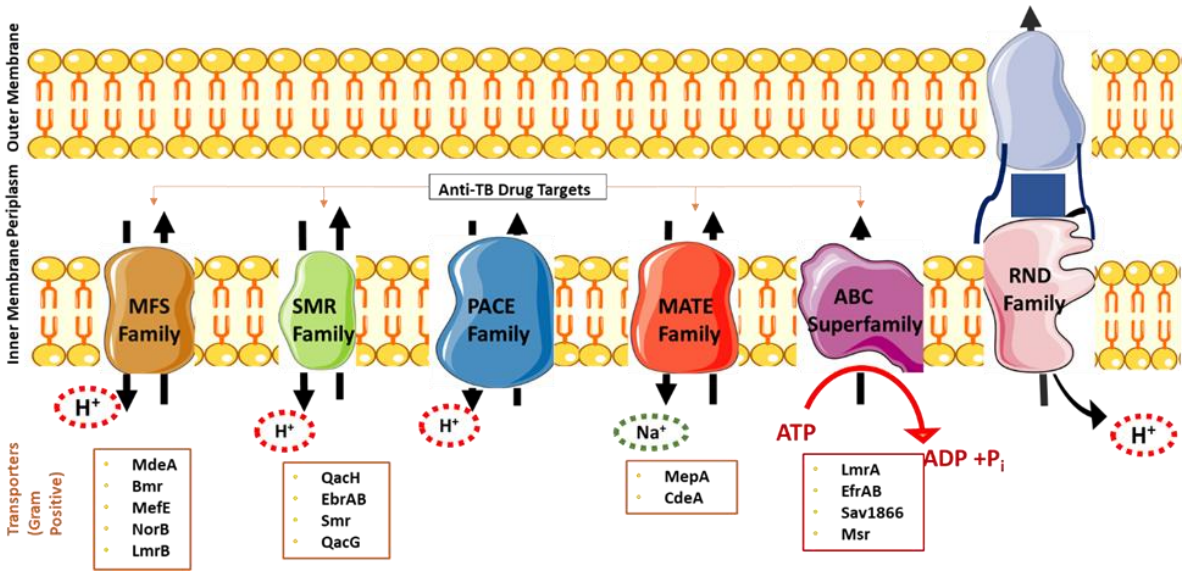

Figure 1 Illustration showing the different classes of efflux transporters: proteins that expel harmful substances and antibiotics from bacterial cells. This image highlights major efflux systems identified in Gram-positive organisms, which are significant in antimicrobial resistance (As per Athar et al. 2023; Henderson et al. 2021; Hassan et al. 2018)).

Rv2836c could potentially enhance the efficacy of antimicrobials by modifying intracellular drug concentrations. Therefore, the current study aims to investigate the molecular interactions between potential drug candidates and the Mtb MATE transporter using an *in-silico* approach.

2 Materials &Methods

2.1 Homology Modeling and Validation of Protein

After completing the literature review, it was found that the tuberculosis Multidrug and Toxic Compound Extruder (MATE) crystal structure is not available in the PDB database. Consequently, homology modeling was performed using Modeller and the MODBASE tool (https://modbase.compbio.ucsf.edu/modweb/) based on the existing structure of the MATE family multidrug resistance transporter Aq_128 (PDB ID: 6FV6). The modeled structure was then minimized using the SPDBV software tool version 4.1.0. Next, structure alignment was conducted in Biovia Discovery Studio Visualiser client version 2021 to ensure no significant deviations from the reference structure. The modeled Protein was validated using the SAVES server (https://saves.mbi.ucla.edu/) and the Ramachandran plot. Finally, the binding cavity of the modeled Protein was analyzed using a depth server (https://cospi.iiserpune.ac.in/depth).

2.2 Ligand Data Set Preparation

A selection of compounds was developed to target proton channels and multidrug transporters, emphasizing inhibiting efflux pumps. However, only a limited number of existing efflux pump inhibitors were identified, and none have been tested against the MATE transporter in *M. tuberculosis* (Mtb). Consequently, computational screening was conducted to evaluate the efficacy of these inhibitors on the catalytic site of the MATE protein. The SDF files for these compounds were retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/). Legend files were generated using the Open Babel software (O'Boyle et al. 2011) and a Raccoon Python script (Forli et al. 2016).

2.3 Molecular Docking and Screening

The receptor protein was processed using AutoDock tools for grid generation. The residues from the DEPTH server were a guiding reference for this grid generation. An exhaustiveness setting of 9 was applied. The binding pose exhibiting the most favorable negative binding energy (BE) value identified the most promising results. A Python script was utilized to calculate the optimal docking score of the MATE transporter protein with the transporter inhibitor to screen potential drug molecules. This analysis and screening aimed to establish a dataset of potential drug candidates.

2.4 Drug-Likeness and ADMET Prediction

Web tools with effective internal methods, such as pkCSM (https://biosig.lab.uq.edu.au/pkcsm/) and BOILED-Egg from SWISS ADME (http://www.swissadme.ch/), have been utilized to create robust predictive models for the physicochemical properties, pharmacokinetics, and drug-like characteristics of top-screened compounds. The SMILES representation of the lead molecule was obtained from the PubChem database, while these servers provided the values for Absorption, Distribution, Metabolism, and Excretion.

2.5 Molecular Dynamics

Molecular dynamics simulations were performed on the Google Colab server using NAMD GPU 2.0 (Phillips et al. 2020; Gopi et al. 2023). The docked pose with the highest affinity, indicated by the lowest docking score, was selected for simulation. Topology and parameter files for the Protein and ligand were generated using CHARMM GUI and VMD 1.9.3 (Lee et al. 2016). The complexes were solvated with the TIP3P water model and neutralized with sodium and chloride ions. The protein-drug complex was minimized for 10000 steps, followed by NVT equilibration and NPT equilibration for 1 ns each while maintaining a pressure of 1.02 atm and a temperature of 310 K. A final production run of 100 ns was conducted for both systems using the CHARMM36 force fields, and VMD 1.9.3 was used to analyze the trajectories (Humphrey et al. 1996).

2.6 MM/PBSA

The Poisson-Boltzmann Surface Area (PBSA) algorithm was employed to analyze the protein-drug complex's binding free energy (ΔG_bind). We utilized 1,500 snapshots from the trajectories over a 100 ns simulation, conducting the calculations with the CaFE plugin in VMD 1.9.3 (Humphrey et al. 1996). The internal dielectric constant for MM/PB was set to 1.0, while the external dielectric constant was set to 80.0. Poisson-Boltzmann calculations used the Adaptive Poisson-Boltzmann Solver (APBS) to define the boundary conditions and charges. The solvent-accessible surface area was computed with a surface tension of 0.00542 and a surface offset of 0.92 (Table 2).

3 Results and Discussion

3.1 Homology Modeling of MATE transporter protein and ligand preparation

After analyzing various transporters, we found that the MATE protein is conserved across different mycobacterial species. We conducted modeling of the MATE transporter protein based on the existing structure of the MATE family transporter Aq_128 (PDB ID: 6FV6) (Zhao et al. 2021). We performed a sequence alignment

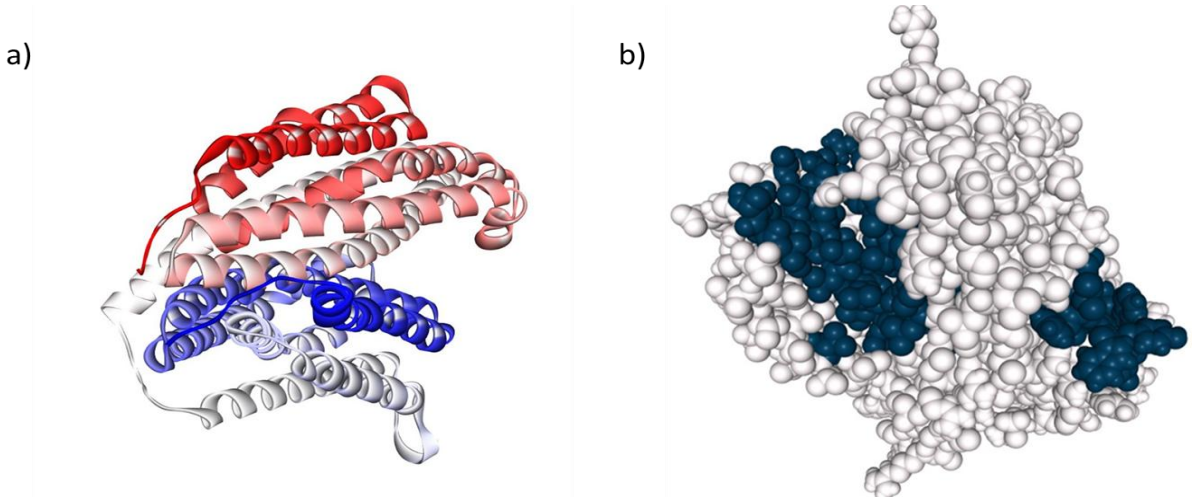

Figure 2 a) Modeled Tb-Multidrug transporter, Modbase image colored in N to C terminus, minimized structure, b) CPK space fill image of modeled transporter protein from DEPTH server showing the binding site pockets on the submitted receptor surface.

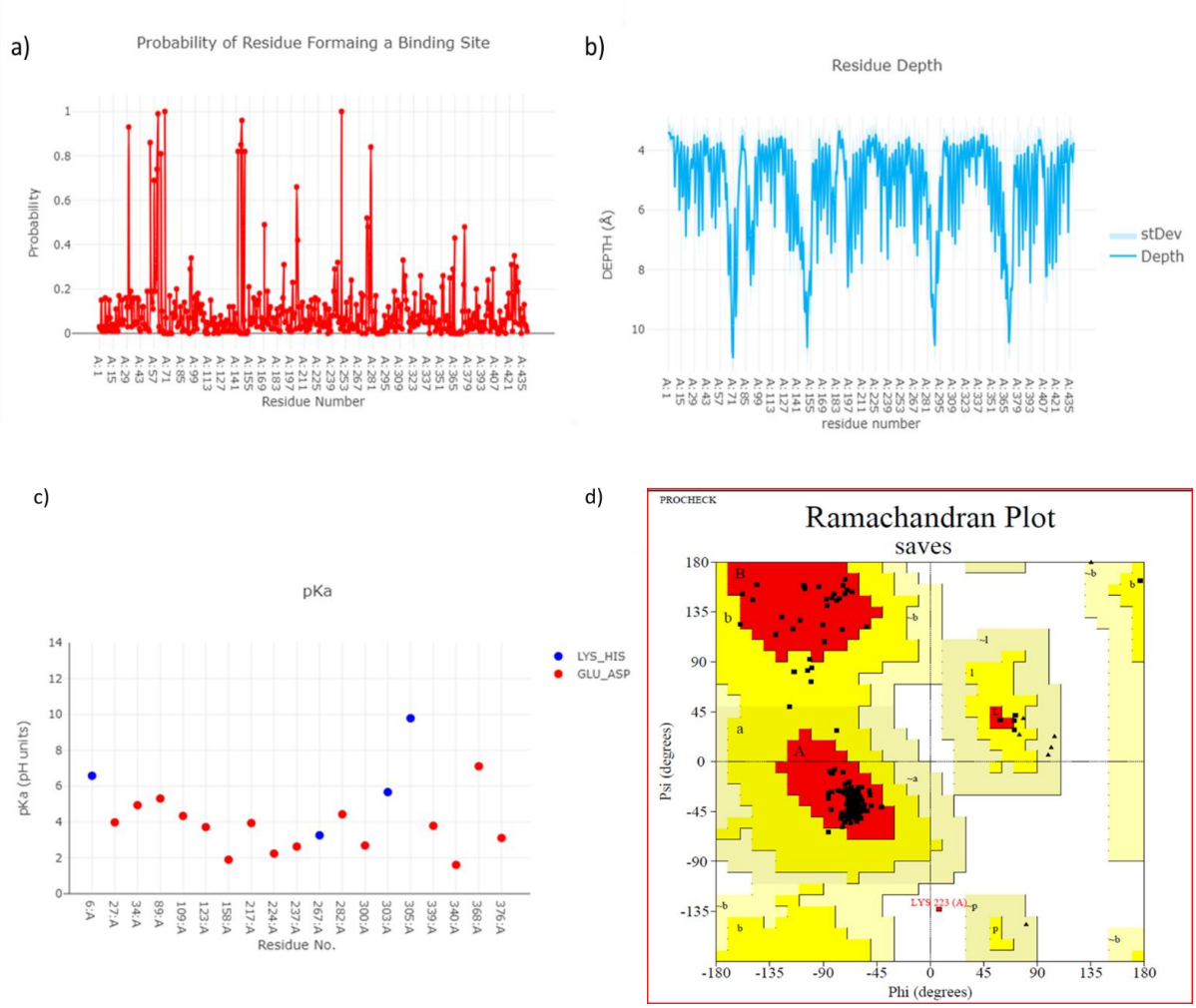

Figure 3 a) probability of residue forming binding site in modelled protein b) Residue depth of modelled protein c) predicted pKa of amino acid residues of modelled protein d) Ramachandran Plot Analysis of Protein Structure Model (MATE transporter protein)

between the template and the target protein, Rv2836c, and identified conserved regions that strongly support using 6FV6 as a template for homology modeling. The conservation of these sequences suggests that the resulting model will enhance our understanding of the structural features of the mycobacterial MATE transporter. This alignment is a foundation for further computational analyses and structural predictions, potentially leading to significant advancements in our understanding of MATE transporter function and developing novel antimicrobial strategies targeting this Protein in mycobacterial species. We prepared the proteins using homology modeling, taking 6FV6 as the template. The three-dimensional structure of the resulting receptor is shown in Figure 2, which displays the MODELLER-modeled MATE transporter protein and an image of the modeled Protein from the DEPTH server.

The protein structure was validated using a Ramachandran plot. According to the plot statistics, 95.4% of the residues are located in favored regions, indicating that the model is of good quality and has accurate protein geometry and conformation, as shown in Figure 3. The presence of only a few outliers in disallowed regions suggests that the overall structural quality is satisfactory. Additionally, the protein structure underwent minimization using SPDBV software to eliminate clashes. The minimized Protein was then aligned with the original structure to check for significant distortions. Table 1 lists the ligands selected for the study and their corresponding PubChem IDs. These ligands were converted into PDB and PDBQT formats using Open Babel and a Raccoon Python script. This conversion was necessary to prepare the ligands for successful docking experiments.

3.2 Molecular Docking and Screening

The grid was generated using ADT software based on the residues provided by the DEPTH server. The modeled Protein was then docked against known inhibitors of a similar class of proteins, with the exhaustiveness parameter set to 9. Table 1 lists compounds that have demonstrated proven activity against related receptors. In total, 33 compounds were docked, and 28 showed binding affinity toward the transporter protein. Some compounds exhibited structural distortion during docking; however, these results were not considered for further analysis.

Our analysis of the docked compounds identified five potential leads: Hoechst (-11.1 kcal/mol), Zosuquidar (-10.4 kcal/mol), 5'-Methoxyhydnocarpin (5'MHC) (-9.6 kcal/mol), Amitriptylinoxide (-8.7 kcal/mol), and Ethidium Bromide (-8.4 kcal/mol).

Table 1 Activity and Binding Affinity of Docked Compounds

S. N.	Compound name	Activity	Binding affinity (kcal/mol)
1	Zosuquidar (LY335979)	3rd generation modulators of P-gp inhibit ATP hydrolysis activity	-10.4
2	Hoechst [CID-1464]	Indicate the efflux pump activity in bacteria	-11.1
3	5'-Methoxyhydnocarpin (5'MHC) [CID-5281879]	Inhibits the NorA in *S. aureus*	-9.6
4	Amitriptylinoxide [CID-20313]	Antibacterial activity	-8.7
5	Amitriptyline [CID-2160]	Inhibit AcrB-mediated efflux by interfering with substrate binding	-7.2
6	Verapamil [CID-2520]	EPI, inhibits *M.tuberculosis* rifampicin efflux	-5.9
7	Carbonyl Cyanide m-Chlorophenylhydrazone [CID-2603]	Disrupts ATP Synthesis, Interfering with the proton gradient	-7.5
8	Ciprofloxacin [CID-2764]	NorA inhibitor	-6.2
9	4',6-Diamidino-2-phenylindole [CID-2954]	Binds to adenine–thymine-rich regions in DNA	-7.2
10	Ethidium bromide [CID-3624]	Substrate for efflux pumps	-8.4
11	Paroxetine [CID-43815]	inhibits both NorA and MepA	-6.2
12	Reserpine [CID-5770]	Inhibitor of both mammalian and gram-positive bacterial efflux, Inhibit Bmr efflux pump in *Bacillus subtilis*, NorA pump in *S. aureus*	-6.7
13	Sertraline [CID-68617]	Inhibit general bacterial efflux pumps	-8.2
14	Citalopram	Potentiate the activity of fluoroquinolones	-7.3
15	Venlafaxine [CID-5656]	Potential efflux pump inhibitor in bacteria	-5.6
16	Escitalopram [CID-146570]	Intrinsic antimicrobial activity against Gram-positive bacteria	-7.5

S. N.	Compound name	Activity	Binding affinity (kcal/mol)
17	Nortriptyline [CID-4543]	Potential efflux pump inhibition	-8.3
18	Trimipramine	Inhibition potencies	-6.3
19	Norfloxacin [CID-4539]	Antimicrobial activity	-7.1
20	Kanamycin [CID-6032]	Inhibiting protein synthesis in bacteria	-7.1
21	Ampicillin [CID-6249]	Beta-lactam antibiotic that inhibits cell wall synthesis	-7.8
22	Acriflavine	Multidrug pump inhibitor	-1.2
23	Rhodamine 6G [CID13807]	Potential efflux pump inhibitor	-6
24	Pyronin Y [CID-7068]	Target cell structures like RNA, DNA, and organelles	-7.8
25	Benzalkonium chloride [CID-8754]	Antibacterial activity	-4.9
26	Triton X-100 [CID-5590]	Inhibits the AcrB transporter	-5.7
27	Crystal violet [CID-3468]	Potential biofilm inhibition	-7.1
28	Berberine [CID-2353]	Inhibits MdfA	-6.2

Figure 4 shows the 3D docking poses of the lead compounds. Among these options, Hoechst, a blue fluorescent dye commonly used for DNA staining, was not selected for further study. Instead, we have chosen Zosuquidar as a candidate due to its significant binding affinity of -10.4 kcal/mol to our modeled protein structure. The docking pose is illustrated in Figure 5. The arrangement of amino acid residues surrounding the ligand indicates a favorable binding pocket or cavity. Key residues such as ILE A.204, GLN A.207, and HIS A.177 likely participate in potential hydrogen bonding interactions, which can enhance the binding affinity and specificity. Additionally, residues like LEU A.49, MET A.173, and TRP A.63 may play a role in hydrophobic interactions with the non-polar regions of the ligand, further stabilizing the binding complex. Overall, the docking results suggest that the ligand can effectively bind to the Protein's binding pocket, forming specific interactions with critical amino acid residues through both hydrogen bonding and hydrophobic interactions. Based on the docking score of Zosuquidar, the protein-ligand complex was subjected to a 100 ns molecular dynamics simulation to evaluate the ligand's stability within the receptor cavity.

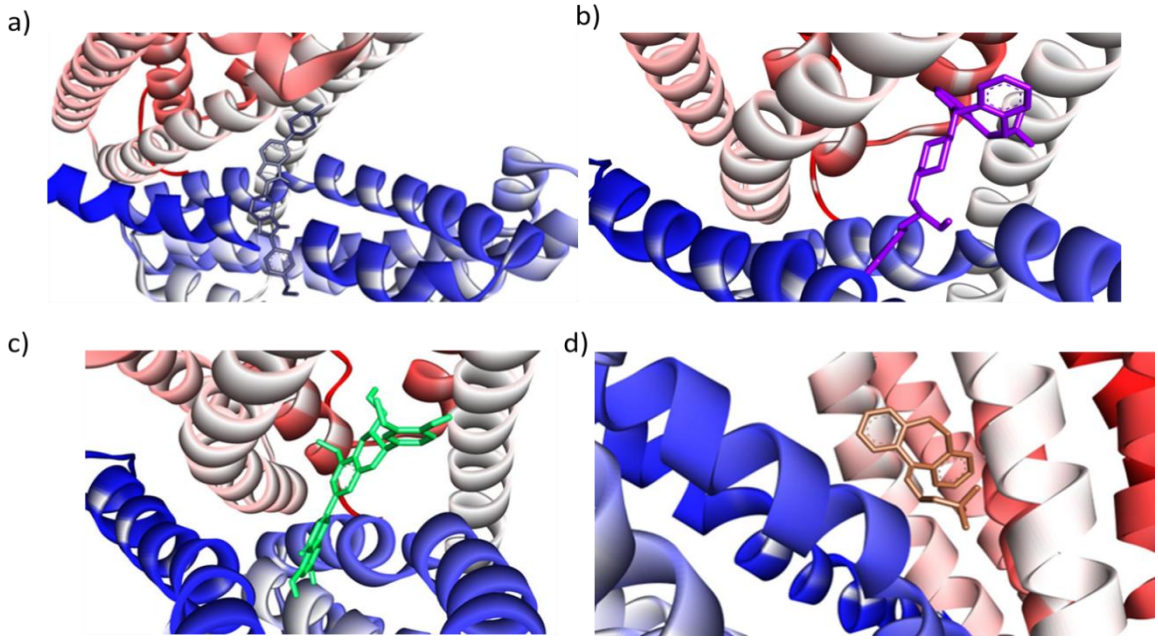

Figure 4 3D docking poses of lead compounds with modelled mycobacterial MATE transporter protein a) Hoechst (-11.1) b) Zosuquidar (-10.4) c) 5' Methoxyhydnocarpin (-9.6) d) Amitriptylinoxide (-8.7)

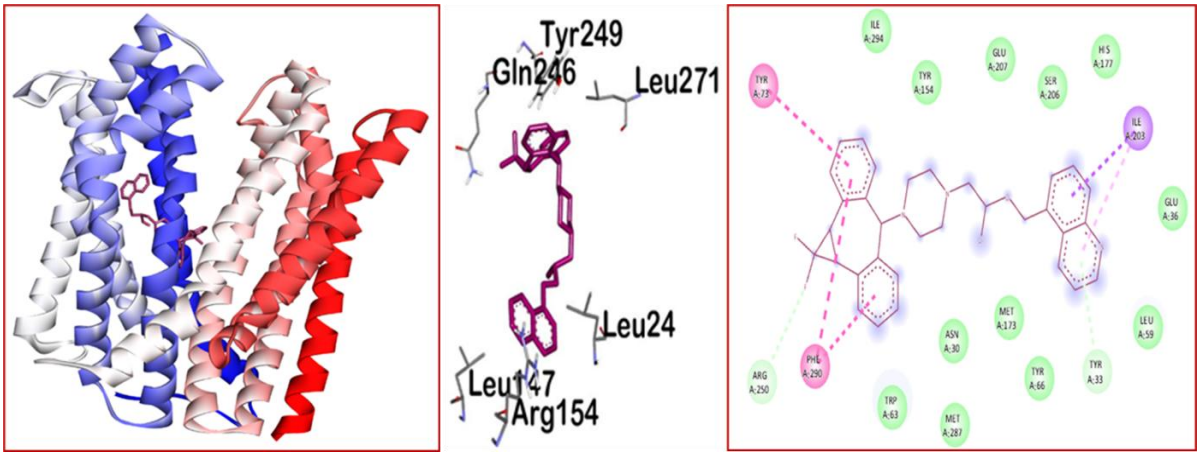

Figure 5 Representation of 3D and 2D binding pose of Zosuquidar bound to modelled MATE transporter protein indicating key binding interactions

3.3 ADMET Analysis of Zosuquidar

The top-ranking compound, Zosuquidar, was evaluated for its ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties using the pKCSM server. The predicted ADMET parameters for Zosuquidar show favorable profiles. Its molecular weight is 636.998 g/mol, which is desirable for drug-like compounds, suggesting good membrane permeability and bioavailability potential. The calculated LogP value of 6.5721 indicates relatively high lipophilicity, a factor that can enhance membrane permeability and absorption. Zosuquidar contains six rotatable bonds, contributing to its conformational flexibility and may affect its interactions with the target protein. The compound has five hydrogen bond acceptors and one hydrogen bond donor, indicating the potential for forming favorable interactions within the binding site. The topological polar surface area (TPSA) of 262.662 Å² is relatively high, suggesting greater polarity than typical drug-like compounds. This property can influence solubility, membrane permeability, and the ability to cross the blood-brain barrier. These molecular characteristics provide valuable insights into the compound's drug-like nature and potential pharmacokinetic behavior, aiding in evaluating and optimizing lead candidates in our drug discovery process. Furthermore, the BOILED-Egg model assessed compounds' drug-likeness and gastrointestinal absorption based on their lipophilicity. The LogP values suggest that Zosuquidar has moderate to high lipophilicity, favorable for permeability across biological membranes and potential oral absorption.

3.4 Molecular Dynamics

The protein-drug binding profile of *M. tuberculosis* (Mtb) was obtained through simulations of the drug-receptor complex. Unraveling the dynamic intricacies of these stable interactions sheds light on the nuances of the binding process, contributing to a deeper understanding of the molecular mechanisms that underlie the drug's efficacy. This comprehensive insight into the dynamic interplay between proteins and drugs could potentially inform targeted drug design strategies for more effective therapeutics.

3.5 Unravelling Complex Formation: Insights into Binding Dynamics and Stability

The trajectory of the protein-drug complex was systematically analyzed to evaluate the stability of these complexes within a solvent-rich dynamic environment, using Root Mean Square Deviation (RMSD) as a measure. We conducted a comparative assessment of the RMSD values for the complex and the Protein in the absence of the drug to estimate the effectiveness of complex formation and its ability to maintain stability throughout the 100 ns simulations. Figure 6a shows that the drug remained in the binding site and maintained complex stability over the 100 ns simulations, with an average RMSD of 0.3016 nm. A slight decrease in fluctuations was noted between 49 and 58 ns. Further examination of the trajectory revealed that this minor drop in fluctuations could be attributed to a slight change in the conformation of the drug within the binding site.

The Radius of Gyration (Rg) was calculated to evaluate the effect of drug binding on the structural compactness of the mycobacterial MATE transporter. The results showed minor differences in Rg values for tRNA, both with and without the drug, ranging from 0.3 to 0.5 nm (Figure 6b). These findings indicate that drug binding caused slight structural changes, which affected the overall shape and increased the structural compactness of the receptor, ultimately contributing to its stability.

3.6 Root Mean Square Fluctuations

The root mean square fluctuations (RMSF) of the base pairs in the mycobacterial MATE transporter were systematically evaluated to understand the drug's impact on their flexibility and stability. The

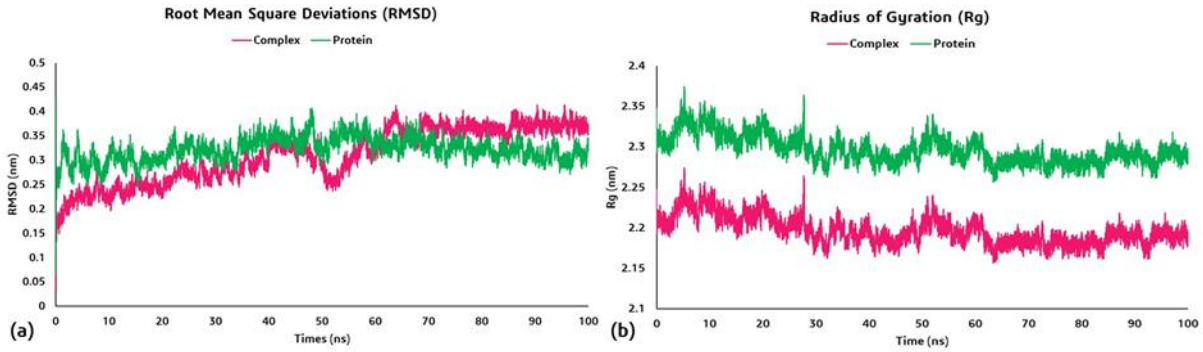

Figure 6a) Root Mean Square Deviations (RMSD), b) Radius of Gyration (Rg)

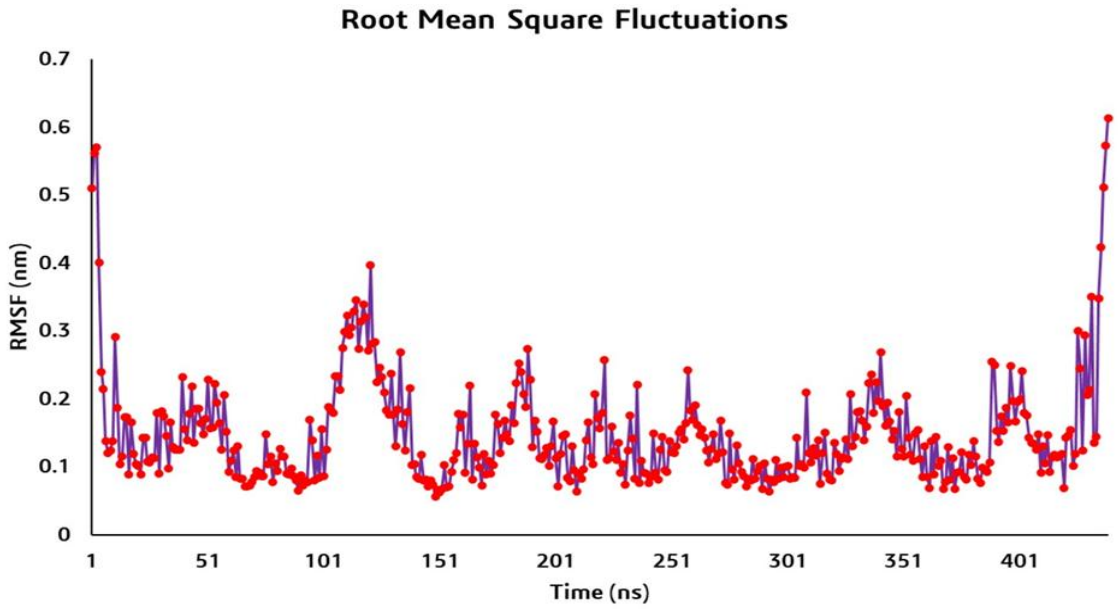

Figure 7 This graph represents the structural flexibility of MATE transporter protein during the simulation, Root Mean Square Fluctuations (RMSF). Higher RMSF values indicate greater flexibility or movement in specific regions or time points.

results indicated that upon binding to the receptor, the drug significantly decreased the flexibility of the base pairs, leading to increased interactions and promoting the formation of a stable complex (Figure 7). Notably, minor fluctuations were observed in some areas of *M. tuberculosis* (Mtb), particularly in the loops and random coils of the secondary structure. However, these fluctuations were likely due to the dynamic nature of the environment rather than direct interactions with the drug. In contrast, amino acid residues within the binding domain showed reduced fluctuations, indicating their active role in forming stable interactions with the drug. This finding suggests that these residues are crucial in decreasing flexibility, thus enhancing the stability of the protein-drug complex.

3.7 Investigation of Key Binding Interactions

The trajectories were further analyzed to evaluate the drug's binding mode and interactions with the mycobacterial MATE transporter protein. A visual inspection of the protein-drug complex trajectory revealed that the initial binding site was consistently maintained throughout the trajectory, with only minor changes in the drug's conformation within the binding site (Figure 8). Stable hydrophobic interactions were observed in the complex. The higher hydrophobicity of drugs with multiple aromatic rings may contribute to these hydrophobic interactions.

3.8 Energy Evaluation of the Complexation

The trajectories of the MATE protein-drug complex were systematically analyzed to determine the binding free energy of the complex. Notably, the primary contributors to the binding free energies were van der Waals interactions, solvent effects, Poisson-Boltzmann (PB) energies, and non-polar energies (Table 2). The total binding free energy for the protein-ligand complex was -8.54 kcal/mol, indicating a favorable and strong drug-binding interaction with the multidrug transporter. The van der Waals interactions were

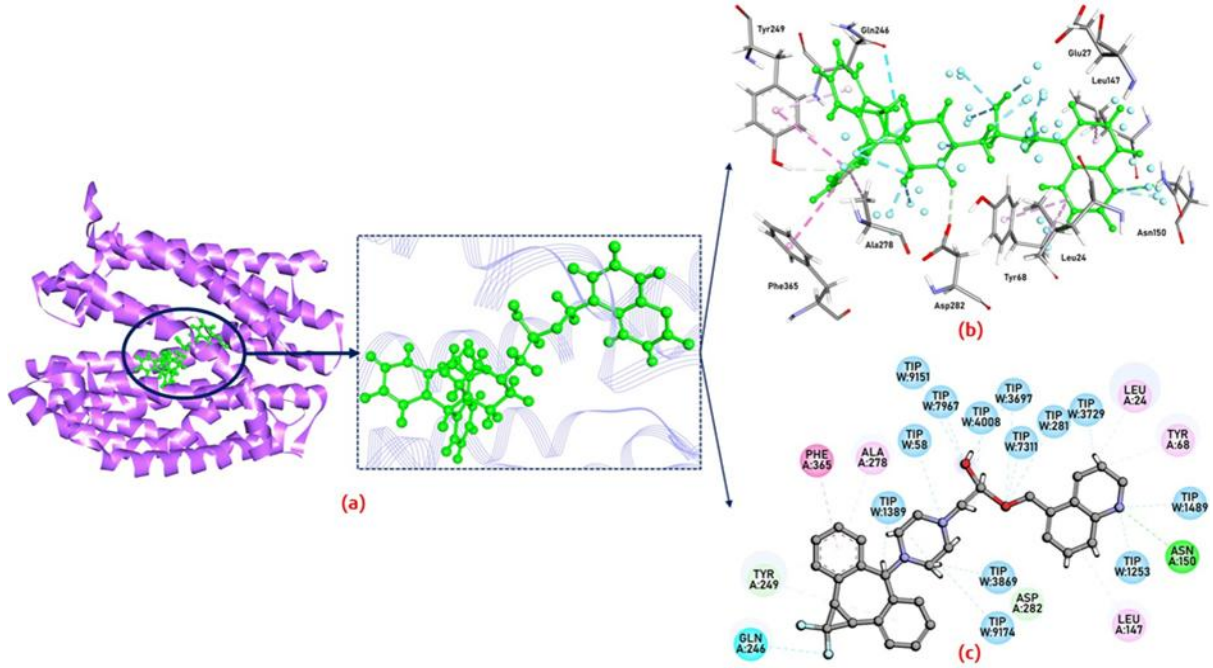

Figure 8 illustrates the binding interaction between a MATE transporter protein and the drug zosuquidar. It comprises three interconnected panels: (a) A broad view of the protein-drug complex. The transporter protein is depicted in purple using a ribbon representation, showing its overall secondary structure. The bound zosuquidar molecule is highlighted in green within a dark circular outline, nestled in a binding pocket of the Protein, (b) An enlarged view of the binding site, detailing the molecular interactions between Zosuquidar (green) and the surrounding amino acid residues of the Protein (gray). Dotted lines indicate potential hydrogen bonds or other non-covalent interactions. Key amino acid residues are labelled, (c) A 2D schematic representation of the binding site. It shows the chemical structure of Zosuquidar (in black) surrounded by interacting amino acid residues. These residues are depicted as coloured circles with their three-letter codes and position numbers. The colour coding represents different types of amino acids or their roles in the interaction.

Table 2 Binding free energy components for the protein-ligand complex calculated using MM/PBSA

Binding Free Energy	Complex (kcal/mol)
Electrostatic (Elec)	8.2202
Van der Waals (Vdw)	-41.6351
Polar Solvation (PB)	28.9779
Non-polar Solvation (SA)	-4.103
Gas Phase Energy (Gas)	-33.4149
Solvation Free Energy (Sol)	24.8749
Polar Contribution (Pol)	37.198
Non-polar Contribution (Npol)	-45.738
Total Binding Free Energy	-8.54

major contributors to this binding affinity, which accounted for -41.64 kcal/mol. This suggests that hydrophobic interactions play a critical role in stabilizing the complex. Breaking down the components of the binding free energy provides valuable insights into the forces governing the protein-ligand interactions. The significant contribution from van der Waals interactions and a favorable non-polar solvation term indicate that hydrophobic effects and structural complementarity primarily drive the binding. These binding free energy calculations, combined with molecular docking and dynamics simulations, offer a comprehensive understanding of the binding mechanisms and assist in optimizing Zosuquidar as a potent inhibitor targeting the mycobacterial MATE transporter protein. The sequence of the multidrug transporter was threaded to known structural templates using

MODBASE, which generated comparative models. The highest-scoring model was refined through energy minimization in SPDBV. Cavity detection, performed using the DEPTH server, identified potential drug-binding regions. A focused library of twenty-eight reported efflux pump inhibitors, including Zosuquidar, was docked against the MATE transporter model using AutoDock Tools. Ligands were prepared in BIOVIA Discovery Studio by generating isomers and ionization variants at a pH of 7.0 ± 2.0. Polar hydrogens were added to the prepared multidrug transporter structure, and partial charges were assigned using the Gasteiger method before generating the docking grid focused on the detected cavity. Autodock Vina executed rigid dockings with an exhaustiveness level of 9, providing predicted free energies and binding poses. The top-scoring compound, Zosuquidar, exhibited a favorable predicted affinity of -10.4 kcal/mol. This complex subsequently underwent explicit-solvent molecular dynamics simulations using Amberleap with the ff14SB force field, neutralized with NaCl ions. After minimization and equilibration, a 100 ns production simulation was conducted with NAMD 2.14 under constant 310 K temperature and 1 bar pressure. An ensemble of 2500 trajectory snapshots was generated for analysis using cpptraj. Key metrics, including root mean square deviation (RMSD), hydrogen bonds, and solvent accessibility, were assessed to evaluate binding stability and identify interacting residues.

Zosuquidar is an efflux pump inhibitor that targets P-glycoprotein and ABC transporters, which play a crucial role in multidrug resistance in cancer. Sandler et al. (2004) studied the safety and tolerability of combining Zosuquidar with doxorubicin in patients with advanced malignancies. Another study by Morrish et al. (2020) showed that Zosuquidar inhibits Hepatitis B Virus (HBV) replication in liver cells when combined with birinapant. As an ABC efflux pump inhibitor, Zosuquidar shows promise in reversing transporter-mediated chemoresistance, as highlighted by Robey et al. (2018). This compound increases drug accumulation in tumors expressing P-glycoproteins, improving etoposide absorption, especially when paired with non-ionic surfactants (Nielsen et al. 2023). Additionally, Zosuquidar has been observed to inhibit multidrug-resistant bacterial strains, including *Acinetobacter baumannii* and *E. coli* (Cripe et al. 2010; Alenazy 2022; Pelegrinova et al. 2024). It also increases the efficacy of polymyxins by enhancing bacterial susceptibility, which underscores its potential as an antibiotic adjuvant (Turner et al. 2020). Our findings suggest that Zosuquidar could be repurposed as an efflux pump inhibitor targeting Rv2836c. This is supported by a previous study indicating that sertraline can inhibit mycobacterial efflux activity, significantly enhancing the potency of bedaquiline (Shankaran et al. 2023). Multidrug transporters in mycobacteria are believed to significantly contribute to antimicrobial resistance (Srivastav et al. 2019; Datta et al. 2024). Transporters like Rv2836c in *M. tuberculosis* (Mtb) are known to extrude various drugs and antimicrobials (Mishra and Daniels 2013; Long et al. 2024). The overexpression of these genes has been demonstrated to promote fluoroquinolone efflux, thereby reducing drug efficacy in multidrug-resistant tuberculosis (Kim et al. 2021). Environmental factors and exposure to sublethal antibiotic doses can upregulate these multidrug transporters, enhancing bacterial resistance (Spengler et al. 2017; Machado et al. 2017; Miotto et al. 2022).

Despite existing research, the potential of Zosuquidar as an efflux pump inhibitor in bacteria has not been fully explored. This study evaluated its activity as a potential inhibitor targeting the mycobacterial multidrug transporter. We investigated its binding affinity and inhibitory potential against the mycobacterial efflux transporter using molecular docking and molecular dynamics. The protein structure was modeled using homology and docked with Zosuquidar, predicting a strong binding affinity at the drug-binding pocket, where we identified significant interactions responsible for the stability of the complex. The simulation indicates that Zosuquidar maintains stable binding while reducing transporter flexibility, suggesting potential allosteric inhibition.

Conclusions

Our analysis identifies various binding modes and key sites on the MATE transporter, enhancing our understanding of the mechanisms behind efflux pump inhibition. We characterized the molecular interactions that could impact the efficacy of the inhibitors. This study offers new insights into inhibiting the mycobacterial MATE efflux transporter. Further research is necessary to assess the effectiveness of these inhibitors against mycobacterial efflux pumps to tackle multidrug and antimicrobial resistance.

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Data Availability Statement

All the data generated during the work has been provided in the manuscript.

Funding Declaration

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Clinical Trial number

Not Applicable

Conflicts of Interest

No conflicts

Ethical Clearance

Not Applicable

References

Alenazy R. (2022). Drug Efflux Pump Inhibitors: A Promising Approach to Counter Multidrug Resistance in Gram-Negative Pathogens by Targeting AcrB Protein from AcrAB-TolC Multidrug Efflux Pump from *Escherichia coli*. *Biology*, *11*(9), 1328. https://doi.org/10.3390/biology11091328.

Andre, E., Goeminne, L., Cabibbe, A., Beckert, P., Kabamba Mukadi, B., et al. (2017). Consensus numbering system for the rifampicin resistance-associated rpoB gene mutations in pathogenic mycobacteria. *Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, *23*(3), 167–172. https://doi.org/10.1016/j.cmi.2016.09.006.

Athar, M., Gervasoni, S., Catte, A., Basciu, A., Malloci, G., Ruggerone, P., & Vargiu, A. V. (2023). Tripartite efflux pumps of the RND superfamily: what did we learn from computational studies?. *Microbiology (Reading, England)*, *169*(3), 001307. https://doi.org/10.1099/mic.0.001307.

Black, P. A., Warren, R. M., Louw, G. E., van Helden, P. D., Victor, T. C., & Kana, B. D. (2014). Energy metabolism and drug efflux in *Mycobacterium tuberculosis*. *Antimicrobial agents and chemotherapy*, *58*(5), 2491–2503. https://doi.org/10.1128/AAC.02293-13.

Campolattano, N., D'Abrosca, G., Russo, L., De Siena, B., Della Gala, M., et al. (2023). Insight into the on/off switch that regulates expression of the MSMEG-3762/63 efflux pump in *Mycobacterium smegmatis*. *Scientific reports*, *13*(1), 20332. https://doi.org/10.1038/s41598-023-47695-4.

Cripe, L. D., Uno, H., Paietta, E. M., Litzow, M. R., Ketterling, R. P., et al. (2010). Zosuquidar, a novel modulator of P-glycoprotein, does not improve the outcome of older patients with newly diagnosed acute myeloid leukemia: a randomized, placebo-controlled trial of the Eastern Cooperative Oncology Group 3999. *Blood*, *116(*20*)*, *4077–4085*. doi:10.1182/blood-2010-04-277269

Datta, D., Jamwal, S., Jyoti, N., Patnaik, S., & Kumar, D. (2024). Actionable mechanisms of drug tolerance and resistance in *Mycobacterium tuberculosis*. *The FEBS journal*, *291*(20), 4433–4452. https://doi.org/10.1111/febs.17142.

Day, N. J., Santucci, P., & Gutierrez, M. G. (2024). Host cell environments and antibiotic efficacy in tuberculosis. *Trends in microbiology*, *32*(3), 270–279. https://doi.org/10.1016/j.tim.2023.08.009.

Forli, S., Huey, R., Pique, M. E., Sanner, M. F., Goodsell, D. S., & Olson, A. J. (2016). Computational protein-ligand docking and virtual drug screening with the AutoDock suite. *Nature protocols*, *11*(5), 905–919. https://doi.org/10.1038/nprot.2016.051.

Ghajavand, H., Kargarpour Kamakoli, M., Khanipour, S., Pourazar Dizaji, S., Masoumi, M., et al. (2019). Scrutinizing the drug resistance mechanism of multi- and extensively-drug resistant *Mycobacterium tuberculosis:* mutations versus efflux pumps. *Antimicrobial resistance and infection control*, *8*, 70. https://doi.org/10.1186/s13756-019-0516-4.

Goletti, D., Meintjes, G., Andrade, B. B., Zumla, A., & Shan Lee, S. (2025). Insights from the 2024 WHO Global Tuberculosis Report - More Comprehensive Action, Innovation, and Investments required for achieving WHO End TB goals. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*, *150*, 107325. https://doi.org/10.1016/j.ijid.2024.107325.

Gopi, P., Gurnani, M., Singh, S., Sharma, P., & Pandya, P. (2023). Structural aspects of SARS-CoV-2 mutations: Implications to plausible infectivity with ACE-2 using computational modeling approach. *Journal of biomolecular structure & dynamics*, *41*(14), 6518–6533. https://doi.org/10.1080/07391102.2022.2108901.

Hassan, K. A., Liu, Q., Elbourne, L. D. H., Ahmad, I., Sharples, D., et al. (2018). Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens. *Research in microbiology*, *169*(7-8), 450–454. https://doi.org/10.1016/j.resmic.2018.01.001.

Henderson, P. J. F., Maher, C., Elbourne, L. D. H., Eijkelkamp, B. A., Paulsen, I. T., & Hassan, K. A. (2021). Physiological Functions of Bacterial "Multidrug" Efflux Pumps. *Chemical reviews*, *121*(9), 5417–5478. https://doi.org/10.1021/acs.chemrev.0c01226.

Huang, L., Wu, C., Gao, H., Xu, C., Dai, M., Huang, L., Hao, H., Wang, X., & Cheng, G. (2022). Bacterial Multidrug Efflux Pumps at the Frontline of Antimicrobial Resistance: An Overview. *Antibiotics (Basel, Switzerland)*, *11*(4), 520. https://doi.org/10.3390/antibiotics11040520.

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: visual molecular dynamics. *Journal of molecular graphics*, *14*(1), 33–28. https://doi.org/10.1016/0263-7855(96)00018-5.

Kim, J., Cater, R. J., Choy, B. C., & Mancia, F. (2021). Structural Insights into Transporter-Mediated Drug Resistance in Infectious Diseases. *Journal of molecular biology*, *433*(16), 167005. https://doi.org/10.1016/j.jmb.2021.167005.

Klukovits, A., & Krajcsi, P. (2015). Mechanisms and therapeutic potential of inhibiting drug efflux transporters. *Expert opinion on drug metabolism & toxicology*, *11*(6), 907–920. https://doi.org/10.1517/17425255.2015.1028917.

Lee, J., Cheng, X., Swails, J. M., Yeom, M. S., Eastman, P. K., et al. (2016). CHARMM-GUI Input Generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM Simulations Using the CHARMM36 Additive Force Field. *Journal of chemical theory and computation*, *12*(1), 405–413. https://doi.org/10.1021/acs.jctc.5b00935.

Long, Y., Wang, B., Xie, T., Luo, R., Tang, J., Deng, J., & Wang, C. (2024). Overexpression of efflux pump genes is one of the mechanisms causing drug resistance in *Mycobacterium tuberculosis*. *Microbiology spectrum*, *12*(1), e0251023. https://doi.org/10.1128/spectrum.02510-23.

Machado, D., Coelho, T. S., Perdigão, J., Pereira, C., Couto, I., Portugal, I., Maschmann, R. A., Ramos, D. F., von Groll, A., Rossetti, M. L. R., Silva, P. A., & Viveiros, M. (2017). Interplay between Mutations and Efflux in Drug Resistant Clinical Isolates of *Mycobacterium tuberculosis*. *Frontiers in microbiology*, *8*, 711. https://doi.org/10.3389/fmicb.2017.00711.

Maphasa, R. E., Meyer, M., & Dube, A. (2021). The Macrophage Response to *Mycobacterium tuberculosis* and Opportunities for Autophagy Inducing Nanomedicines for Tuberculosis Therapy. *Frontiers in cellular and infection microbiology*, *10*, 618414. https://doi.org/10.3389/fcimb.2020.618414.

Miotto, P., Sorrentino, R., De Giorgi, S., Provvedi, R., Cirillo, D. M., & Manganelli, R. (2022). Transcriptional regulation and drug resistance in *Mycobacterium tuberculosis*. *Frontiers in cellular and infection microbiology*, *12*, 990312. https://doi.org/10.3389/fcimb.2022.990312.

Mishra, M. N., & Daniels, L. (2013). Characterization of the MSMEG_2631 gene (mmp) encoding a multidrug and toxic compound extrusion (MATE) family protein in *Mycobacterium smegmatis* and exploration of its polyspecific nature using biolog phenotype microarray. *Journal of bacteriology*, *195*(7), 1610–1621. https://doi.org/10.1128/JB.01724-12.

Monedero-Recuero, I., Gegia, M., Wares, D. F., Chadha, S. S., & Mirzayev, F. (2021). Situational analysis of 10 countries with a high burden of drug-resistant tuberculosis 2 years post-UNHLM declaration: progress and setbacks in a changing landscape. *International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases*, *108*, 557–567. https://doi.org/10.1016/j.ijid.2021.06.022.

Morrish, E., Mackiewicz, L., Silke, N., Pellegrini, M., Silke, J., Brumatti, G., & Ebert, G. (2020). Combinatorial Treatment of Birinapant and Zosuquidar Enhances Effective Control of HBV Replication In Vivo. *Viruses*, *12*(8), 901. https://doi.org/10.3390/v12080901.

Nielsen, R. B., Holm, R., Pijpers, I., Snoeys, J., Nielsen, U. G., & Nielsen, C. U. (2023). Combinational Inhibition of P-Glycoprotein-Mediated Etoposide Transport by Zosuquidar and Polysorbate 20. *Pharmaceutics*, *15*(1), 283. https://doi.org/10.3390/pharmaceutics15010283.

O'Boyle, N. M., Banck, M., James, C. A., Morley, C., Vandermeersch, T., & Hutchison, G. R. (2011). Open Babel: An open chemical toolbox. *Journal of cheminformatics*, *3*, 33. https://doi.org/10.1186/1758-2946-3-33.

Peddireddy, V., Doddam, S. N., & Ahmed, N. (2017). Mycobacterial Dormancy Systems and Host Responses in Tuberculosis. *Frontiers in immunology*, *8*, 84. https://doi.org/10.3389/fimmu.2017.00084.

Pelegrinova, L., Sofrankova, L., Spaldova, J., Stefik, P., Sulova, Z., Breier, A., & Elefantova, K. (2024). Zosuquidar: An Effective Molecule for Intracellular Ca^{2+} Measurement in P-gp Positive Cells. *International journal of molecular sciences*, *25*(6), 3107. https://doi.org/10.3390/ijms25063107.

Phillips, J. C., Hardy, D. J., Maia, J. D. C., Stone, J. E., Ribeiro, J. V., et al. (2020). Scalable molecular dynamics on CPU and GPU architectures with NAMD. *The Journal of chemical physics*, *153*(4), 044130. https://doi.org/10.1063/5.0014475.

Poulton, N. C., & Rock, J. M. (2022). Unraveling the mechanisms of intrinsic drug resistance in *Mycobacterium tuberculosis*. *Frontiers in cellular and infection microbiology*, *12*, 997283. https://doi.org/10.3389/fcimb.2022.997283.

Roberts A. G. (2021). The Structure and Mechanism of Drug Transporters. *Methods in molecular biology (Clifton, N.J.)*, *2342*, 193–234. https://doi.org/10.1007/978-1-0716-1554-6_8.

Robey, R. W., Pluchino, K. M., Hall, M. D., Fojo, A. T., Bates, S. E., & Gottesman, M. M. (2018). Revisiting the role of ABC transporters in multidrug-resistant cancer. *Nature reviews. Cancer*, *18*(7), 452–464. https://doi.org/10.1038/s41568-018-0005-8.

Rodrigues, L., Cravo, P., & Viveiros, M. (2020). Efflux pump inhibitors as a promising adjunct therapy against drug resistant

tuberculosis: a new strategy to revisit mycobacterial targets and repurpose old drugs. *Expert review of anti-infective therapy*, *18*(8), 741–757. https://doi.org/10.1080/14787210.2020.1760845.

Rodrigues, L., Cravo, P., & Viveiros, M. (2020). Efflux pump inhibitors as a promising adjunct therapy against drug resistant tuberculosis: a new strategy to revisit mycobacterial targets and repurpose old drugs. *Expert review of anti-infective therapy*, *18*(8), 741–757. https://doi.org/10.1080/14787210.2020.1760845.

Seung, K. J., Keshavjee, S., & Rich, M. L. (2015). Multidrug-Resistant Tuberculosis and Extensively Drug-Resistant Tuberculosis. Cold Spring Harbor perspectives in medicine, 5(9), a017863. https://doi.org/10.1101/cshperspect.a017863.

Chang, D. P. S., & Guan, X. L. (2021). Metabolic Versatility of *Mycobacterium tuberculosis* during Infection and Dormancy. *Metabolites*, *11*(2), 88. https://doi.org/10.3390/metabo11020088.

Shankaran, D., Singh, A., Dawa, S., Arumugam, P., Gandotra, S., & Rao, V. (2023). The antidepressant sertraline provides a novel host directed therapy module for augmenting TB therapy. *eLife*, *12*, e64834. https://doi.org/10.7554/eLife.64834.

Sharma, K., Ahmed, F., Sharma, T., Grover, A., Agarwal, M., & Grover, S. (2023). Potential Repurposed Drug Candidates for Tuberculosis Treatment: Progress and Update of Drugs Identified in Over a Decade. *ACS omega*, *8*(20), 17362–17380. https://doi.org/10.1021/acsomega.2c05511.

Spengler, G., Kincses, A., Gajdács, M., & Amaral, L. (2017). New Roads Leading to Old Destinations: Efflux Pumps as Targets to Reverse Multidrug Resistance in Bacteria. *Molecules, 22* (3), 468. https://doi.org/10.3390/molecules22030468.

Srivastav, R., Kumar, D., Grover, A., Singh, A., Manjasetty, B. A., Sharma, R., & Taneja, B. (2014). Unique subunit packing in mycobacterial nanoRNase leads to alternate substrate recognitions in DHH phosphodiesterases. *Nucleic acids research*, *42*(12), 7894–7910. https://doi.org/10.1093/nar/gku425.

Srivastav, R., Sharma, R., Tandon, S., & Tandon, C. (2019). Role of DHH superfamily proteins in nucleic acids metabolism and stress tolerance in prokaryotes and eukaryotes. *International journal of biological macromolecules*, *127*, 66–75. https://doi.org/10.1016/j.ijbiomac.2018.12.123.

Traoré, A. N., Rikhotso, M. C., Mphaphuli, M. A., Patel, S. M., Mahamud, H. A., Kachienga, L. O., Kabue, J. P., & Potgieter, N. (2023). Isoniazid and Rifampicin Resistance-Conferring Mutations in *Mycobacterium tuberculosis* Isolates from South Africa. *Pathogens (Basel, Switzerland)*, *12*(8), 1015. https://doi.org/10.3390/pathogens12081015.

Turner, A. P., Alam, C., & Bendayan, R. (2020). Efflux Transporters in Cancer Resistance: Molecular and Functional Characterization of P-Glycoprotein. In A. Sosnik, & R. Bendayan (Eds.) *Drug Efflux Pumps in Cancer Resistance Pathways: From Molecular Recognition and Characterization to Possible Inhibition Strategies in Chemotherapy*, *Cancer Sensitizing Agents for Chemotherapy vol.* 7 (pp. 1–30). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-816434-1.00001-2.

Tyagi, R., Srivastava, M., Jain, P., Pandey, R. P., Asthana, S., Kumar, D., & Raj, V. S. (2022). Development of potential proteasome inhibitors against *Mycobacterium tuberculosis*. *Journal of biomolecular structure & dynamics*, *40*(5), 2189–2203. https://doi.org/10.1080/07391102.2020.1835722.

WHO. (2024). *Global Tuberculosis Report 2024*. Retrieved from https://www.who.int/teams/global-tuberculosis-programme/tb-reports/global-tuberculosis-report-2024.

WHO Results Report 2020-2021. n.d. Retrieved from https://www.who.int/about/accountability/results/who-results-report-2020-2021. Accessed February 19, 2024.

Wilczek, N. A., Brzyska, A., Bogucka, J., Sielwanowska, W. E., Żybowska, M., Piecewicz-Szczęsna, H., & Smoleń, A. (2023). The Impact of the War in Ukraine on the Epidemiological Situation of Tuberculosis in Europe. *Journal of clinical medicine*, *12*(20), 6554. https://doi.org/10.3390/jcm12206554.

Yang, J., Zhang, L., Qiao, W., & Luo, Y. (2023). *Mycobacterium tuberculosis*: Pathogenesis and therapeutic targets. *MedComm*, *4*(5), e353. https://doi.org/10.1002/mco2.353.

Zgurskaya H. I. (2021). Introduction: Transporters, Porins, and Efflux Pumps. *Chemical reviews*, *121*(9), 5095–5097. https://doi.org/10.1021/acs.chemrev.1c00010.

Zhao, J., Xie, H., Mehdipour, A. R., Safarian, S., Ermler, U., et al. (2021). The structure of the *Aquifex aeolicus* MATE family multidrug resistance transporter and sequence comparisons suggest the existence of a new subfamily. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(46), e2107335118. https://doi.org/10.1073/pnas.2107335118.

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Genome-wide identification and expression analysis of DOF transcription factor in tomato (*Solanum lycopersicum*) and its effect against developmental and stress condition

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ABSTRACT

The transcription factor known as DNA-binding with one finger (DOF) is a plant-based regulator involved in stress responses, growth, and development. Specifically, DOFs play key roles in essential biological processes, including signal transduction, cellular morphogenesis, and reactions to environmental stress. We aim to identify and characterize the DOF transcription factors in tomato (*Solanum lycopersicum*) and examine their expression under various developmental and stress conditions. In this study, we conducted a genome-wide identification of the DOF family in tomato, which involved phylogenetic analysis, conserved motif identification, predictions of sub-cellular localization, gene structure analysis, gene expression profiling, and protein-protein interaction studies. We identified, classified, and analyzed the expression of 8 DOF genes in tomato. The sequences of these genes showed similarity to those in *S. lycopersicum*, including DOF5.1, DOF3.1, DOF2.4-like, DOF2.5-like, DOF3.4-like, DOF1.4, DOF3.4-like, and DOF3.1. The zf-DOF (pfam ID: pfam02701) and the zf-DOF superfamily (pfam Cl: 03664) were identified as two common superfamily domains across all eight genes. Through phylogenetic analysis, we identified two genes associated with stress response and six genes related to developmental processes. Notably, DOF1.4 was found to be expressed in both stress and developmental contexts. The distinct expression profiles of DOF genes in response to abiotic stimuli suggest their significant involvement in the plant's defense mechanisms. These findings enhance our understanding of the mechanisms underlying plant growth, development, and stress responses, providing valuable insights that could improve crop productivity and resilience in agricultural practices.

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1 Introduction

The transcription process and the role of transcription factors are the primary regulatory mechanisms for various plant processes, including cell growth, stress responses, and environmental signaling. Transcription factors (TFs) are essential for regulating gene expression by binding to cis-regulatory elements located within the promoter region of genes (Waschburger et al. 2022). Plants have developed an extraordinary ability to respond to internal and external signals, allowing them to adapt to changing environmental conditions and support their growth and development (Tabassum et al. 2022). A crucial aspect of this regulatory mechanism is the precise control of gene expression, in which TFs play a significant role.

The DOF transcription factor family is unique to plants and is recognized for its involvement in several biological processes, including signal transduction, cellular morphogenesis, and stress responses (Li et al. 2022). TFs are proteins that bind to specific DNA sequences in the promoter regions of their target genes, modulating transcriptional activity by either enhancing or suppressing gene expression (Jiao et al. 2022). The DNA-binding domains of the DOF family are characterized by a single, consistent zinc finger motif that enhances their ability to connect with specific cis-acting regions found in the promoters of target genes (Li et al. 2022).

The roles of DOF family members in leaf vein development have attracted significant attention. Arabidopsis VDOF1 (VASCULAR-RELATED DOF1) and VDOF2 (VASCULAR-RELATED DOF2) may inhibit cotyledon vein formation and lignin deposition by regulating brassinosteroid (BR) signaling and the transcription of genes related to lignin in inflorescence stems (Ramachandran et al. 2020). Recent studies by Zhang et al. (2022) indicate that CDF4 regulates cotyledon vein development. Additionally, DOFs have been implicated in jasmonic acid (JA)-induced leaf senescence in both monocots and dicots. For instance, OsDOF24 in rice delays leaf senescence by suppressing the activity of the OsAOS gene, which is associated with JA biosynthesis (Renard et al. 2020). Conversely, Arabidopsis AtDOF2.1 has been shown to actively contribute to JA-induced leaf senescence through a MYC2-DOF2.1-MYC2 actively feed-forward transcription loop (Negi et al. 2013). These findings suggest that plant-specific DOF TFs may regulate various processes associated with developmental stages in plants or their roles in long-distance signaling.

In the context of stress conditions, several studies indicate that DOF TFs respond to biotic stresses by enhancing the ability of plants to defend against pathogens. For instance, the transient expression of the DOF genes BBF2 and BBF3 in tobacco has increased plant resistance to pathogens (Sasaki et al. 2015).

S. lycopersicum, a significant economic crop worldwide, requires a deep understanding of the regulatory mechanisms governing its growth, development, and stress responses for agricultural improvement (Bedinger et al. 2011). While research on DOF transcription factors in various plant species is growing, comprehensive studies on this family in tomatoes remain sparse (Zou and Sun 2023). The TF TMO6, a member of the DOF family that responds to auxin signals, is central to the pathway affected by MP 77. Additionally, several members of the DOF family have been demonstrated to play vital roles in promoting cell divisions in vascular tissues. Specific DOF genes, like TMO6, are regulated by cytokinin, making TMO6 responsive to both cytokinin and auxin signals (Smit et al. 2020).

Given these considerations, we aim to identify and characterize the DOF transcription factor in tomatoes and study its expression during developmental and stress conditions to understand its role fully. This information is crucial for crop improvement strategies, including genetic engineering approaches to enhance stress tolerance, fruit development, and crop productivity in tomatoes and related species.

2 Material and methods

2.1 Discovery of Potential DOF Genes Associated with Stress and Developmental Conditions in Tomato Plants

Nucleotide sequences of DOF genes, which are responsible for responses to stress and developmental conditions, were retrieved from the TAIR Database (https://www.arabidopsis.org). The TAIR (The Arabidopsis Information Resource) is a comprehensive web-based database with complete genetic and molecular biology information for the *Arabidopsis thaliana* model. A total of 39 DOF nucleotide sequences were obtained from the TAIR database. Following the retrieval, BLAST (with an e-value of 1e-10 and a minimum identity of 50%) was performed using the nucleotide sequences to identify homologous genes in *S. lycopersicum* (Table 1). This process resulted in eight blast sequences that exhibited more than 50% similarity (Li et al. 2022). The BLAST sequences (with an e-value of 1e-10 and a minimum identity of 50%) from the *S. lycopersicum* genome were employed as queries against the total expressed sequence tags (ESTs) of *S. lycopersicum* to identify the expressed genes in that genome. Additionally, NCBI's Conserved Domain search tool was utilized to calculate and analyze conserved motifs (https://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi).

2.2 The Analysis of Phylogenetic Trees

The Blast DOF gene sequences identified from the *S. lycopersicum* genome were aligned using Clustal W2. A phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 11

software (Larkin et al. 2007; Kumar et al. 2018). Bootstrap values were calculated to explore the relationships among the DOF genes of *S. lycopersicum*, which were identified through the Blastn similarity search. Additionally, using phylogeny.fr (https://www.phylogeny.fr/), a separate phylogenetic tree was created to examine the relatedness of eight genes based on stress and developmental characteristics derived from the Blastn analysis of the *S. lycopersicum* genome.

2.3 Gene Ontology Study

2.3.1 Sub-cellular localization

We used Busca (Bioinformatic Utility for Species Context Analysis) (https://busca.biocomp.unibo.it/) to predict specific subcellular localization that starts from the protein sequence, which was translated using the ExPaSy translation tool from the gene obtained through BLASTn.

2.3.2 Protein-protein interaction

We investigated complex pathways by analyzing protein-protein interaction (PPI) networks to enhance our understanding of the relationships between the identified DOF genes and uncover unknown proteins' roles. STRING (https://string-db.org/) performed the PPI interactions, focusing on the stress-related functions associated with the DOF genes. Additionally, we annotated and integrated functional files detailing various functions, from which we categorized the genes according to their respective developmental and stress-related conditions.

2.4 Plant material, pathogen inoculation, and biotic stress treatments

Two tomato cultivars, *ArkaSamrat* (resistance to BW) and *Pusa Ruby* (susceptible to BW) were selected for the present study. These cultivars were chosen based on their known resistance and susceptibility to *Ralstonia solanacearum* bacteria. *R. solanacearum* was grown in separate growth conditions to ensure its availability for plant inoculation. The growth conditions involved a suitable culture medium, such as nutrient agar or broth, and appropriate temperature and humidity for bacterial growth. A temperature-controlled growth chamber was set up to maintain specific temperature conditions necessary for the growth of tomato plants and *R. solanacearum*. The chamber had temperature and humidity sensors and control systems to maintain the desired conditions. Tomato plants of both AS and PR cultivars were selected for inoculation. The bacterial suspension was prepared by diluting the culture to an appropriate concentration. The tomato plants were inoculated with *R. solanacearum* root immersion. Care was taken to avoid cross-contamination between cultivars during inoculation. The inoculated tomato plants were placed inside the temperature-controlled growth chamber. The chamber was set to maintain the required temperature, typically around 28-30°C, which is favorable for *R. solanacearum* infection and disease development. The chamber's humidity level was also adjusted to create an optimal environment for the plants and the bacterial pathogen. The tomato plants were regularly monitored for disease progression and symptoms caused by *R. solanacearum* infection. Disease severity and symptoms, such as wilting, leaf yellowing, necrosis, or stunting, were recorded for each cultivar separately.

2.5 Inoculation of R. solanacearum

The bacteria were cultivated in TTC (Triphenylterazolium chloride) medium, which contains 3 g of sucrose, 5–10 g of beef extract, 7 g of tryptone, and 7 g of agar per liter. The medium was treated with 1% TTC for two days and maintained at 30 °C. *R. solanacearum* was injected into plants at the six-leaf stage through root wounds. The plants underwent a mock inoculation using sterile water and were allowed to incubate for 30 minutes in a bacterial solution containing 10^8 colony-forming units (cfu)/ml. All inoculated plants were then placed in plastic containers. The Arka Samrat plants inoculated with pathogens and mock-inoculated were labeled R-5dpi, R-10dpi, R-15dpi, and R-mock (control). Similarly, the Pusa Ruby plants subjected to pathogen injection and mock inoculation were designated S-5dpi, S-10dpi, S-15dpi, and S-mock (control). We maintained both susceptible and resistant plant varieties at three distinct developmental stages: the vegetative state (40 days after germination), the blooming stage (90 days after germination), and the seedling stage (20 days after germination). Tissue samples from plants at different developmental stages were then quickly frozen in liquid nitrogen and stored at –80 °C for further examination. Each experiment was conducted three times to ensure reliability.

2.6 Isolation of RNA and Analysis of Gene Expression

Five stem tissue samples were collected at each designated time point and developmental stage to obtain RNA. Total RNA was isolated from the ice-frozen control and treatment samples using Trizol reagent (Invitrogen, Darmstadt, Germany). DNAse I (supplied by Promega, Madison, USA) was then utilized to purify the resulting RNA according to the manufacturer's instructions. The concentration of the RNA was evaluated using the NanoDrop spectrophotometer (Thermo Scientific, Waltham, USA). RNA samples with a 260/280 nm ratio between 2.0 and 2.1 were selected for further examination. Following the instructions, 2 µg of RNA were transcribed using the high-capacity cDNA synthesis kit (Life Technologies, Burlington, CA) to synthesize the first strand of cDNA. After diluting the cDNA tenfold, it served as the template for quantitative real-time PCR (qRT-PCR). A set of gene-based primers developed from the conserved regions of eight DOF genes was used for the qRT-PCR. Before qPCR, RT-PCR was performed to verify the specificity of the primers, and agarose gel

electrophoresis was used to separate the amplified cDNA products. Each RT-PCR reaction was conducted in a total volume of 10 mL, which included 5 µL of FASTSYBR Green mix from Kappa Biosystems (D Mark, Toronto, CA), 1 µL of each forward (F) and reverse (R) primer (at a concentration of 5 µM), 1 µL of reverse-transcribed cDNA (with a concentration of 5 ng), and 2 µL of nuclease-free (NF) water.

Using the Step One Plus real-time PCR equipment (Life Technologies, Burlington, Canada), the reactions underwent rapid qPCR with the following thermal cycling conditions: an initial denaturation step for one minute at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 seconds and annealing at 60 °C for 30 seconds. A melting curve analysis was performed by first holding at 65 °C for 15 seconds, then gradually increasing the temperature to 95 °C at a rate of 0.2 °C per second to confirm the accuracy of the PCR product. Nine reactions, consisting of three biological and three technical replicates, were used to calculate the Ct values for each sample. Glyceraldehyde-3-phosphate dehydrogenase (SlGADPH) and Solanum lycopersicum Ubiquitin-containing enzyme 3 (SlUBC3) were endogenous controls.

Using the comparative 2−ΔΔCt method, the threshold cycle values were converted into relative expression values for the genes, with the control transcript set at 1 (Bedinger et al. 2011). The results of the qRT-PCR were statistically significant, as evidenced by a two-way analysis of variance (ANOVA) and numerous evaluations using the uncorrected Fisher's LSD test. A p-value of less than 0.05 was used to determine the significance of the differences in mean values.

3 Results

3.1 Computational discovery of genes associated with developmental and stress conditions

We downloaded 39 nucleotide sequences of *A. thaliana* from the TAIR database to identify the DOF genes associated with developmental and stress conditions. A nucleotide BLAST was performed against the whole genome of *S. lycopersicum* using NCBI BLASTn. Out of the 39 sequences from *A. thaliana*, only 8 exhibited similarity with *S. lycopersicum*: DOF5.1, DOF3.1, DOF2.4-like, DOF2.5-like, DOF3.4-like, DOF1.4, DOF3.1, and DOF3.4-like. Their identity and query coverage are detailed in Table 1. The stress and developmental conditions for these sequences were assigned based on a literature review, as indicated in Table 1.

Out of 8 DOF sequences, one was categorized under stress conditions, while the remaining were associated with developmental conditions. To assess the expression of the derived DOF sequences of *S. lycopersicum* from BLASTn, we conducted an analysis using Expression Sequence Tags (EST). It was found that DOF2.4-like, DOF3.4-like, and DOF1.4 did not show any expression in the EST database, as shown in Table 2.

3.2 Motif and conserved domain analysis

The MEME analysis of DOF genes identified three significant motifs that reflect potential regulatory elements crucial for their function. The genes analyzed include DOF5_1, DOF3_1, DOF2_4_like, DOF2_5_like, DOF3_4_like, DOF1_4, DOF3_1_1, and DOF3_4. These motifs were found at various positions across the genes, with highly significant p-values ranging from 1.49e-4 for DOF5_1 to 8.16e-49 for DOF3_4, indicating strong statistical support for their presence. Motif 1, which has a consensus sequence of "AACA CAAAG TTYGT TACTA CAACA AYTYA RYYKT CTCAG CCAGC CACGCCA," is prominently found in all the analyzed genes, suggesting a conserved functional role. In addition, Motif 2, with the sequence "AGGT AYTGGC ATYRA GGNGG AACTY TAMG BAAY RTHC CWGT ITGG IGWGG," and Motif 3, with the sequence "YTTY TGCA AAGACT YG," were also identified. Each of these motifs contributes to the regulatory landscape of the DOF genes. The motif logos illustrate

Table 1 Identity/similarity between *A. thaliana* and *S. lycopersicum* Dof sequences.

Est– results			
Gene name	Identity	Similarity	e-value
DOF5.1	100%	100%	5e-06
DOF3.1	100%	100%	4e-89
DOF2.4-like	-	-	-
DOF2.5-like	100%	100%	3e-91
DOF3.4-like	-	-	-
DOF1.4	-	-	-
DOF3.1	98.73%	98%	2e-33
DOF3.4-like	100%	100%	5e-62

Table 2 Represents similarity/identity and e value. Where Accession no. (AT) represents the *A. thaliana*, and Accession no. (sol) represents the *S. lycopersicum.*

Gene name	Accession no. (AT)	Accession no. (sol)	Identity	E value	Condition
DOF5.1	AT1G29160.1	XM_004250915.4	100%	4e-04	Seed coat Development (Renard et al. 2020)
DOF3.1	AT2G28510.1	XM_004235567.4	77.53%	1e-19	Tissue regeneration development (Zhang et al. 2022)
DOF2.4-like	AT2G37590.1	XM_004253279.1	96.19%	0.0	Radial growth development (Miyashima et al., 2019)
DOF2.5-like	AT2G46590.2	XM_004229950.4	82.87%	3e-37	Stress (Zou and Sun 2023)
DOF3.4-like	AT3G52440.2	XM_004233894.4	81.61%	1e-09	Positive regulator of light-mediated seed germination l (Santopolo et al. 2015)
DOF1.4	AT4G24060.1	XM_004232850.4	78.99%	1e-16	Regulate vascular cell differentiation and lignin biosynthesis (Ramachandran et al. 2020)
DOF3.1	AT5G60850.1	XM_004241893.4	79.84%	4e-16	Control Vascular Development in Arabidopsis (Smit et al. 2020)
DOF3.4-like	AT5G65590.1	XM_004233894.4	80.77%	5e-19	Stomatal guard cell maturation and differentiation (Negi et al. 2013)

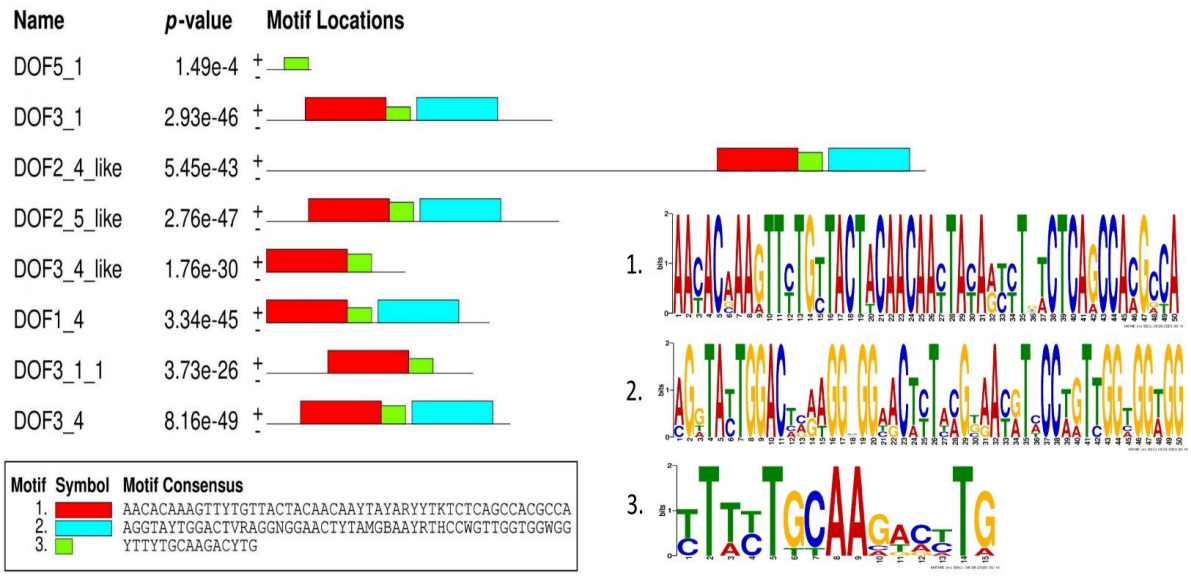

Figure 1 Conserved motif analyses of DOF protein sequences using the MEME 4 program. The length of each distinct protein sequence is shown by a solid line in the graphical representation, and the various discovered motifs are shown as colour boxes.

the conservation of specific nucleotide positions within each motif, highlighting their potential importance in gene regulation (Figure 1). DOF3_1 exhibited Motifs 1, 2, and 3 at multiple locations, with a highly significant p-value of 2.93e-46, underscoring its complex regulatory functions. Similarly, DOF2_5_like, which also contains Motifs 1, 2, and 3, had a p-value of 2.76e-47, suggesting robust regulatory roles as well. The presence of these motifs across different DOF genes indicates their involvement in essential regulatory mechanisms that could influence gene expression and functional specificity. The high conservation of these motifs among various DOF genes and their significant p-values emphasize their critical role in the regulatory networks governing gene expression.

The conserved region analysis of the DOF genes revealed significant insights into their structural domains, particularly the zinc finger-Dof (zf-Dof) domain, which is crucial for DNA binding and gene regulation. The gene DOF5.1 (XM_004250915.4) spans residues 69 to 127, with an E-value of 3.6e-35, indicating a highly conserved domain (pfam02701). DOF3.1 (XM_004235567.4) spans residues 1 to 144, with an E-value of 1.61e-28, also showing a conserved zf-Dof domain. DOF2.4-like (XM_004253279.1), covering residues 250 to 408, presents an E-value of 4.78e-36 and belongs to the zf-Dof superfamily (cl03664), indicating a broader conserved region. DOF2.5-like (XM_004229950.4) spans residues 3 to 164 with an E-value of 2.56e-32, confirming its conserved zf-Dof domain. DOF3.4-like (XM_004233894.4) spans residues 1 to

Table 3 Representation of interval conserved part in the sequence of the DOF gene their 'e' value with pfam id.

Conserved region of genes	Gene Name	Accession no.	Interval	E-value	Gene name (cd)	pfam id
	Dof 5.1	XM_004250915.4	69-127	3.6e-35	zf-Dof	pfam02701
	Dof 3.1	XM_004235567.4	1-144	1.61e-28	zf-Dof	pfam02701
	Dof 2.4-like	XM_004253279.1	250-408	4.78e-36	zf-Dof superfamily	Cl03664
	DOF2.5-like	XM_004229950.4	3-164	2.56e-32	zf-Dof	pfam02701
	DOF3.4-like	XM_004233894.4	1-84	7.28e-15	zf- Dof superfamily	cl03664
	DOF1.4	XM_004232850.4	1-138	2.41e-27	zf-Dof	pfam02701
	DOF3.1-like	XM_004241893.4	9-128	1.71e-23	zf-Dof superfamily	cl03664
	DOF3.4-like	XM_004233894.4	1-150	4.44e-32	zf-Dof	pfam02701

84 with an E-value of 7.28e-15 and belongs to the zf-Dof superfamily (cl03664), indicating a shorter conserved region. DOF1.4 (XM_004232850.4) spans residues 1 to 138 with an E-value of 2.41e-27, highlighting a conserved zf-Dof domain (pfam02701). DOF3.1-like (XM_004241893.4) spans residues 9 to 128 with an E-value of 1.71e-23, belonging to the zf-Dof superfamily (cl03664). Finally, DOF3.4 (XM_004233894.4) spans residues 1 to 150 with an E-value of 4.44e-32, indicating a highly conserved zf-Dof domain (pfam02701) (Table 3). These conserved regions across the DOF genes underscore their evolutionary conservation and functional importance in DNA binding and gene regulation.

3.3 Phylogenetic analysis

The phylogenetic analysis revealed that the DOF genes can be categorized into two main groups: development-related genes and stress-related genes. In the development-related cluster, the genes XM004250915.4 (DOF5.1) and XM004253279.1 (DOF2.4-like) had branch lengths of 0.00 and 0.14, respectively. XM004233894.4 (DOF3.4-like) and its variant, DOF3.4-like (2), exhibited a branch length of 0.00, indicating a close relationship between them. The genes XM004235567.4 (DOF3.1) and XM004241893.4 (DOF3.1) showed branch lengths of 0.12 and 0.04, respectively. In the stress-related gene cluster, XM004229950.4 (DOF2.5-like) and XM004232850.4 (DOF1.4) exhibited branch lengths of 0.07 and 0.14, respectively. The tree's root connected these two clusters, with branch lengths of 0.05 for the development-related genes and 0.02 for the stress-related genes. This analysis emphasizes these genes' evolutionary relationships and genetic distances, highlighting their functional divergence in response to developmental processes and stress conditions (Figure 2).

3.4 Functional Characterization of the DOF Genes

3.4.1 Subcellular localization

The physiological positions of the genes were determined using subcellular localization data (Table 3) with the assistance of BUSCA. Most genes, represented as 5 DOF, are located in the chloroplast thylakoid lumen, except DOF 2.4-like, which is found in the extracellular space and scored 0.67 (Table 4).

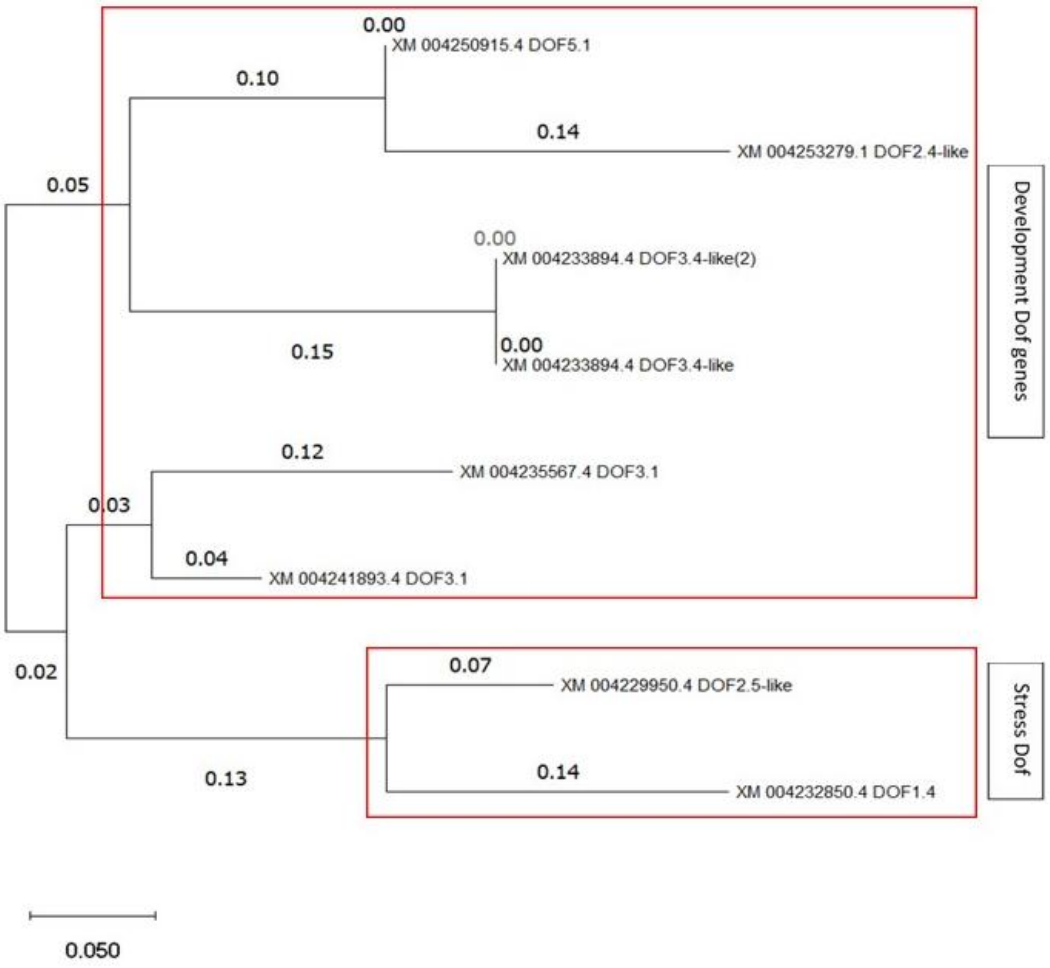

Figure 2 Phylogenetic analysis of *S. lycopersicum* DOF genes differentiate with development and stress condition.

Table 4 Dof genes with accession and location, with the score mentioned for identifying the location.

Gene Name	Accession	GOids	GOterms	Score
Dof 3.1	XM_004235567.4 479-655	GO:0009543	C:chloroplast thylakoid lumen	0.75
Dof 2.4-like	XM_004253279.1	GO:0005615	C:extracellular space	0.67
DOF2.5-like	XM_004229950.4 303-483	GO:0009543	C:chloroplast thylakoid lumen	0.8
DOF3.4-like	XM_004233894.4 449-534	GO:0009543	C:chloroplast thylakoid lumen	0.77
DOF1.4	XM_004232850.4 309-446	GO:0009543	C:chloroplast thylakoid lumen	0.78
DOF3.1-like	XM_004241893.4 176-303	GO:0009543	C:chloroplast thylakoid lumen	0.74

3.4.2 Protein-protein interaction

To better understand the functional relationships among the derived DOF genes, a protein-protein interaction (PPI) network analysis was conducted. We calculated and identified the different functions of the stress-related genes, DOF2.5-like and DOF1.4 (Figure 3).

Based on the STRING protein-protein interaction network, the analysis revealed significant interactions among several proteins. The central protein, Solyc00g024680.1.1, interacts with multiple other proteins, each supported by various types of evidence. The interaction with Solyc01g005300.2.1 is robustly supported by text mining data (light green line), curated database information (ocean blue line), and experimental data (magenta line), indicating a well-

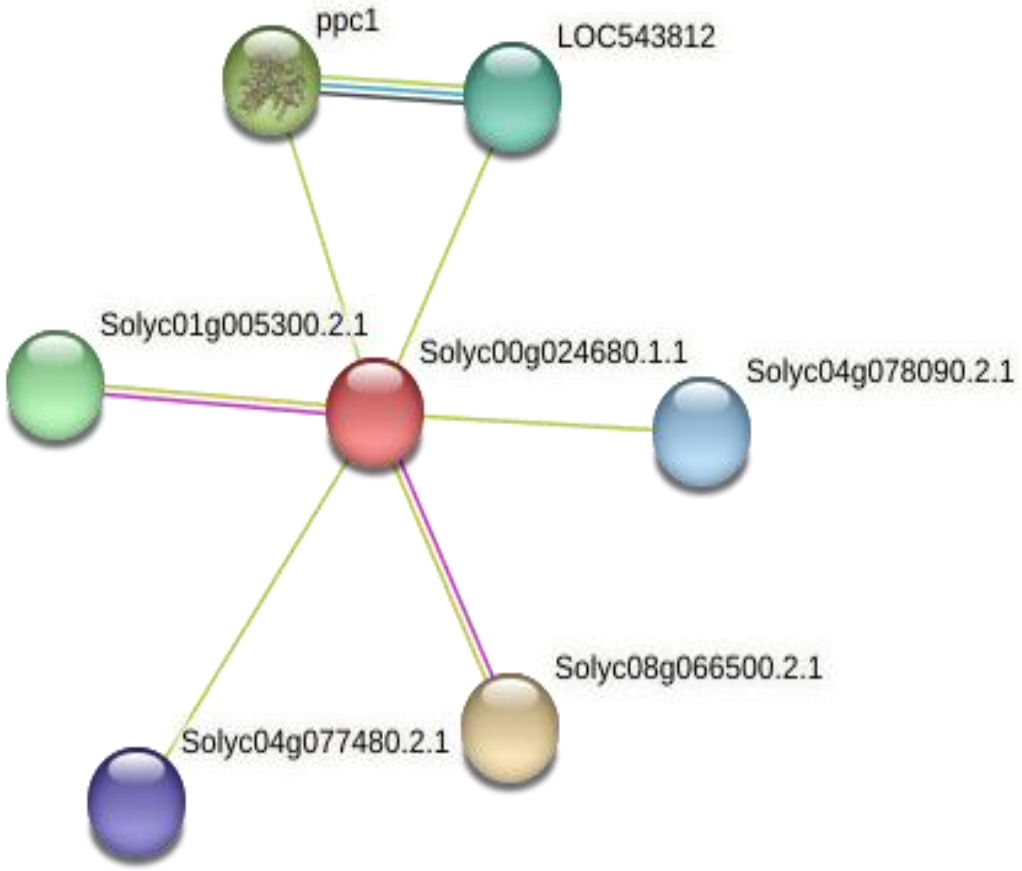

Figure 3 Protein-protein interaction analysis between the genes and their interaction pattern is represented.

documented relationship. Other proteins, such as ppc1, LOC543812, Solyc04g078090.2.1, and Solyc08g066500.2.1, also show potential interactions with Solyc00g024680.1.1 based solely on text mining data. Additionally, Solyc04g077480.2.1 is linked to Solyc00g024680.1.1 through text mining data and co-expression evidence (black line), suggesting these proteins are co-expressed in the same or different species. This comprehensive network illustrates the intricate web of protein interactions, underpinned by diverse evidence sources, providing a deeper understanding of the functional connections between these proteins.

3.5 Expression of DOF-related gene under biotic stress in resistance and susceptible Plant

In this study, we analyzed the 8DOFs gene for gene expression. Under biotic stress conditions, the genes Solyc04g077480.2.1 (DOF 1.4) and Solyc01g005300.2.1 (DOF2.5-like) were differentiated according to gene ontology in Table 4.

After exposure to stress, changes in the transcriptome of the plants were monitored. The comparison was conducted in two steps. In the first step, to characterize the role of DOF genes in disease management, a transcriptome comparison was made between three stages of the resistant Arka Samrat line (Figure 4) and the susceptible Pusa Ruby line (Figure 5). The RNA expression was studied at 5-day intervals to monitor the gradual changes in DOF expression in response to the induction of disease (Figure 6). The results showed that the genes resembling DOF 2.5 and DOF 1.4 exhibited significant downregulation, while genes like DOF 3.1, DOF 3.4, and DOF 2.4 showed moderate downregulation. In contrast, the genes resembling DOF 5.1, DOF 3.4.2, and DOF 3.1.2 displayed minimal changes compared to the control in the resistant Arka Samrat line. The changes in RNA expression in response to abiotic stress were different in the susceptible Pusa Ruby line. The DOF 2.5 and DOF 1.4 genes exhibited only minor changes, while the other genes showed substantial to moderate downregulation compared to the control.

In the second step, similar trends were observed in the gradual changes in DOF expression. The DOF 2.5 and DOF 1.4 genes showed significant changes throughout the infection period, whereas the other six DOF genes displayed little to moderate changes when compared to the control data taken at 0 hours post-infection.

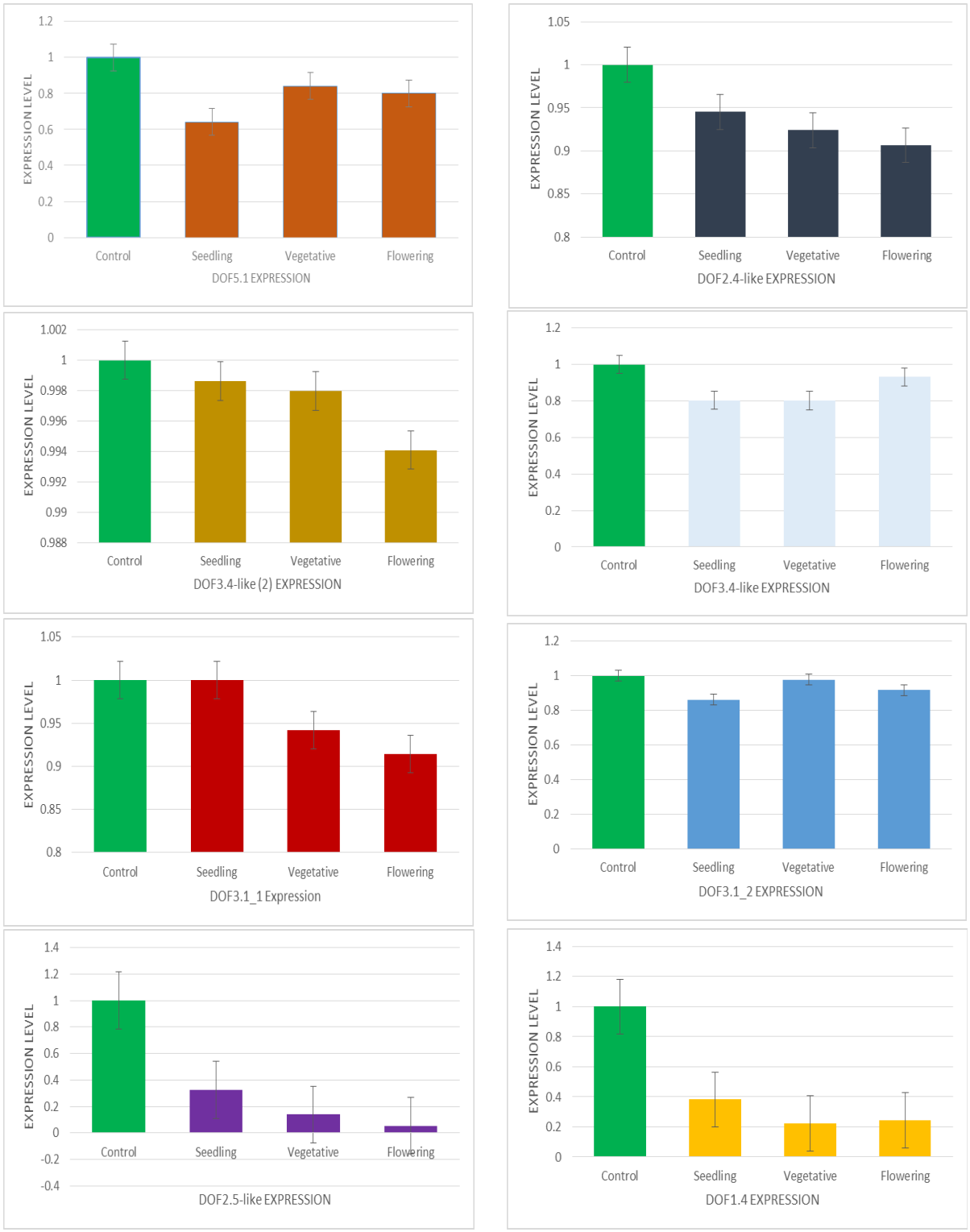

Figure 4 shows the expression analysis of eight selected DOF genes in the resistant tomato variety Arkasamrat under abiotic stress. After the stress treatment, RNA was extracted from plants collected at three developmental stages. GAPDH was used as the housekeeping gene to normalize the mRNA levels compared to the untreated sensitive line, ArkaSamrat. The error bars represent the standard deviations from three separate real-time PCR tests.

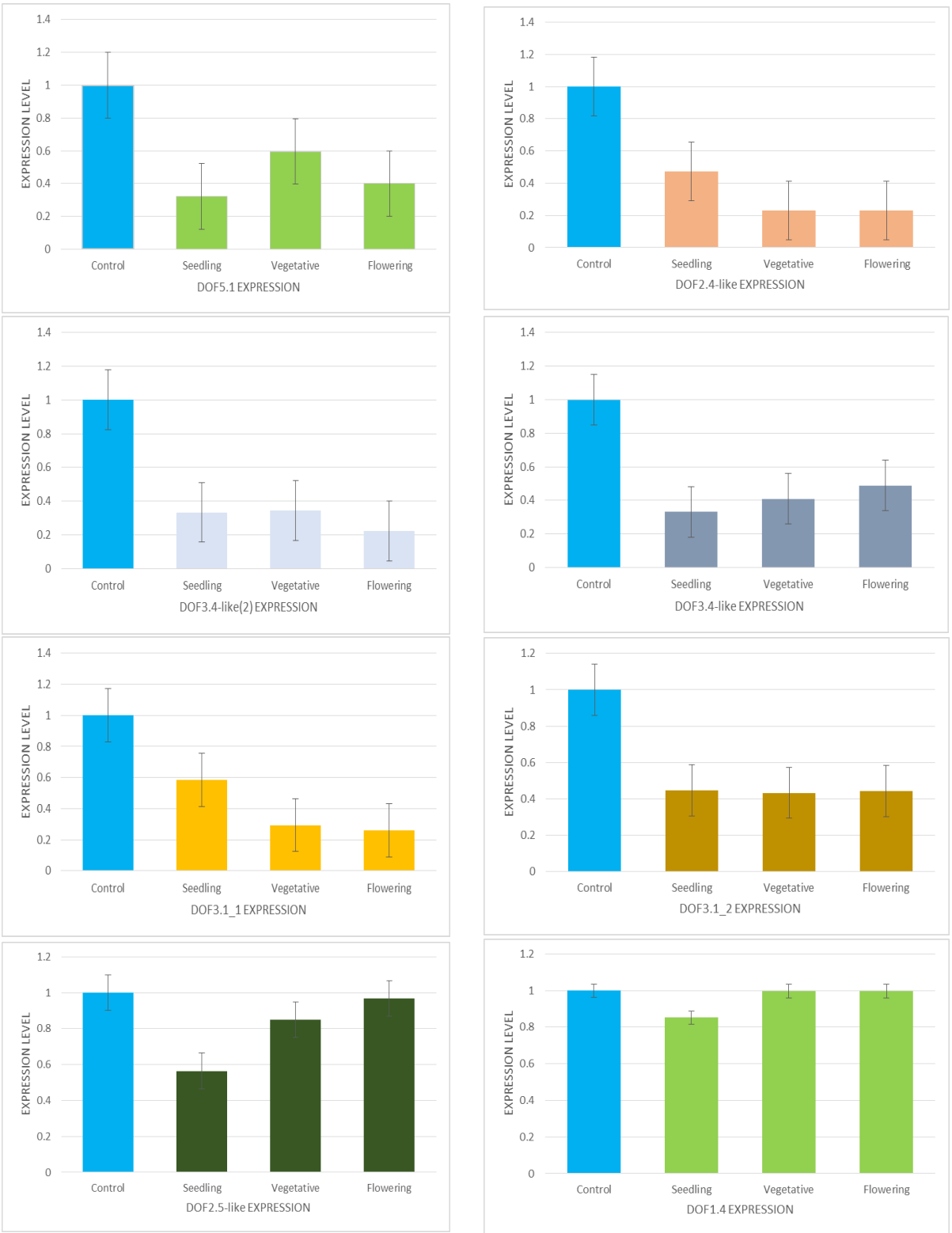

Figure 5 shows the expression analysis of eight selected tomato DOF genes in the susceptible variety Pusa Ruby under abiotic stress. After the stress treatment, RNA was extracted from plants collected at three developmental stages. The mRNA levels were normalized against those in the untreated sensitive line, Pusa Ruby. The housekeeping gene used for normalization was GAPDH.

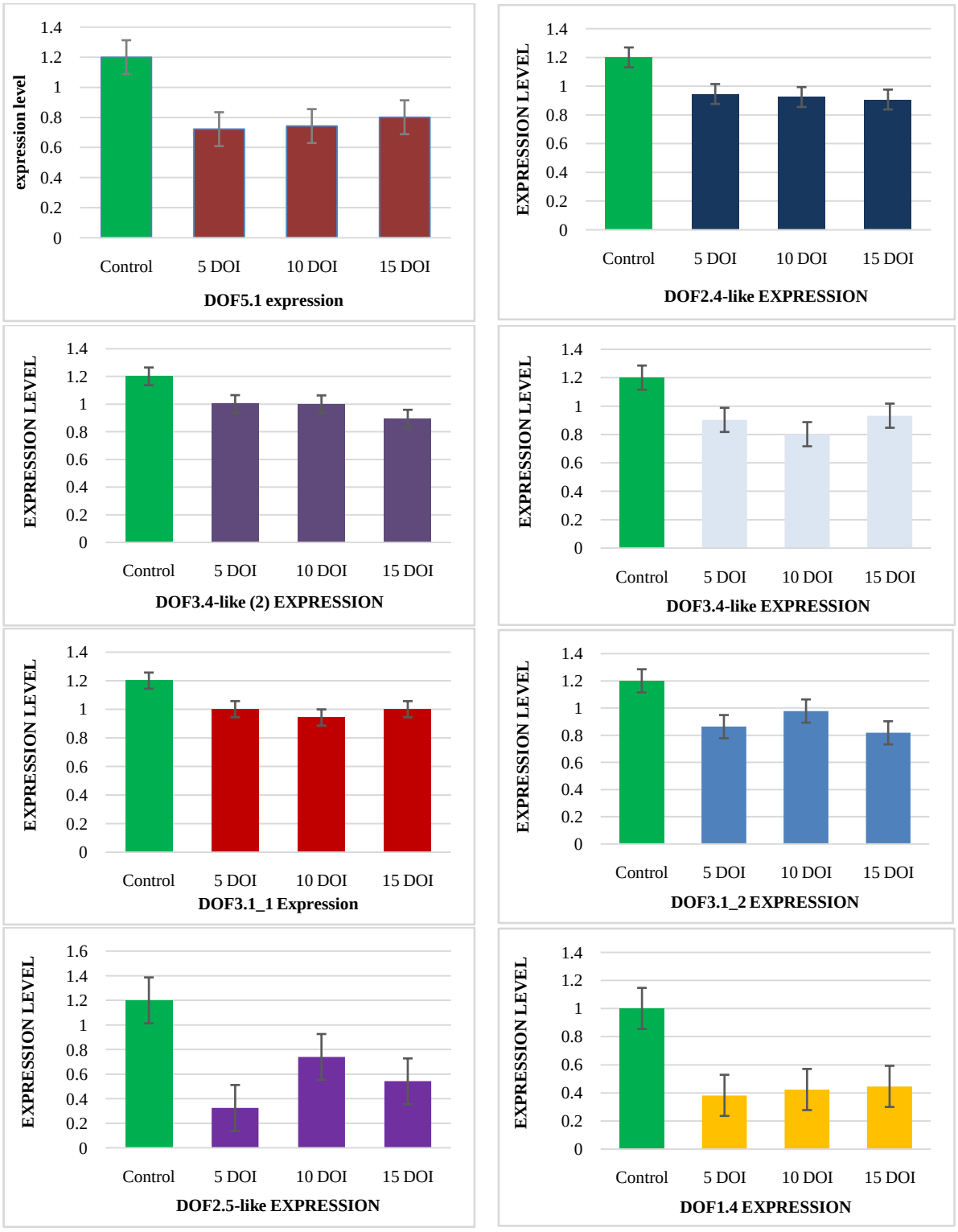

Figure 6 shows the expression analysis of eight selected DOF genes in the susceptible tomato variety Pusa Ruby under abiotic stress. RNA was extracted from the plants collected at 5, 10, and 15 days after inoculation (DAI). The mRNA levels were normalized to the untreated sensitive line Pusa Ruby (0 hours), setting the relative expression at 1 for this time point. The housekeeping genes used for normalization were GAPDH and UBI3.

4 Discussion

Transcription factors are crucial in plant growth and development, significantly influencing crop yield under cultivation. Their importance has increased in the context of changing climatic conditions. This research aims to identify the transcription factor gene DOF in *S. lycopersicum* (tomato). The identified DOF genes from *S. lycopersicum* include DOF5.1, DOF3.1, DOF2.4-like, DOF2.5-like, DOF3.4-like, DOF1.4, and DOF3.4-like, all derived from the selected 39 DOF gene in the TAIR database of *A. thaliana*. The selection of these candidate genes in the present study was based on information provided by Li et al. (2022). A literature survey of the eight identified genes indicated that, except for DOF2.5-like, all are involved in plants' biotic and abiotic stress conditions. This work focuses on identifying the DOF genes in the tomato genome and differentiating their roles in developmental and stress conditions. Analysis of the conserved domains indicates that, except for DOF5.1, all other genes fall under the conserved region of the zinc-finger family and superfamily group (Rao et al. 2013).

In this phylogenetic analysis, our primary objective was to elucidate the relationship between DOF genes identified in Solanum lycopersicum (commonly known as tomato) and their association with developmental and stress-related genes. Our investigation revealed intriguing findings regarding the evolutionary divergence and functional categorization of these DOFs. Specifically, we identified DOF1.4, a known transcription factor associated with developmental processes (Ramachandran et al. 2020), which is situated on the same branch as DOF2.5-like, a transcription factor linked to stress responses (Santopolo et al. 2015). This proximity in the phylogenetic tree suggests a potential relationship or shared ancestry between these two distinct DOFs despite their involvement in different biological contexts. These results provide insights into the intricate interplay between developmental pathways and stress responses in *S. lycopersicum*. The shared evolutionary lineage between DOF1.4 and DOF2.5-like indicates a possible functional overlap or co-regulation, suggesting a potential cross-talk between developmental programs and stress-related signaling pathways (Santopolo et al. 2015). Further exploring these findings may offer valuable insights into the molecular mechanisms governing plant growth, development, and adaptation to environmental stresses. Understanding the functional implications and regulatory networks associated with these DOFs could pave the way for developing novel strategies for crop improvement, such as enhancing stress tolerance while maintaining optimal developmental processes in tomato and other related plant species (Mohanty et al. 2019). Our investigation also uncovered potential interactions among proteins encoded by DOF genes through protein-protein network analysis. This analysis provided insightful information about the regulatory networks involving DOF transcription factors and their potential roles in mediating plant responses to environmental stimuli, as indicated by the gene ontology function in the PPI network analysis. By analyzing protein-protein interactions, we better understood how DOF genes may collaborate with other proteins to modulate plant responses to various environmental cues. The interactions revealed valuable insights into the molecular mechanisms underlying the regulatory networks governing plant stress responses. Our research focused on analyzing the expression patterns of DOF genes in the plant variety under biotic stress conditions, as these stress-related genes respond to such stimuli. We aimed to identify genes associated with resistance or susceptibility. We employed the qRT-PCR technique for gene expression validation (Mohanty et al. 2017). Our analysis identified distinct expression profiles of DOF genes responding to biotic stimuli, indicating their potential involvement in the plant's defense mechanisms. These findings suggest that specific DOF genes may significantly confer resistance or susceptibility traits in the examined plant variety.

However, it is important to note that additional experimental investigations are necessary to validate and expand upon these initial findings. Nevertheless, this phylogenetic analysis serves as a solid foundation for future studies, paving the way for deeper exploration of the intricate relationships between DOFs and their roles in developmental and stress conditions in *S. lycopersicum* and other plant systems. These DOF genes have been shown to play significant roles in several plants' development and responses to biotic and abiotic stresses. All identified genes exhibited essential features characteristic of the selected genes in question. Furthermore, there are variations in tomato transcription factor genes compared to those in the model plant Arabidopsis, illustrating phylogenetic divergence and distinct characteristics. With the availability of genomic sequences for several crop plants and advances in bioinformatics, new avenues are opening to decipher the genetic elements involved in controlling complex traits such as flowering.

Conclusion

The current research aimed to identify and analyze DOF and DOF-like genes in the tomato genome (*S. lycopersicum*) using sequence homology searches and various bioinformatics methods. We successfully identified eight putative DOF transcription factors. Our comprehensive genome-wide analysis investigated conserved domain architectures, evolutionary relationships, and expression patterns across tissues and stress conditions. The results of our study significantly enhance our understanding of the functional roles that DOF transcription factors play in tomatoes. By clarifying their involvement in various biological processes, we have gained insights into how these regulatory proteins contribute to tomato growth, development, and stress responses. This knowledge is crucial for

understanding the signaling pathways involved in DOF transcription factor-mediated regulation, particularly concerning resistance to *C. truncatum* in chili pepper. Moreover, our findings open new avenues for crop improvement strategies, including genetic engineering approaches to enhance stress tolerance, fruit development, and overall crop productivity in tomatoes and related species. The fundamental information provided by this study will serve as a valuable resource for future research and breeding programs focused on improving tomato resilience and yield.

Ethics approval and consent to participate

Not applicable

Declaration of competing interest

The authors declare that there is no conflict of interest and has approved for publication.

Availability of data and material

All the data generated or analyzed during this study are included in this article.

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The research received no external funding.

Author's contributions

Conceptualization,J.N.M., S.J.S. and A.P.; Validation, S.J.S., B.L.P., A.J.,M.B., R.M, A.P and J.N.M.; Original draft preparation, J.N.M.,S.J.S., R.M. and A.P.; Review and editing, AP,MB, J.N.M.; Supervision, J.N.M., and A.P.

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References

Bedinger, P. A., Chetelat, R. T., McClure, B., Moyle, L. C., Rose, J. K., et al. (2011). Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation. *Sexual plant reproduction*, *24*(3), 171–187. https://doi.org/10.1007/s00497-010-0155-7 .

Jiao, Z., Tang, D., Fan, K., Zhang, Q., Liu, M.Y., & Ruan, J. (2022). Genome-Wide Identification Of The Dna-Binding One Zinc Finger (Dof) Transcription Factor Gene Family And Their Potential Functioning In Nitrogen Remobilization In Tea Plant (*Camellia Sinensis* L.). *Scientia Horticulturae, 292*, 110615. Http://Dx.Doi.Org/10.1016/J.Scienta.2021.110615 .

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular biology and evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics (Oxford, England)*, *23*(21), 2947–2948. https://doi.org/10.1093/bioinformatics/btm404.

Li, T., Wang, X., Elango, D., Zhang, W., Li, M., Zhang, F., Pan, Q., & Wu, Y. (2022). Genome-wide identification, phylogenetic and expression pattern analysis of Dof transcription factors in blueberry (*Vaccinium corymbosum* L.). *PeerJ*, *10*, e14087. https://doi.org/10.7717/peerj.14087.

Rao, X., Huang, X., Zhou, Z., & Lin, X. (2013). An improvement of the 2ˆ(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. *Biostatistics, bioinformatics and biomathematics*, *3*(3), 71–85.

Mohanty, J.N., Chand, S.K. & Joshi, R.K. (2019). Multiple microRNAs Regulate the Floral Development and Sex Differentiation in the Dioecious Cucurbit *Coccinia grandis* (L.) Voigt. *Plant Molecular Biology Reporter*, *37*, 111–128. https://doi.org/10.1007/s11105-019-01143-8.

Mohanty, J. N., Nayak, S., Jha, S., & Joshi, R. K. (2017). Transcriptome profiling of the floral buds and discovery of genes related to sex-differentiation in the dioecious cucurbit Coccinia grandis (L.) Voigt. *Gene*, *626*, 395–406. https://doi.org/10.1016/j.gene.2017.05.058.

Negi, J., Moriwaki, K., Konishi, M., Yokoyama, R., Nakano, T., Kusumi, K., et al. (2013). A Dof transcription factor, SCAP1, is essential for the development of functional stomata in Arabidopsis. *Current biology : CB*, *23*(6), 479–484. https://doi.org/10.1016/j.cub.2013.02.001.

Ramachandran, V., Tobimatsu, Y., Masaomi, Y., Sano, R., Umezawa, T., Demura, T., & Ohtani, M. (2020). Plant-specific Dof transcription factors VASCULAR-RELATED DOF1 and VASCULAR-RELATED DOF2 regulate vascular cell differentiation and lignin biosynthesis in Arabidopsis. *Plant molecular biology*, *104*(3), 263–281. https://doi.org/10.1007/s11103-020-01040-9.

Renard, J., Martínez-Almonacid, I., Sonntag, A., Molina, I., Moya-Cuevas, J., et al. (2020). PRX2 and PRX25, peroxidases regulated by COG1, are involved in seed longevity in Arabidopsis. *Plant, cell & environment*, *43*(2), 315–326. https://doi.org/10.1111/pce.13656.

Santopolo, S., Boccaccini, A., Lorrai, R., Ruta, V., Capauto, D., et al. (2015). DOF AFFECTING GERMINATION 2 is a positive regulator of light-mediated seed germination and is repressed by DOF AFFECTING GERMINATION 1. *BMC plant biology*, *15*, 72. https://doi.org/10.1186/s12870-015-0453-1.

Sasaki, N., Matsumaru, M., Odaira, S., Nakata, A., Nakata, K., Nakayama, I., Yamaguchi, K., & Nyunoya, H. (2015). Transient Expression Of Tobacco Bbf1-Related Dof Proteins, Bbf2 And Bbf3, Upregulates Genes Involved In Virus Resistance And Pathogen Defense. *Physiological and Molecular Plant Pathology*, *89*, 70-77. https://Doi.Org/10.1016/J.Pmpp.2014.12.005.

Smit, M. E., McGregor, S. R., Sun, H., Gough, C., Bågman, A. M., et al. (2020). A PXY-Mediated Transcriptional Network Integrates Signaling Mechanisms to Control Vascular Development in Arabidopsis. *The Plant cell*, *32*(2), 319–335. https://doi.org/10.1105/tpc.19.00562.

Tabassum, J., Raza, Q., Riaz, A., Ahmad, S., Rashid, M. A. R., et al. (2022). Exploration of the genomic atlas of Dof transcription factor family across genus *Oryza* provides novel insights on rice breeding in changing climate. *Frontiers in plant science*, *13*, 1004359. https://doi.org/10.3389/fpls.2022.1004359.

Waschburger, E. L., Guzman, F., & Turchetto-Zolet, A. C. (2022). Genome-Wide Identification and Analysis of *DOF* Gene Family in *Eugenia uniflora* L. (Myrtaceae). *Genes*, *13*(12), 2235. https://doi.org/10.3390/genes13122235.

Zhang, A., Matsuoka, K., Kareem, A., Robert, M., Roszak, P., et al. (2022). Cell-wall damage activates DOF transcription factors to promote wound healing and tissue regeneration in Arabidopsis thaliana. *Current biology*, *32*(9), 1883–1894.e7. https://doi.org/10.1016/j.cub.2022.02.069.

Zou, X., & Sun, H. (2023). DOF transcription factors: Specific regulators of plant biological processes. *Frontiers in plant science*, *14*, 1044918. https://doi.org/10.3389/fpls.2023.1044918.

Miyashima, S., Roszak, P., Sevilem, I., Toyokura, K., Blob, B., et al. (2019). Mobile PEAR transcription factors integrate positional cues to prime cambial growth. *Nature*, *565*(7740), 490-494.

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Optimized in vitro micropropagation and microtuber production in potato (*Solanum tuberosum* L.) through apical buds using hormone regulation and tissue culture techniques

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Benzylaminopurine (BAP)

Gibberellic acid (GA3)

In vitro organogenesis

Micropropagation

Hormone optimization

Microtuber production

ABSTRACT

Potato is an essential crop worldwide, and optimizing micropropagation techniques is important for enhancing germplasm conservation and large-scale production. This study focuses on the in vitro propagation of two potato varieties, *Agata* and *Fianna*, emphasizing optimizing sterilization protocols, shoot induction, rooting, and microtuber production. Apical buds from healthy, disease-free plants were selected as explants. These buds were surface-sterilized using 70% ethanol and sodium hypochlorite (NaOCl) with Tween-20. The explants were excised from tuber sprouts and cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of plant growth regulators, including benzylaminopurine (BAP) at 0.10–0.40 mg/L, gibberellic acid (GA3) at 0.20–1.00 mg/L, and naphthalene acetic acid (NAA) at 0.01 and 0.04 mg/L to promote root development. The study also explored the effects of these hormonal treatments on shoot induction, proliferation, and rooting. Significant differences were observed between the two varieties regarding oxidation, contamination, and aseptic conditions, with *Fianna* demonstrating better resistance to oxidation and contamination than *Agata*. Shoot multiplication was most efficient with BAP concentrations of 0.40 mg/L for *Fianna* and 0.30 mg/L for *Agata*. Root induction was maximized with higher sucrose concentrations (110 g/L) and GA3 (1.00 mg/L) for *Agata*, while moderate concentrations of these compounds produced optimal results

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for *Fianna*. Microtuber formation was most successful with moderate sucrose (80–100 g/L) and GA3 (0.25–0.75 mg/L) concentrations. This study provides valuable insights into optimizing tissue culture practices for potato propagation, enhancing both microtuber production and the overall efficiency of potato production systems.

1 Introduction

Potato (*Solanum tuberosum* L.) is among the most important tuber crops worldwide, serving as a significant source of food and income for many regions, including Nuevo Leon, Mexico (Aksoy et al. 2021; SIAP 2024). The potato plant is highly valued for its nutritional content, which includes carbohydrates, vitamins, and minerals, as well as its adaptability to various climatic conditions and soil types (Dolničar 2021; Heuberger et al. 2022). Given potatoes' economic and nutritional importance, understanding the mechanisms of organogenesis, mainly through apical buds, can provide critical insights for improving propagation techniques and crop yields (Navarro et al. 2015; De Koeyer and Harding 2019). Plant organogenesis involves forming and developing organs such as roots, shoots, and leaves from undifferentiated cells (Smet and Beeckman 2019). This process is essential for plant regeneration and is of particular interest in the context of vegetative propagation, a common practice in potato cultivation (O'Brien and McCleary 2023). Apical buds, located at the tip of the stem, play a crucial role in the growth and development of new shoots, which are vital for clonal propagation (Vlahova et al. 2022).

Numerous studies have investigated the factors influencing organogenesis in potatoes. For instance, García-González and Quiroz (2018) examined the impact of plant growth regulators on shoot regeneration from potato explants, emphasizing the importance of hormonal balance for successful organogenesis. Similarly, Bustos and Carrillo (2017) explored the genetic and environmental factors that affect the differentiation of apical meristems into various organ systems in potato plants. These studies highlight the complexity of organogenesis and the need for a comprehensive understanding of intrinsic and extrinsic factors.

Hormonal regulation, particularly the roles of cytokinins and auxins, remains a primary focus in organogenesis research. Cytokinins, such as benzylaminopurine (BAP), have been found to stimulate shoot proliferation, while auxins like indole-3-acetic acid (IAA) are essential for root development (Šmeringai et al. 2023). Furthermore, gibberellic acid (GA_3) is often used to enhance elongation and reduce apical dominance, underscoring the interplay of growth regulators in tissue culture systems (Ritonga et al. 2023).

Environmental factors also significantly influence outcomes in organogenesis. Light quality, temperature, and photoperiod conditions have been shown to affect the efficiency of organogenesis in potato explants (Develi and Miler 2023). Studies indicate that red and blue light spectra positively influence microtuberization, while green light inhibits these processes (Abu-Zeid et al. 2022). Additionally, the composition of the growth medium, including macronutrients, micronutrients, and gelling agents, plays a crucial role in the success of organogenesis (Anjum and Ali 2004). For instance, adding activated charcoal has been reported to reduce oxidative stress and phenolic exudation, common challenges in potato tissue culture (Thomas 2008; Permadi et al. 2024).

Genetic factors must also be considered, as different potato cultivars respond variably to the same growth conditions and hormonal treatments (Coleman et al. 1990). Advances in transcriptomics and proteomics have provided more profound insights into gene expression patterns during organogenesis, identifying key regulatory pathways and candidate genes responsible for shoot and root differentiation (Dobránszki et al. 2019). Collectively, these studies illustrate the multifaceted nature of organogenesis in potatoes and underscore the necessity of optimizing both biotic and abiotic conditions for successful micropropagation.

In Nuevo León, potato cultivation faces water scarcity, soil salinity, and temperature fluctuations. These environmental stressors significantly affect potato growth, yield, and quality, making adopting innovative strategies to enhance crop resilience essential. To tackle these challenges, improving propagation techniques, including the efficient use of apical buds, can bolster the resilience and productivity of potato crops in this region. Recent advancements in tissue culture and molecular biology offer new avenues for optimizing organogenesis in potatoes, providing potential solutions to overcome local agricultural constraints (Hasnain et al. 2022). However, despite significant progress in potato tissue culture research, a critical gap remains in developing region-specific protocols tailored to the unique environmental conditions of Nuevo León. Most existing studies focus on generalized propagation techniques without considering the specific physiological and environmental responses of potato varieties grown under local stress conditions.

The novelty of this research lies in its targeted approach to optimizing hormonal combinations and culture conditions specifically for potato varieties cultivated in arid and semi-arid environments like Nuevo León. This study aims to establish a robust and reproducible protocol for organogenesis and microtuber production by analyzing the interaction between growth regulators, environmental factors, and potato genotypes. Additionally, the

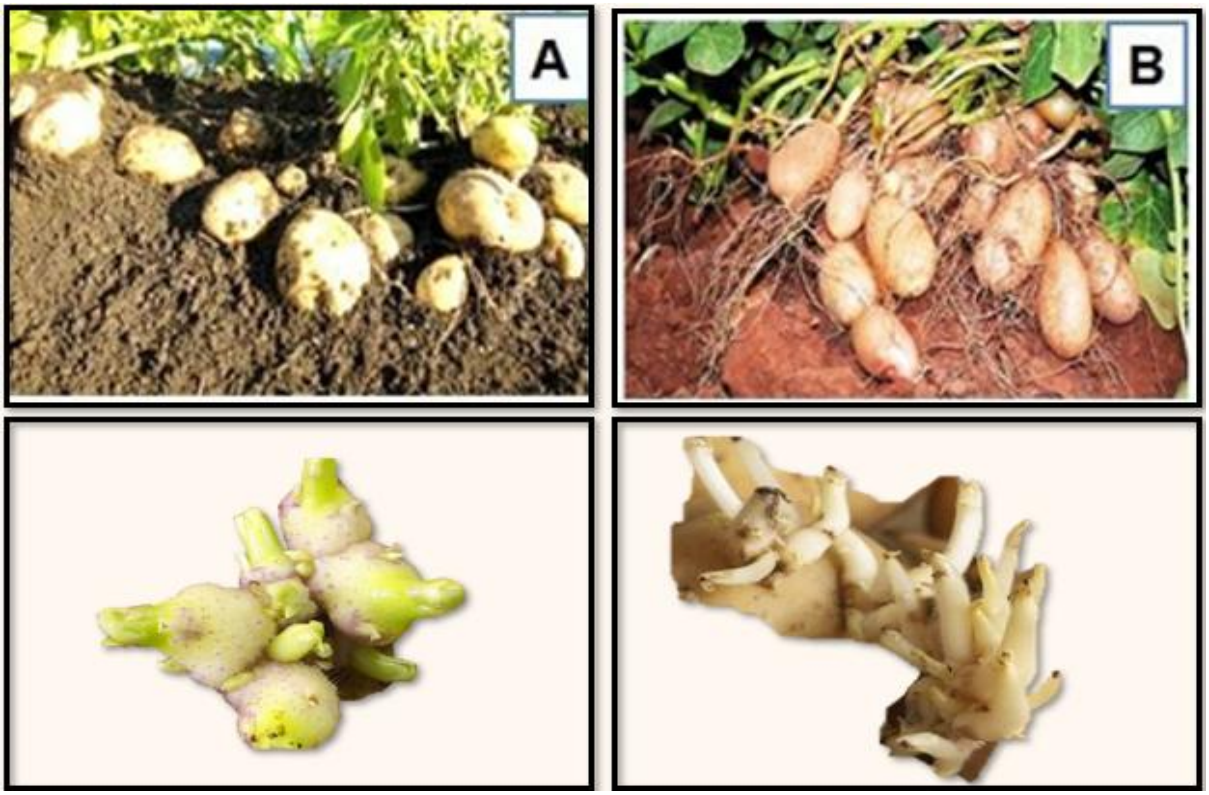

Figure 1 Potato tubers from three varieties of *S. tuberosum* (A) Agata: Smooth, oval-shaped tuber with light brown skin and shallow eyes, (B) Fianna: Round to oval-shaped tuber with a slightly rough texture and medium-depth.

research explores the influence of different hormonal treatments on various organ development stages, addressing the knowledge gap concerning variety-specific responses to tissue culture conditions. The findings are expected to enhance the scientific understanding of potato organogenesis and provide practical solutions for sustainable potato production in regions facing environmental stressors.

2 Materials and Methods

2.1 Plant Material and Sterilization

Apical buds from two potato varieties, *'Agata'* and *'Fianna*,' were selected as explants for in vitro propagation. These explants were carefully chosen from healthy, disease-free plants grown in greenhouse conditions. To prepare the buds, they were pre-washed with running tap water for 30 minutes to remove surface contaminants. Next, they underwent surface sterilization by being immersed in a 70% ethanol solution for 1 minute, followed by treatment with a sodium hypochlorite solution (0.5%) containing two drops of Tween-20 for 15 minutes. Benomyl (0.1 g/L) was included in the sterilization process to reduce fungal contamination further. After sterilization, the explants were rinsed three times with sterile distilled water to eliminate residual sterilizing agents. The effectiveness of the sterilization process was evaluated by assessing contamination rates, and the samples were examined under a light microscope to confirm their cleanliness (Figure 1).

2.2 Culture Medium Preparation

Murashige and Skoog (MS) medium was used as the basal culture medium due to its well-documented efficacy in supporting potato tissue culture. The medium was supplemented with varying concentrations of benzylaminopurine (BAP) (0.10, 0.20, 0.30, and 0.40 mg/L) and gibberellic acid (GA3) (0.25, 0.50, 0.75, and 1.00 mg/L) to evaluate their effects on shoot induction, proliferation, and microtuber formation. The medium also contained 3% (w/v) sucrose as a carbon source and 0.8% (w/v) agar as a gelling agent. The pH was adjusted to 5.8 using 0.1 N NaOH or HCl before autoclaving at 121°C for 20 minutes.

2.3 Pre-disinfestation process of Plant Material

Before the disinfection process of the plant material, 2 centimeter shoots from the varieties *Ágata* and *Fianna* were cultivated for six weeks under controlled conditions, which included a 16-hour light cycle (54 µmol m^{-2} s^{-1}) and 8 hours of darkness at a temperature of 24 ± 2 °C. The cleaning procedure began with washing the shoots' apical meristem, followed by brushing them with liquid soap and rinsing them with potable water. A final rinse with purified water

was then conducted. The shoots were subsequently transferred to an antifungal solution containing benomyl at a concentration of 1.0 mg/L for 30 minutes. After this time, the explants were rinsed with purified water to remove any residue from the antifungal solution. Finally, the plant material underwent disinfection under aseptic conditions (Forest et al. 2023).

2.4 *In Vitro* Aseptic Establishment of Explants

The pre-disinfested explants were placed in a laminar flow hood, where the disinfection process took place under aseptic conditions. The explants were immersed in a sodium hypochlorite (NaOCl) solution (Cloralex® at 6% active ingredient) at concentrations of 15% and 20% (v/v), supplemented with 0.02% Tween-20, for 10 minutes. Following this, the explants underwent three rinses with sterile double-distilled water. Each experimental unit consisted of four explants placed in a Magenta™ box, and they were incubated under controlled conditions: a 16-hour light cycle (at 54 µmol m^{-2} s^{-1}) followed by 8 hours of darkness, maintaining a temperature of 24 ± 2 °C. The experimental design for this establishment phase utilized a completely randomized design with a 3x2 factorial arrangement. Factor A consisted of the potato varieties, specifically *Fianna* and *Ágata*, while Factor B represented the concentration of the disinfecting agent. This resulted in a total of five treatments with 10 replications each. Over eight weeks, the variables of oxidation, contamination, and asepsis of the explants were evaluated. The results were analyzed using non-parametric statistics, mainly through Contingency Tables, and the Chi-square test (X^2) was employed for evaluation.

2.5 Shoot Induction and Proliferation

Sterilized explants were cultured in sterile glass containers containing MS medium with varying concentrations of BAP and GA3. Cultures were maintained in a growth chamber under controlled conditions: a photoperiod of 16 hours of light and 8 hours of darkness, a temperature of 24±2°C, and a light intensity of 50 µmol m^{-2} s^{-1} provided by cool-white, fluorescent tubes. Shoot induction was monitored weekly, and data were recorded for parameters such as shoot length, the number of shoots per explant, and oxidation levels. Oxidation severity was visually assessed, and strategies to minimize oxidation, such as frequent sub-culturing and antioxidant treatments, were applied when necessary.

2.6 Rooting Stage

Once shoots reached a 3-4 cm height, they were excised and transferred to MS medium supplemented with naphthalene acetic acid (NAA) at concentrations of 0.01 and 0.04 mg/L to promote root development (Table 1). Cultures were maintained under similar environmental conditions as the shoot induction stage. Root length, the number of roots per shoot, and overall root morphology were recorded after three weeks.

2.7 Microtuber Production

Shoots from well-rooted plants were transferred to MS medium containing 8% sucrose and varying concentrations of BAP and GA3 to promote microtuber formation. The cultures were kept under reduced light conditions, with 8 hours of light and 16 hours of darkness, at a temperature of 20±2°C to encourage tuberization (Table 2).

2.8 Data Analysis

All experiments were carried out using a completely randomized design, consisting of five treatments with ten replications for each treatment. Quantitative data was collected on shoot length, the number of shoots, rooting efficiency, oxidation levels, and microtuber yield. Statistical analysis was conducted using ANOVA, and mean comparisons were performed with Tukey's HSD test, set at a significance level of $p < 0.05$.

Table 1 Treatments applied during the multiplication stage for two potato varieties (Fianna and Ágata) of *S. tuberosum.*

Variety	Treatment[#]	NAA (mg/l)	GA_3 (mg/l)	BAP (mg/l)
Ágata	1	0.00	0.00	0.00
	2	0.01	0.25	0.10
	3	0.02	0.50	0.20
	4	0.03	0.75	0.30
	5	0.04	1.00	0.40
Fianna	1	0.00	0.00	0.00
	2	0.01	0.25	0.10
	3	0.02	0.50	0.20
	4	0.03	0.75	0.30
	5	0.04	1.00	0.40

Table 2 Treatments used during the microtuber production stage for two potato varieties (Fianna and Ágata) of *S. tuberosum*.

Variety	Treatment[#]	Sucrose (g/L)	GA_3 (mg/l)	BAP (mg/l)
Ágata	1	30	0.00	0.00
	2	80	0.25	0.10
	3	90	0.50	0.20
	4	100	0.75	0.30
	5	110	1.00	0.40
Fianna	1	30	0.00	0.00
	2	80	0.25	0.10
	3	90	0.50	0.20
	4	100	0.75	0.30
	5	110	1.00	0.40

3 Results and Discussion

3.1 Stage of Aseptic Establishment of Explants

3.1.1 Effect of Oxidation on Potato Varieties

The Chi-square test yielded a value of 8.128, which was higher than the tabulated value for p = 0.05. This led to the conclusion that the varieties displayed significant differences in oxidation levels. Notably, the Ágata variety exhibited the highest oxidation percentage at 63.63%, surpassing the Fianna variety with an oxidation percentage of 46.1% (Table 3). These results align with the findings of Smith et al. (2023) and García and López (2022), who noted that the in vitro establishment of potatoes (*S. tuberosum*) is typically characterized by a low percentage of aseptic explants and a high incidence of obscured explants. Furthermore, contamination and oxidation of donor tissue have been identified as significant challenges in the micropropagation of woody plants (Bettoni et al. 2024; Mohamed and Girgis 2023).

3.1.2 Effect of NaOCl on Oxidation of the Potato Explants

The results of the sodium hypochlorite (NaOCl) treatment on the oxidation of two potato varieties, Fianna and Ágata, reveal significant differences in their effects. This is supported by a calculated Chi-square value of 6.39, which exceeds the tabulated value at p = 0.05. This finding suggests that the doses of NaOCl significantly influence the degree of oxidation in the plants, indicating a dose-dependent response. The treatment with 20% NaOCl prevented oxidation, as 58.3% of the plants remained non-oxidized. In contrast, the 15% NaOCl treatment resulted in only 28.5% of the plants remaining non-oxidized. These results underscore the impact of NaOCl concentration on plant tissue and its ability to minimize oxidation, which is essential for preventing damage during processes such as sterilization or tissue culture propagation (Murashige and Skoog 1962). Previous studies have consistently shown that NaOCl concentration is crucial in determining its effectiveness. For instance, research has demonstrated that higher concentrations of NaOCl are generally more effective at sterilizing plant tissues. However, they can induce oxidative stress or damage if not carefully controlled (Yildiz et al. 2012).

3.1.3 Contamination

The chi-square test results that compare contamination levels among the potato varieties (Fianna and Ágata) and different

Table 3 Comparison of oxidation, contamination, and asepsis rates between *Fianna* and *Ágata* potato varieties under different NaOCl concentrations.

Parameter	Fianna	Ágata	Statistical Results
Oxidation Percentage	46.1%	63.63%	Chi-square = 8.128, $p < 0.05$
Contamination Rate	40%	100%	Chi-square = 35.52, $p < 0.05$
Asepsis Percentage	60%	0%	Chi-square = 35.52, $p < 0.05$
Effect of NaOCl (15% concentration)	28.5% non-oxidized	28.5% non-oxidized	Chi-square = 6.39, $p < 0.05$
Effect of NaOCl (20% concentration)	58.3% non-oxidized	58.3% non-oxidized	Chi-square = 6.39, $p < 0.05$
NaOCl Concentration Effect on Contamination	1.63 (no significant effect)	1.63 (no significant effect)	Chi-square = 1.63, $p > 0.05$

concentrations of NaOCl provide significant insights into the sterilization process. The chi-square value of 35.52 for contamination among the varieties was much higher than the tabulated value of 9.21 (p=0.05), indicating that Fianna had a significantly lower contamination rate of 40% compared to Ágata, which had a contamination rate of 100% (Table 3). This finding aligns with previous research suggesting that different potato varieties may exhibit varying resistance to contamination due to genetic factors, such as surface properties or antimicrobial compounds (Nagy et al. 2023). Conversely, the chi-square test for NaOCl concentrations yielded a value of 1.63, lower than the tabulated value of 3.84 (p=0.05). This indicates that the NaOCl concentrations used were ineffective in preventing contamination, highlighting the need to optimize sterilization protocols. As Nagy et al. (2023) emphasized, sterilization treatments' effectiveness depends on factors such as concentration and exposure time, which must be carefully adjusted to improve outcomes in tissue culture practices.

3.1.4 Asepsis

The chi-square test to assess environmental asepsis among different potato varieties revealed a significant difference in contamination resistance. The variety Fianna exhibited 60% asepsis, while Ágata displayed 0%, indicating that Fianna has a higher resistance to contamination. The calculated chi-square value was 35.52, which surpassed the tabulated value of 9.21 (p=0.05), demonstrating Fianna's superior ability to maintain aseptic conditions. In contrast, no significant difference was found between the tested NaOCl concentrations. The chi-square value for these concentrations was 1.63, lower than the tabulated value of 3.84 (p=0.05). This suggests that the NaOCl concentrations used in the experiment were insufficient to control contamination (Table 3) effectively. These results underscore the need to optimize NaOCl concentrations to ensure effective sterilization in tissue culture. Nagy et al. (2023) recommended that future studies investigate higher NaOCl concentrations or consider alternative sterilization methods to enhance asepsis.

3.2 Shoot Induction, Proliferation, and Multiplication

Under various hormonal treatments, the study observed significant differences in shoot induction, proliferation, and multiplication between the potato varieties Ágata and Fianna. The effects of hormonal combinations consisting of NAA (naphthalene acetic acid), GA3 (gibberellic acid), and BAP (benzylaminopurine) were tested to evaluate their impact on the number of shoots, shoot length, and node number per shoot (Table 4). The number of shoots per explant varied significantly between the two varieties and the treatments applied. Ágata demonstrated the highest shoot number (5.237) under Treatment 4 (0.01 mg/L NAA, 0.20 mg/L GA3, and 0.50 mg/L BAP), while Fianna produced the highest shoot number (6.233) in Treatment 5 (0.01 mg/L NAA, 0.20 mg/L GA3, and 1.00 mg/L BAP). The higher concentration of BAP in Treatment 5 was particularly effective for Fianna, leading to a more significant number of shoots. This finding aligns with previous studies, such as Vázquez-Martínez et al. (2022), which highlighted that increased BAP concentrations in potato cultivars enhance shoot multiplication, indicating that BAP plays a crucial role in shoot proliferation. Regarding shoot length, Fianna exhibited the longest shoots in Treatment 5 (5.293 cm), significantly surpassing those in other treatments. In contrast, Ágata produced the longest shoots in Treatment 1 (control), with an average length of 5.551 cm. The longer shoot length observed in Fianna may be attributed to the hormonal combination in Treatment 5, particularly the higher concentration of BAP, which is known to promote shoot elongation (Srivastava et al., 2012; Hussain et al., 2023). Notably, Ágata did not show significant

Table 4 Shoot induction, proliferation, and length in *Agata* and *Fianna* potato varieties under different hormonal treatments.

Variety	Treatment #	NAA (mg/l)	GA_3 (mg/l)	BAP (mg/l)	Shoots number/ explant	Shoot length (cm)	Nodes number/shoot
Ágata	1	0.00	0.00	0.00	3.485^{b}	5.551^{a}	4.740^{a}
	2	0.01	0.25	0.10	4.342^{b}	4.688^{b}	4.829^{a}
	3	0.02	0.50	0.20	3.683^{b}	4.400^{b}	4.987^{a}
	4	0.03	0.75	0.30	5.237^{a}	4.077^{b}	4.775^{a}
	5	0.04	1.00	0.40	3.977^{b}	4.560^{b}	5.507^{a}
Fianna	1	0.00	0.00	0.00	3.844^{c}	4.928^{b}	2.962^{b}
	2	0.01	0.25	0.10	3.447^{c}	2.133^{d}	3.740^{b}
	3	0.02	0.50	0.20	4.173^{b}	3.267^{c}	2.015^{c}
	4	0.03	0.75	0.30	5.080^{a}	4.113^{b}	4.167^{a}
	5	0.04	1.00	0.40	6.233^{a}	5.293^{a}	4.693^{a}

†Averages with the same letter in each column show no significant difference, Tukey sig. $P \leq 0.05$.

differences in shoot length across the treatments, although Treatment 4 (0.01 mg/L NAA, 0.20 mg/L GA3, and 0.50 mg/L BAP) resulted in relatively longer shoots than the other treatments. Regarding node number, Ágata produced the highest number of nodes per shoot (5.507) in Treatment 5, although no significant differences were observed across the other treatments for node production. For Fianna, Treatment 5 also resulted in the highest node count (4.693). These results suggest that Treatment 5 promoted greater shoot multiplication and facilitated better node formation. The increase in nodes per shoot in both varieties under Treatment 5 supports the beneficial effect of BAP on node production, as reported in previous studies (Sota et al. 2020).

3.3 Rooting Efficiency

The effects of sucrose, GA3 (gibberellic acid), and BAP (benzylaminopurine) on root formation in the potato varieties Ágata and Fianna were evaluated, focusing on root number and root length (Table 5). For Ágata, Treatment 5 (sucrose 110 g/L, GA3 1.00 mg/L, BAP 0.40 mg/L) resulted in the highest root formation, with 7.100 roots and a root length of 5.260 cm, indicating that the combination of higher sucrose concentration and hormones was most effective in promoting root growth. Treatment 4 (sucrose 100 g/L, GA3 0.75 mg/L, BAP 0.30 mg/L) also supported significant root growth, producing 5.810 roots and a root length of 4.125 cm. In comparison, Treatment 3 (sucrose 90 g/L, GA3 0.50 mg/L, BAP 0.20 mg/L) resulted in fewer and shorter roots (3.956 roots and a length of 2.780 cm), suggesting that higher BAP concentrations may inhibit root formation, as found by Amghar et al. (2021). For Fianna, Treatment 4 (sucrose 100 g/L, GA3 0.75 mg/L, BAP 0.30 mg/L) resulted in the highest root number (6.254 roots) and root length (3.837 cm), demonstrating that moderate GA3 and BAP concentrations were optimal for root development in this variety. Treatment 2 (sucrose 80 g/L, GA3 0.25 mg/L, BAP 0.10 mg/L) also promoted good root growth (4.785 roots and a root length of 3.402 cm), though less effective than Treatment 4. Treatment 3 (sucrose 90 g/L, GA3 0.50 mg/L, BAP 0.20 mg/L) showed weaker root growth (5.134 roots and a length of 2.931 cm), further supporting the idea that excessive BAP may not always be beneficial for rooting, as indicated by Zhang et al. (2005). In summary, Treatment 5 consistently produced the best root formation in Ágata (7.100 roots, 5.260 cm). In comparison, Treatment 4 was optimal for Fianna (6.254 roots, 3.837 cm), highlighting the importance of balancing sucrose and plant hormone concentrations to promote efficient root growth. These results support the role of sucrose as an energy source and GA3 and BAP in regulating root development (Kumlay 2014; Pasternak and Steinmacher 2024).

3.4 Optimizing Microtuber Formation: Influence of sucrose, GA_3, BAP, and culture medium on *Ágata* and *Fianna* varieties

The study examined how different concentrations of sucrose, gibberellic acid (GA3), and benzylaminopurine (BAP) affect microtuber production in the Ágata and Fianna varieties. Key factors evaluated included the average number of microtubers, microtuber diameter, and average microtuber weight. The data revealed significant variation in microtuber formation among the different treatments and varieties (Figure 2).

In the Ágata variety, Treatment 2 (sucrose 80 g/L, GA3 0.25 mg/L, BAP 0.10 mg/L) yielded the highest average number of microtubers (2.159), with the largest microtuber diameter (21.7 mm) and the highest average microtuber weight (1.45 g). This indicates that a moderate combination of sucrose and growth hormones significantly enhanced microtuber production in quantity and size. Treatment 4 (sucrose 100 g/L, GA3 0.75 mg/L, BAP 0.30 mg/L) also resulted in a relatively high production of microtubers

Table 5 Effect of different sucrose, GA3, and BAP concentrations on root formation in *Ágata* and *Fianna* varieties.

Variety	Treatment #	Sucrose (g/L)	GA_3 (mg/l)	BAP (mg/l)	Number of Roots	Root length (cm)
Ágata	1	30	0.00	0.00	4.501^{c}	2.325^{d}
	2	80	0.25	0.10	6.233^{b}	3.000^{c}
	3	90	0.50	0.20	3.956^{c}	2.780^{d}
	4	100	0.75	0.30	5.810^{b}	4.125^{b}
	5	110	1.00	0.40	7.100^{a}	5.260^{a}
Fianna	1	30	0.00	0.00	3.234^{d}	2.012^{d}
	2	80	0.25	0.10	4.785^{c}	3.402^{b}
	3	90	0.50	0.20	5.134^{b}	2.931^{d}
	4	100	0.75	0.30	6.254^{a}	3.837^{a}
	5	110	1.00	0.40	4.032^{c}	3.102^{c}

†Averages with the same letter in each column show no significant difference, Tukey sig. $P \leq 0.05$.

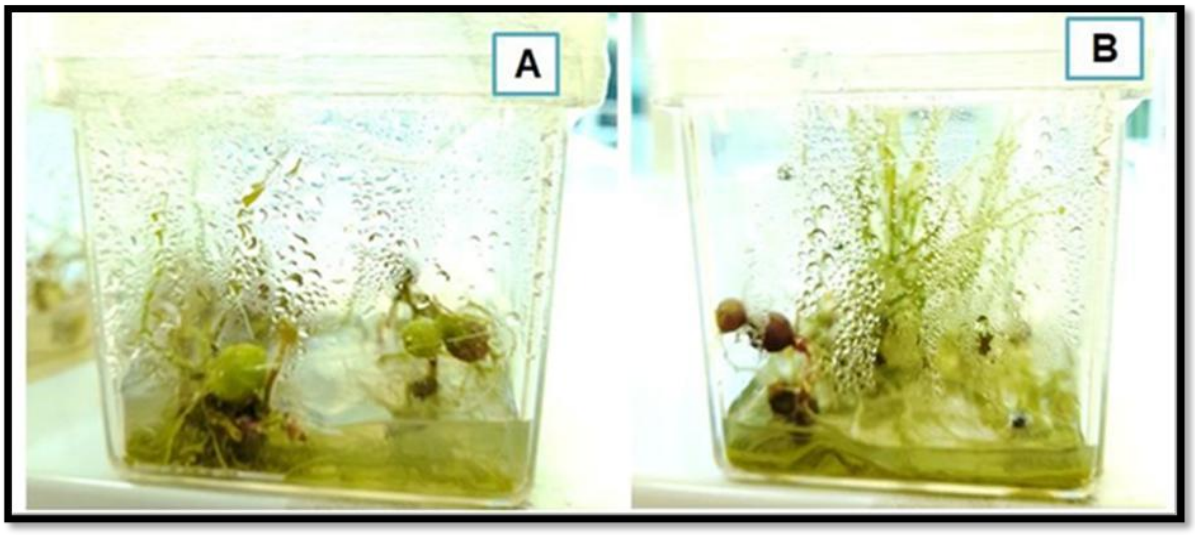

Figure 2 Effect of sucrose, GA_3, and BAP on microtuber number, diameter, and weight in *Ágata* and *Fianna* varieties.

Table 6 Effect of sucrose, GA_3, and BAP on microtuber number, diameter, and weight in *Ágata* and *Fianna* varieties.

Variety	Treatment #	Sucrose (g/L)	GA3 (mg/l)	BAP (mg/l)	Average microtuber number	Microtuber Diameter (mm)	Average microtuber weight (g)
Ágata	1	30	0.00	0.00	1.173^{c}	18.5^{b}	1.23^{ab}
	2	80	0.25	0.10	2.159^{a}	21.7^{a}	1.45^{a}
	3	90	0.50	0.20	1.692^{b}	16.9^{c}	0.98^{c}
	4	100	0.75	0.30	1.404^{c}	20.3^{a}	1.35^{a}
	5	110	1.00	0.40	1.303^{c}	19.1^{ab}	1.10^{b}
Fianna	1	30	0.00	0.00	1.201^{c}	15.2^{d}	0.85^{d}
	2	80	0.25	0.10	2.284^{b}	18.7^{c}	1.10^{c}
	3	90	0.50	0.20	2.692^{a}	22.3^{b}	1.65^{b}
	4	100	0.75	0.30	1.392^{b}	25.0^{a}	2.02^{a}
	5	110	1.00	0.40	1.311^{b}	19.6^{c}	1.35^{c}

†Averages with the same letter in each column show no significant difference, Tukey sig. $P \leq 0.05$.

(1.404) and a microtuber diameter of 20.3 mm, with an average weight of 1.35 g. These results align with previous studies suggesting that increased sucrose concentration, combined with GA3 and BAP, promotes microtuber growth (Mohamed and Girgis 2023). In contrast, Treatment 3 (sucrose 90 g/L, GA3 0.50 mg/L, BAP 0.20 mg/L) produced a significantly lower number of microtubers (1.692) and a smaller diameter (16.9 mm), with a lower average weight (0.98 g). This finding suggests that higher levels of BAP may inhibit microtuber formation, consistent with the observed cytokinin-induced inhibition of tuberization (Kumlay 2014; García-García et al. 2019).

For the Fianna variety, Treatment 3 (sucrose 90 g/L, GA3 0.50 mg/L, BAP 0.20 mg/L) produced the highest number of microtubers (2.692) and the largest microtuber diameter (22.3 mm), along with an average weight of 1.65 g. This indicates that a lower sucrose concentration and a higher BAP concentration effectively promoted tuberization in the Fianna variety. Treatment 4 (sucrose 100 g/L, GA3 0.75 mg/L, BAP 0.30 mg/L) yielded a large microtuber diameter of 25.0 mm and an average weight of 2.02 g, although the average number of microtubers was lower (1.392) compared to Treatment 3. This suggests that higher concentrations of sucrose and GA3 promote fewer microtubers, resulting in larger sizes. In contrast, Treatment 1 (sucrose 30 g/L, GA3 0.00 mg/L, BAP 0.00 mg/L) produced the lowest number of microtubers (1.201) and the smallest diameter (15.2 mm), with the lowest average weight (0.85 g). This outcome shows that low sucrose levels and the absence of growth regulators are not conducive to microtuber development (Hossain et al. 2017; Gautam et al. 2021).

3.5 Varietal Differences

The study found significant differences between the Ágata and Fianna potato varieties. Ágata exhibited higher oxidation rates (63.63%) than Fianna (46.1%) and was more susceptible to contamination, with a 100% contamination rate in contrast to Fianna's 40%. Additionally, Fianna demonstrated better aseptic performance, achieving 60% asepsis, while Ágata recorded 0%. Regarding shoot induction, Fianna responded more effectively to higher concentrations of BAP, whereas Ágata performed better with more balanced hormone treatments. Ágata also showed superior root formation when exposed to higher sucrose treatments, while Fianna thrived with moderate hormone concentrations.

Conclusions

In conclusion, *in vitro* propagation of the potato varieties *Ágata* and *Fianna* demonstrated significant differences in response to various sterilization, hormonal treatments, and culture conditions. The pre-disinfestation and sterilization treatments, particularly with sodium hypochlorite (NaOCl), revealed that *Fianna* exhibited lower oxidation and contamination rates than *Ágata*, which showed higher susceptibility to oxidation and contamination. Fianna responded better to higher BAP concentrations for shoot induction and proliferation, particularly in moderate GA_3 levels, producing more shoots and longer shoots than *Ágata*. Rooting efficiency was also optimized in both varieties with higher sucrose and hormone concentrations, with *Ágata* showing the best results at a higher sucrose concentration of 110 g/L. Microtuber production was most successful in both varieties with moderate sucrose and BAP concentrations, with *Fianna* producing the highest number of microtubers under specific treatment conditions. The results underscore the importance of optimizing sterilization protocols and hormonal treatments to improve the efficiency of *in vitro* propagation and microtuber production for both potato varieties, contributing valuable insights to the micropropagation field.

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Ethical Approval

Not applicable to this study.

References

Abu Zeid, S. M., Soliman, H. I. A., & Metwali, E. M. R. (2022). In vitro evaluation of some high yield potato (*Solanum tuberosum* L.) cultivars under imposition of salinity at the cellular and organ levels. *Saudi Journal of Biological Sciences*, *29*(4), 2541-2551. https://doi.org/10.1016/j.sjbs.2021.12.040.

Aksoy, E., Orozco, J. M., López, J., Yildirim, N., & Acar, T. (2021). Recent advances in potato (Solanum tuberosum L.) breeding. In J. M. Al-Khayri, S. M. Jain, & D. V. Johnson (Eds.), *Advances in Plant Breeding Strategies: Vegetable Crops* (pp. 169-203). Springer, Cham. https://doi.org/10.1007/978-3-030-66965-2_10.

Amghar, I., Diria, G., Boumlik, I., El Mouden, E. H., Khouya, E., El Modafar, C., & Ait Barka, E. (2021). An efficient regeneration pathway through adventitious organogenesis for the endangered *Argania spinosa* (L.) Skeels. *Vegetos*, *34*(3), 355–367. https://doi.org/10.1007/s42535-021-00208-y.

Anjum, M. A., & Ali, H. (2004). Effect of culture medium on direct organogenesis from different explants of various potato genotypes. *Biotechnology*, *10*, 187-193. https://doi.org/10.3923/biotech.2004.187.193.

Bettoni, J. C., Wang, M. R., & Wang, Q. C. (2024). In vitro regeneration, micropropagation and germplasm conservation of horticultural plants. *Horticulturae*, *10*(1), 45. https://doi.org/10.3390/horticulturae10010045.

Bustos, R., & Carrillo, L. (2017). Genetic and environmental factors affecting the differentiation of apical meristems into various organ systems in potato (*Solanum tuberosum* L.). *Journal of Plant Physiology*, *216*, 58-67. https://doi.org/10.1016/j.jplph.2017.07.008.

Coleman, M., Waugh, R., & Powell, W. (1990). Genetical analysis of in vitro cell and tissue culture response in potato. *Plant Cell, Tissue and Organ Culture*, *23*, 181-186. https://doi.org/10.1007/BF00034429.

De Koeyer, D., & Harding, K. (2019). The potato tuber: From plant to table. In J. M. Bradeen & S. M. De Jong (Eds.), *Genetics and Genomics of Potato* (pp. 261-278). Springer, Cham. https://doi.org/10.1007/978-3-030-23134-3_12.

Develi, B. E., & Miler, N. (2023). Impact of light quality on in vitro potato microtuber formation. *International Journal of Horticultural Science and Technology*, *10*(special issue, light in horticulture), 89-100. https://doi.org/10.22059/ijhst.2023.353476.613.

Dobránszki, J., Hidvégi, N., Gulyás, A., & Teixeira da Silva, J. A. (2019). mRNA transcription profile of potato (*Solanum tuberosum* L.) exposed to ultrasound during different stages of in vitro plantlet development. *Plant Molecular Biology*, *100*(4-5), 511-525. https://doi.org/10.1007/s11103-019-00876-0.

Dolničar, P. (2021). Importance of potato as a crop and practical approaches to potato breeding. In D. Dobnik, K. Gruden, Ž. Ramšak, & A. Coll (Eds.), *Solanum tuberosum: Methods in Molecular Biology* (Vol. 2354, pp. 1-17). Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1609-3_1.

Forest, M. J., Ojeda Zacarías, M. D. C., Saldaña, H. L., Alvarado, R. E. V., Sáenz, E. O., & Cham, A. K. (2023). The impact of elicitation on potato (*Solanum tuberosum* L.) production, enzymatic and antioxidant activity in Nuevo León, Mexico. *Journal of Experimental Biology and Agricultural Sciences*, *11*(3), 572–580. https://doi.org/10.18006/2023.11(3).572.580.

García, J., & López, E. (2022). In vitro propagation and establishment of nodal segments in guava (*Psidium guajava* L.). *Journal of Plant Biotechnology and Microbiology*, *9*(1), 45-52. https://doi.org/10.5897/JPBM2021.0076.

García-García, J. A., Azofeifa-Bolaños, J. B., Solano-Campos, F., & Orozco-Rodríguez, R. (2019). Effect of two cytokinins and a growth inhibitor on the in vitro tuberization of two genotypes of *Solanum tuberosum* L. cvs. Atlantic and Alpha. *Uniciencia*, *33*(2), 1-12. https://dx.doi.org/10.15359/ru.33-2.1.

García-González, R., & Quiroz, K. A. (2018). Impact of plant growth regulators on shoot regeneration from potato (*Solanum tuberosum* L.) explants. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *134*(3), 439-451. https://doi.org/10.1007/s11240-018-1435-2.

Gautam, S., Solis-Gracia, N., Teale, M. K., Mandadi, K., da Silva, J. A., & Vales, M. I. (2021). Development of an in vitro microtuberization and temporary immersion bioreactor system to evaluate heat stress tolerance in potatoes (*Solanum tuberosum* L.). *Frontiers in Plant Science*, *12*, 700328. https://doi.org/10.3389/fpls.2021.700328.

Hasnain, A., Naqvi, S. A. H., Ayesha, S. I., Khalid, F., Ellahi, M., et al. (2022). Plants in vitro propagation with its applications in food, pharmaceuticals, and cosmetic industries; current scenario and future approaches. *Frontiers in Plant Science*, *13*, 1009395. https://doi.org/10.3389/fpls.2022.1009395.

Heuberger, A. L., Joshi, J. R., & Toulabi, S. (2022). Potatoes. In J. P. Miller & C. Van Buiten (Eds.), *Superfoods: Food and Health* (pp. 201-220). Springer, Cham. https://doi.org/10.1007/978-3-030-93240-4_13.

Hossain, M. S., Hossain, M. M., Hossain, T., Haque, M. M., Zakaria, M., & Sarkar, M. D. (2017). Varietal performance of potato on induction and development of microtuber in response to sucrose. *Annals of Agricultural Sciences*, *62*(1), 75–81. https://doi.org/10.1016/j.aoas.2017.05.002.

Hussain, S., Khan, A., Ahmad, N., Shabbir, G., Aziz, M. A., Rauf, M. A., & Riaz, M. (2023). In vitro micropropagation and micro-tuberization potential of selected potato (*Solanum tuberosum* L.) varieties. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *135*(2), 289-302. https://doi.org/10.1007/s11240-023-02125-8.

Kumlay, A. M. (2014). Combination of the auxins NAA, IBA, and IAA with GA3 improves the commercial seed-tuber production of potato (*Solanum tuberosum* L.) under in vitro conditions. *Biomed Research International*, *2014*, 439259. https://doi.org/10.1155/2014/439259.

Mohamed, A. E. S., & Girgis, N. D. (2023). Factors affecting in vitro tuberization of potato. *Bulletin of the National Research Centre*, *47*, 80. https://doi.org/10.1186/s42269-023-00720-6.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473-494. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nagy, A. M., Oros, P., Cătană, C., Antofie, M. M., & Sand, C. S. (2023). In vitro cultivation of purple-fleshed potato varieties: Insights into their growth and development. *Horticulturae*, *9*(4), 425. https://doi.org/10.3390/horticulturae9040425.

Navarro, C., Abelenda, J. A., Cruz-Oró, E., & Cuéllar, C. A. (2015). Control of potato tuberization by StSP6A. *Nature*, *482*(7384), 232-235. https://doi.org/10.1038/nature10703.

O'Brien, L., & McCleary, M. (2023). Advances in vegetative propagation techniques for potato: A review. *Potato Research*, *66*(1), 1-18. https://doi.org/10.1007/s11540-022-09479-3.

Pasternak, T. P., & Steinmacher, D. (2024). Plant growth regulation in cell and tissue culture in vitro. *Plants*, *13*(2), 327. https://doi.org/10.3390/plants13020327.

Permadi, N., Akbari, S. I., Prismantoro, D., Indriyani, N. N., Nurzaman, M., Alhasnawi, A. N., Doni, F., & Julaeha, E. (2024). Traditional and next-generation methods for browning control in plant tissue culture: Current insights and future directions. *Current Plant Biology*, *38*, 100339. https://doi.org/10.1016/j.cpb.2024.100339.

Ritonga, F. N., Zhou, D., Zhang, Y., Song, R., Li, C., Li, J., & Gao, J. (2023). The roles of gibberellins in regulating leaf development. *Plants (Basel)*, *12*(6), 1243. https://doi.org/10.3390/plants12061243.

SIAP. (2024). Nuevo León Agri-Food Infographic. [Online]. Retrieved from https://www.gob.mx/siap/acciones-y-programas/produccion-agricola-33119.

Šmeringai, J., Schrumpfová, P. P., & Pernisová, M. (2023). Cytokinins – regulators of de novo shoot organogenesis. *Frontiers in Plant Science*, *14*. https://doi.org/10.3389/fpls.2023.1239133.

Smet, W., & Beeckman, T. (2019). Organogenesis in plants: Initiation and elaboration of leaves. *Trends in Plant Science*, *24*(6), 512-526. https://doi.org/10.1016/j.tplants.2019.03.004.

Smith, A., Jones, B., & Johnson, C. (2023). In vitro culture of potato (Solanum tuberosum L.): Recent advances and applications. *Plant Cell Reports*, *42*(5), 701-715. https://doi.org/10.1007/s00299-023-02468-z.

Sota, V., Bekheet, S., & Kongjika, E. (2020). Effect of growth regulators on micropropagation and in vitro tuberization of Solanum tuberosum L. cv. Vermosh. *South Western Journal of Horticulture, Biology and Environment*, *11*(2), 67-81. https://doi.org/10.17660/swjhbe.2020.11.2.e20106.

Srivastava, A. K., Diengdoh, L. C., Rai, R., Bag, T. K., & Singh, B. P. (2012). In vitro micropropagation and micro-tuberization potential of selected potato varieties. *Indian Journal of Hill Farming*, *25*(2), 14-17. Retrieved from https://kiran.nic.in/pdf/IJHF/Vol25_2/In_Vitro_Micropropagation.pdf.

Thomas, T. D. (2008). The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, *26*(6), 618-631. https://doi.org/10.1016/j.biotechadv.2008.08.003.

Vázquez-Martínez, F. F., González-Aguilar, G. A., Hernández-López, M., Martínez-Sánchez, L. M., & Torres-Pacheco, I. (2022). In vitro propagation and optimization of culture conditions for enhanced shoot proliferation in potato cultivars. *Journal of Plant Growth Regulation*, *41*(3), 479-489. https://doi.org/10.1007/s00344-022-10755-4.

Vlahova, M., Kondakova, V., & Atanassov, A. (2022). Role of apical dominance in clonal propagation of potato (Solanum tuberosum L.). *Bulgarian Journal of Agricultural Science*, *28*(1), 45-52. https://doi.org/10.3235/BJAS220712.

Yildiz, M., Ozcan, S. F., Kahramanogullari, T., & Tuna, E. (2012). Title of the article. *The Natural Products Journal*, *2*(4), 328-331. https://doi.org/10.2174/2210315511202040328.

Zhang, Z., Zhou, W., & Li, H. (2005). The role of GA, IAA, and BAP in the regulation of in vitro shoot growth and microtuberization in potato. *Acta Physiologiae Plantarum*, *27*(3), 363–369. https://doi.org/10.1007/s11738-005-0013-7.

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Carbazole alkaloids Koenigicine, Koenigine, Mahanine and Mukonicineas Multi-Target Inhibitors in Triple-Negative Breast Cancer: Insights into MMP9, MMP13, EGFR, and NUDT5 Interactions through Molecular Docking

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MMP9

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ABSTRACT

Plant-based natural products have been widely used for treating and preventing diseases due to their nutritional and pharmacological benefits, significantly improving the health and well-being of individuals. These medicinal plants are also easily accessible and offer a low-cost, less harmful source for developing new medications. Breast cancer is the second most common form of cancer reported in women worldwide. The treatment of triple-negative breast cancer (TNBC) remains challenging, as this subtype lacks targeted therapeutics. TNBC accounts for approximately 15-20% of newly diagnosed breast cancer cases. Because TNBC tumors do not express estrogen receptors (ER), progesterone receptors (PR), or human epidermal growth factor receptor 2 (HER2), patients with TNBC do not benefit significantly from treatments aimed at ER, PR and HER2-positive breast tumors. While TNBC initially responds well to chemotherapy, it often develops resistance over time, complicating disease management and presenting a significant clinical challenge. To address therapy resistance and improve patient outcomes, exploring new therapeutic options for TNBC is essential. This molecular docking study

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EGFR

NUDT5

shows strong interactions between the carbazole alkaloids Koenigicine, Koenigine, Mahanine, and Mukonicine with key oncogenic protein targets such as MMP9, MMP13, NUDT5, and EGFR, which are associated with TNBC progression. The binding energy of these molecules ranges from -7.4 to -9.9 kcal/mol, indicating a very high potential for inhibition. Mahanine exhibits the highest binding affinity for all tested targets, demonstrating strong interactions with NUDT5 (-9.8 kcal/mol) and EGFR (-9.9 kcal/mol). This suggests its potential role as a multi-target inhibitor. The primary non-covalent interactions that contribute to the binding of carbazole alkaloids with target proteins include Van der Waals forces, hydrogen bonds, alkyl interactions, π-alkyl interactions, and π-π stacking. These interactions are crucial for stabilizing the ligand-protein complexes, enhancing binding affinity, and likely influencing the inhibitory effects of the compounds on TNBC-associated oncogenic proteins. The results of this study highlight the potential role of carbazole alkaloids in TNBC treatment, warranting further experimental validation.

1 Introduction

In the modern era, researchers and the general public are increasingly interested in plant-derived natural products for their roles in disease prevention, treatment, and overall health improvement. This surge in interest is mainly due to their nutritional value and significant pharmacological properties. Medicinal plants are widely accessible and provide a low-cost, less harmful alternative for new medication development (Atanasov et al. 2015; Yuan et al. 2016). With a long history dating back to ancient civilizations, plant-based traditional medicine has been a cornerstone of healthcare in many Asian societies. Nearly all traditional medicines rely on plant materials to formulate and synthesize various medications. Current medical practices are profoundly influenced by the rapid development of pharmacologically active drugs derived from medicinal herbs (Thomford et al. 2018). For example, natural ingredients or their analogs account for approximately 60% of all medications currently used to treat cancer (Newman and Cragg 2020). Due to their remarkable pharmacological properties, the use of plants in medicine has grown exponentially in recent years.

The Rutaceae family, which includes 1,600 species and 150 genera, features *Murraya koenigii* (commonly known as curry leaf) (Gaikwad et al. 2025). Research indicates that this plant is native to South Asia, particularly in Bangladesh, India, and Sri Lanka (Gaikwad et al. 2025). *M. koenigii* has been utilized between the first and fourth centuries AD and is traditionally considered stomachic and tonic (Ajay et al. 2011). The leaves, roots, bark, fruits, and seeds of *M. koenigii* contain various natural compounds identified through chromatography and spectroscopy techniques. Phytochemical analyses have isolated bioactive compounds such as terpenoids, alkaloids, flavonoids, coumarins, polyphenols, and essential oils (Balakrishnan et al. 2020).

Experimental studies on animals have shown that *M. koenigii* has hypolipidemic effects, resulting in significant reductions in triglyceride levels when included in their diet. The hypolipidemic properties of *M. koenigii* can be attributed to its phytochemicals, including carbazole alkaloids, terpenoids, flavonoids, saponins, and phenols (Pinto and Cojandaraj 2024). Additionally, *M. koenigii* exhibits hepatoprotective, antioxidant, anti-inflammatory, antimicrobial, nephroprotective, gastroprotective, cardioprotective, neuroprotective, wound-healing, Anticancer, and immunomodulatory properties, which can aid in recovery from liver damage (Gangawat et al. 2024).

Sadwal et al. (2023) demonstrated a significant protective effect against breast cancer induced by DMBA (dimethylbenz[α]anthracene) in rats. The hydroethanolic extract of *M. koenigii* (curry leaves) significantly reduced tumor incidence and volume compared to the group treated with DMBA alone. This extract could be a valuable ingredient in animal feed and medicinal applications. The presence of various bioactive compounds suggests that curry leaves may enhance health and nutrition. Notably, they contain 23.73% alkaloids, 1.24% flavonoids, 8.74% saponins, 4.4% phenolics, and 5.2% tannins (Arif et al. 2024). Alkaloids in plants play a crucial role in defending against biotic and abiotic stressors (Arif et al. 2024). Various parts of the *M. koenigii* plant, including the bark, roots, and leaves, are rich in beneficial compounds, particularly carbazole alkaloids, contributing to its medicinal properties (Patil et al. 2023). Additionally, *M. koenigii* extract is a promising new ingredient for cosmetic formulations to enhance skin elasticity and reduce sagging. Its ability to boost the production of key proteins involved in skin structure positions it as a valuable addition to anti-aging products (Lorion et al. 2023). Research has also found that this extract supports the recovery of biochemical markers and enzymes involved in metabolism, indicating its potential to improve liver function and overall health (Gangawat et al. 2024).

Breast cancer (BC) is the most commonly diagnosed cancer in women worldwide. Molecular subtypes of breast cancer are classified based on varying gene expressions, which influence their prognosis and available treatments. Triple-negative breast cancer (TNBC) is the only BC subtype that currently lacks targeted

therapies, accounting for 15-20% of new breast cancer cases (Zagami and Carey 2022). The standard treatment for TNBC is chemotherapy because the tumors do not express the estrogen receptor (ER), progesterone receptor (PR), or HER2. Although TNBC often shows a good initial response to conventional chemotherapy treatments, it frequently develops resistance to these medications, leading to a clinically challenging situation. Unfortunately, targeted therapies aimed at ER and HER2 are ineffective for these patients (Cheang et al. 2008; Leidy et al. 2014). Furthermore, TNBC has the lowest 5-year survival rate among breast cancer subtypes, underscoring the need for novel treatment options.

Chemotherapy resistance is a significant contributor to breast cancer mortality; despite initial positive responses, 50-80% of women with TNBC may relapse or develop resistance to chemotherapeutic drugs. The lack of targeted treatments and the emergence of chemotherapy resistance are two key factors contributing to higher mortality rates among TNBC patients. Chemoresistance can develop through various mechanisms, including complex interactions between the tumor microenvironment, drug efflux, cancer stem cells, and bulk tumor cells. Specific proteins such as ABC transporters can hinder the effectiveness of chemotherapy drugs by actively transporting the drugs out of cancer cells (Nedeljković and Damjanović 2019). Furthermore, chemotherapy can cause damage that DNA repair pathways can rectify, allowing cancer cells to survive (Bai et al. 2021). The epithelial-mesenchymal transition (EMT) may further enhance cancer cell resistance by enabling them to adopt a more invasive and migratory phenotype (Bai et al. 2021).

There is an urgent need to identify and characterize additional molecular mechanisms and downstream pathways crucial for the development of triple-negative breast cancer (TNBC), chemotherapy resistance, and recurrence. Chemotherapy not only destroys cancer cells but also harms healthy normal cells. Therefore, anticancer medications derived from plants with therapeutic qualities could be better alternatives to current treatment options, as they typically cause fewer side effects (Suvarna et al. 2024). Recently, there has been a growing awareness of the adverse effects of synthetic pharmaceuticals, leading more people to favor natural treatments or those made from plants or alkaloids. Pradhan et al. (2024) demonstrated through *in-silico* docking analysis that flavonoid molecules, particularly rutin, show significant binding energy with butyrylcholinesterase (BChE). This finding supports the potential therapeutic applications of the phytochemical constituents of *M. koenigii* for neurodegenerative diseases (Pradhan et al. 2024). An *in-silico* docking study by Samanta et al. (2024) established a high binding potential between important phytochemical constituents of *M. koenigii*, such as flavonoids and carbazole alkaloids, and caspase-3. Mahanine, a carbazole alkaloid from *M. koenigii*, has shown anticancer activity against most subtypes of breast cancer, likely due to its ability to alter cell cycle genes, particularly CDK4 and CDK6 (Sadwal et al. 2023). These results emphasize the significant potential of carbazole alkaloids to bind with proteins, further validating their biological functions and pharmaceutical applications. The current study aims to provide a viable solution based on the structural, biological, and pharmacological significance of the carbazole derivatives of *M. koenigii*. In this research, we selected four protein targets associated with TNBC: Matrix Metalloproteinase-9 (MMP9), Matrix Metalloproteinase-13 (MMP13), Nudix Hydrolase 5 (NUDT5), and Epidermal Growth Factor Receptor (EGFR). The carbazole alkaloids Koenigicine, Koenigine, Mahanine, and Mukonicine were chosen for docking studies to assess their binding affinity.

2 Computational Methods

In the current study, carbazole alkaloids Koenigicine, Koenigine, Mahanine, and Mukonicinewere taken from PubChem's biological database (NCBI 2024). IR spectra of the titled compounds were computed by using the online computational chemistry server WebMO (Version 24) and Mopac engine on webmo.net (https://www.webmo.net/demoserver/cgi-bin/webmo/jobmgr.cgi)(Polik and Schmidt 2022). Molecular docking was conducted using Mcule.com (Mcule 2024), an online drug discovery platform for molecular design and drug discovery (https://mcule.com/apps/1-click-docking/). The ligand-protein interactions were extracted and visualized using the Discovery Studio 4.5 software (Dassault Systèmes BIOVIA 2016).

3 Results and Discussion

3.1 IR Spectra Analysis

IR spectroscopy is a powerful bioanalytical method that can provide qualitative and quantitative information from various biological samples (Beć et al. 2020). The two most common types of vibrations observed in IR spectra are stretching vibrations, which occur between approximately 4000 and 1000 cm^{-1}, and bending vibrations, which range from around 1500 to 400 cm^{-1}. These vibrations are primarily examined in mid-infrared spectroscopy and are crucial in understanding molecular interactions (Ozaki 2021). For instance, in the case of aromatic rings, C=C stretching vibrations are detected in the range of 1400 to 1600 cm^{-1}. C−H stretching absorption occurs between 3100 and 3000 cm^{-1} for alkenes and aromatic compounds. In alkanes, the C−H stretching is observed around 3000 to 2850 cm^{-1}. Additionally, O−H and N−H stretching vibrations typically appear in the range of 3200 to 3600 cm^{-1} (Wang et al. 2023).

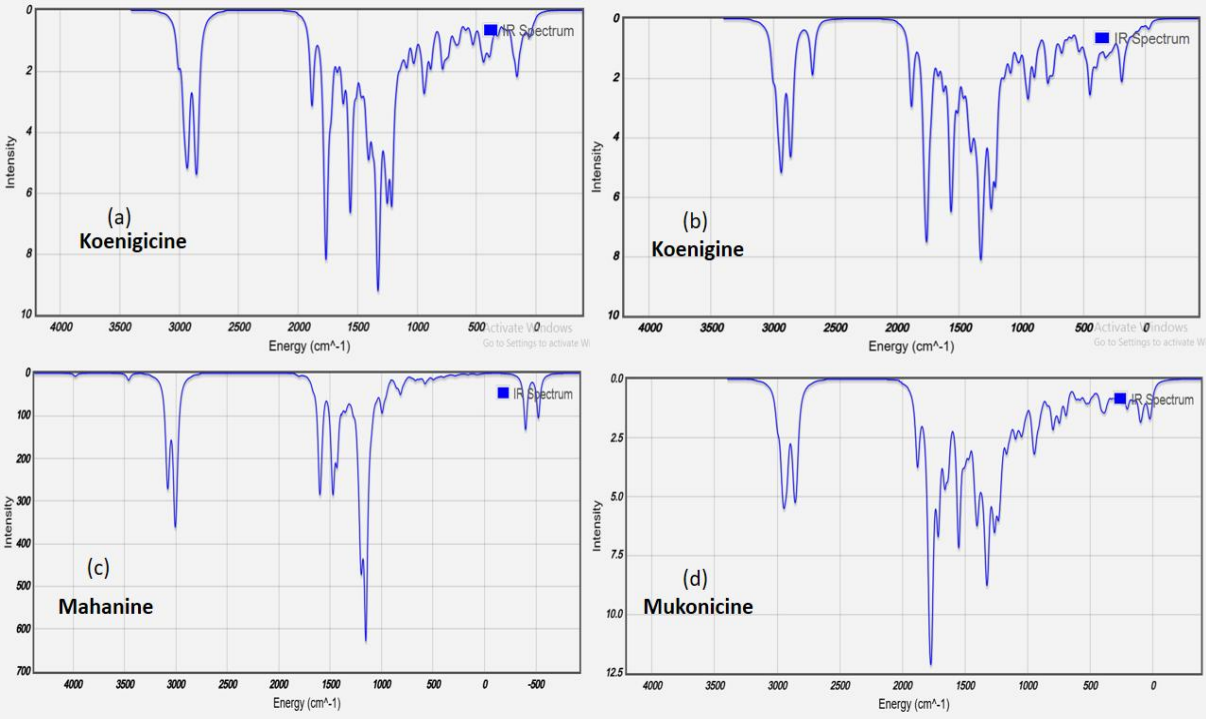


Figure 1 IR (infrared) spectra of the compounds Koenigicine, Koenigine, Mahanine, and Mukonicine. Each plot depicts the IR absorption intensities (y-axis) versus the energy (or wavenumber) in cm^{-1} (x-axis).

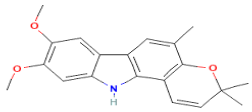
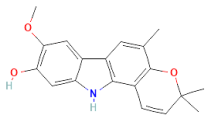
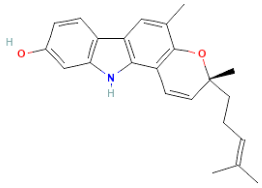
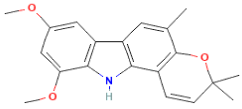
The computed IR spectra provide valuable information about the structural features of each compound, which in turn informs us about their interactions with the MMP9, MMP13, NUDT5, and EGFR proteins related to triple-negative breast cancer (TNBC). The IR spectra illustrated in Figure 1 confirm the presence of functional groups in each compound, suggesting potential binding interactions. The stretching frequencies of C-H, N-H, and C=C bonds observed in the IR spectra are crucial for determining the possibilities of hydrogen bonding, π-π interactions, and van der Waals forces within the molecules (Ghosh et al. 2020; Hansen et al. 2021). All the compounds studied exhibit C-H stretching in the 3000–2800 cm^{-1} region, indicating the presence of aliphatic or aromatic hydrogens. These hydrogens are likely to bind noncovalently within the hydrophobic binding pocket of the proteins. The IR spectra provide clear evidence of C-H, N-H, and C=C stretching, indicating that these molecules can form hydrogen bonds with carbonyl groups or other hydrogen bond acceptors present in the protein framework, as well as engage in π-π stacking interactions with aromatic residues.

3.2 Molecular Docking - Binding Energy

Molecular docking was performed to elucidate the type of interaction and the strength of the binding energy between the identified compounds and the target proteins MMP9, MMP13, NUDT5, and EGFR. The binding energy values for the carbazole alkaloids Koenigicine, Koenigine, Mahanine, and Mukonicine with the proteins MMP9, MMP13, NUDT5, and EGFR are presented in Table 1. These values reflect the strength of the interactions, with more negative values indicating stronger binding affinities. Substantial evidence shows that matrix metalloproteinases (MMPs), a family of secreted, zinc-dependent endopeptidases, play a crucial role at various stages of malignant tumor growth. MMPs can degrade extracellular matrix components, contributing to cancer cell survival (Gonzalez-Avila et al. 2019). Specifically, MMP9 and MMP13 break these matrix components essential for cancer cell invasion and metastasis. Inhibiting MMP9 and MMP13 may reduce triple-negative breast cancer (TNBC) cell dissemination (Mandel et al. 2018). The NUDT5 protein is linked to regulating cellular energy metabolism and stress responses; its inhibition could disrupt the pathways required for rapid cell division, thereby affecting cancer cell viability. Additionally, EGFR is significantly involved in cell signaling and is often overexpressed in TNBC, contributing to aggressive tumor growth. Targeting EGFR could interfere with the proliferation and survival of TNBC cells (Lejeune et al. 2023).

Koenigicine ($C_{20}H_{21}NO_3$) exhibits the highest binding affinity with NUDT5 at -9.2 kcal/mol, followed closely by MMP9 at -9.0 kcal/mol and EGFR at -9.1 kcal/mol. Its interaction with MMP13 is weaker at -7.9 kcal/mol. These results suggest that Koenigicine can form stable complexes with several targets, particularly MMP9, EGFR, and NUDT5. The binding affinity of Koenigicine to EGFR indicates its potential as an anticancer agent. Its affinity

Table 1 Carbazole alkaloids Koenigicine, Koenigine, Mahanine and Mukonicine and their Binding Energy with Protein targets MMP9, MMP13, NUDT5 and EGFR

S. No.	Name of ligand	Molecular formula	2-D Structures	Target Proteins and Binding Affinity (kcal/mol)		Hydrogen bonds Interacting residues
1	Koenigicine	$C_{20}H_{21}NO_3$		MMP9 MMP13 NUDT5 EGFR	-9.0 -7.9 -9.2 -9.1	TYR B132 HIS A148 ARG A38 ASP A146
2	Koenigine	$C_{19}H_{19}NO_3$		MMP9 MMP13 NUDT5 EGFR	-8.5 -9.2 -8.8 -9.2	TYR B132 LEU B130 GLY A134 THR A 142 ARG A38 ASP A146
3	Mahanine	$C_{23}H_{25}NO_2$		MMP9 MMP13 NUDT5 EGFR	-9.2 -9.4 -9.8 -9.9	TYR B132 HIS A148 GLN B2 PHE A154
4	Mukonicine	$C_{20}H_{21}NO_3$		MMP9 MMP13 NUDT5 EGFR	-7.4 -8.3 -8.8 -8.6	GLN B114 GLU B34 LYS A40

for MMP13 and EGFR suggests a multifaceted therapeutic effect, impacting both matrix remodeling and cell proliferation, offering potential implications for cancer treatment (Szczygielski et al. 2024). This indicates that Koenigicine may serve as a multi-target agent, affecting proteins involved in signaling, metabolism, and cancer pathways. Follow-up experiments conducted in vitro and in vivo are needed to support these findings further and clarify the specific mechanisms by which Koenigicine affects these targets (Endo et al. 2018; Almutairi et al. 2023).

In the case of Koenigine ($C_{19}H_{19}NO_3$), a binding affinity of -8.5 kcal/mol was observed for MMP9 and -9.2 kcal/mol for MMP13, indicating a strong interaction with these matrix metalloproteinases, which are key players in extracellular matrix degradation and are associated with increased invasion and metastasis. Notably, the strong binding affinity to MMP13 suggests that Koenigine could inhibit tumor cell migration and invasive pathways, which represent critical mechanisms in the pathobiology of TNBC.

Given the role of NUDT5 in energy homeostasis within the cancer microenvironment, it is noteworthy that Koenigine binds to NUDT5 with an affinity of -8.8 kcal/mol. In triple-negative breast cancer (TNBC), NUDT5 plays a crucial role in preventing oxidative DNA damage, and its inhibition can suppress cancer cell growth (Qian et al. 2024). The interaction between Koenigine and NUDT5 may disrupt these adaptive processes, subsequently inhibiting TNBC progression. Additionally, EGFR has been linked to poor prognosis and resistance to therapy (Zang et al. 2024). The binding affinity of Koenigine to EGFR is -9.2 kcal/mol, indicating that it may act as a potent EGFR inhibitor. Inhibiting EGFR could help shut down proliferative and survival pathways in TNBC cells, making this a rational strategy for limiting their growth and proliferation.

The binding affinity results demonstrate Koenigine's significant interaction with four key protein targets: MMP9, MMP13, NUDT5, and EGFR, all contributing to tumor growth, metastasis, and survival mechanisms in TNBC. By inhibiting MMP9 and MMP13 while downregulating NUDT5 and EGFR, Koenigine may disrupt critical cellular pathways involved in TNBC progression. Its multi-target potential makes it an attractive candidate for drug development, whether alone or combined with other agents targeting various pathways in tumorigenesis.

The present study also examined the molecular interactions of Mahanine ($C_{23}H_{25}NO_2$) with the major pharmaceutical targets MMP9, MMP13, NUDT5, and EGFR. Mahanine's binding

affinities with these targets were measured at -9.2 kcal/mol (MMP9), -9.4 kcal/mol (MMP13), -9.8 kcal/mol (NUDT5), and -9.9 kcal/mol (EGFR). These results indicate a strong interaction between Mahanine and the four targets, essential for its therapeutic effect. In the context of TNBC, MMPs like MMP9 and MMP13 are significantly involved in tumor invasion and metastasis. Mahanine's strong affinity for MMP13 and MMP9 suggests a potential reduction in extracellular matrix degradation, supporting tumor cell migration and positioning Mahanine as an effective anti-metastatic agent. The binding affinity with EGFR indicates that Mahanine may influence pathways contributing to tumor proliferation and survival, particularly in remodeling EGFR-positive TNBC cases (Zhang et al. 2024). Furthermore, NUDT5 correlates with NAD+-related signaling in cancer cell proliferation and survival (Dubey et al. 2024). The strong affinity of Mahanine for NUDT5 could help explain its ability to inhibit metabolic processes necessary for the survival of TNBC cells. This study suggests that Mahanine could be a potential multi-target agent suppressing tumor cell growth and survival in TNBC. Further investigations, including in vitro and in vivo studies, must validate these computational findings and determine Mahanine's effectiveness and the mechanisms involved in TNBC treatment (Dubey et al. 2024).

Additionally, Mukonicine ($C_{20}H_{21}NO_3$) has shown binding affinities of -7.4 kcal/mol and -8.3 kcal/mol for MMP9 and MMP13, respectively. Mukonicine is expected to significantly prevent invasion and metastasis in TNBC cells, as demonstrated by its strong interactions with MMP9 and MMP13. Its affinity for NUDT5 at -8.8 kcal/mol suggests a potent interaction with this target, which could impede regulatory pathways and impact cellular proliferation. Mukonicine also exhibits a binding affinity of -8.6 kcal/mol towards EGFR, indicating a significant inhibitory effect on cell proliferation (Kar et al. 2024).

3.3 Molecular Docking-Interactions Maps

We have examined the 2-D interaction maps of the targeted compounds with specific proteins: MMP9, MMP13, NUDT5, and EGFR. Figure 2 illustrates the molecular interactions of Koenigicine with these protein targets: MMP9 (PDB ID: 2ow2), MMP13 (PDB ID: 1xuc), EGFR (PDB ID: 1xkk), and NUDT5 (PDB ID: 2dsc). The interaction diagrams highlight critical binding characteristics and the relationships between Koenigicine and the active sites of these targeted proteins. They highlight meaningful non-covalent interactions, including van der Waals forces, hydrogen bonds, and π-π stacking interactions. These findings

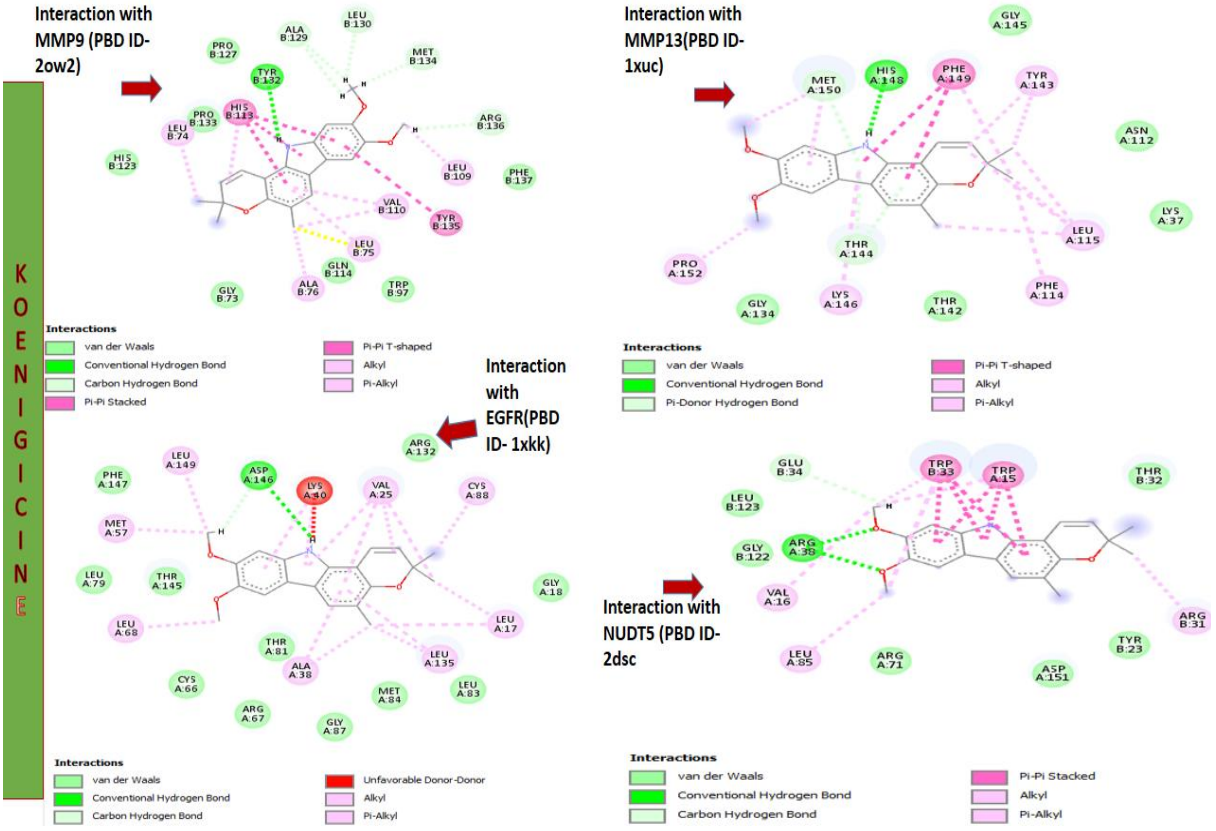


Figure 2 Koenigicine's interaction with MMP9(PBD ID- 2ow2), MMP13(PBD ID- 1xuc),EGFR(PBD ID- 1xkk), NUDT5 (PBD ID- 2dsc ADP-sugar pyrophosphatase).

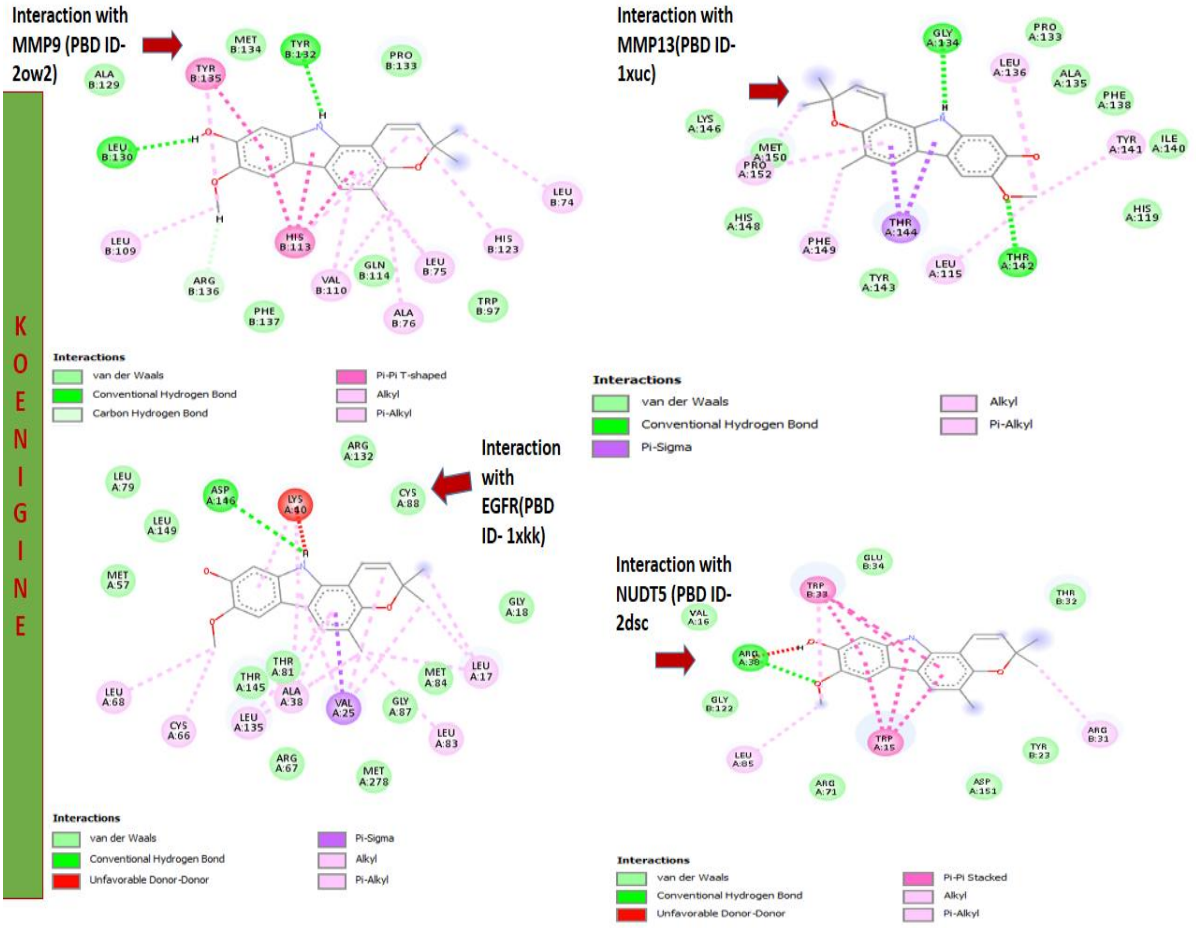


Figure 3 Koenigine interaction with MMP9(PBD ID- 2ow2),MMP13(PBD ID- 1xuc),EGFR(PBD ID- 1xkk),NUDT5 (PBD ID- 2dsc ADP-sugar pyrophosphatase).

demonstrate the binding modes of Koenigicine with the protein targets and suggest that it could serve as a potential inhibitor in therapeutic applications related to triple-negative breast cancer (TNBC).

Additionally, Figure 3 illustrates the molecular docking interactions of Koenigine with four target proteins: MMP9 (PDB ID: 2ow2), MMP13 (PDB ID: 1xuc), EGFR (PDB ID: 1xkk), and NUDT5 (PDB ID: 2dsc). The primary non-covalent binding interactions between Koenigine and the active sites of these proteins include van der Waals forces, hydrogen bonds, and π-π stacking, all depicted in the interaction maps. Together, these docking interactions highlight the binding profile of Koenigine across various targets associated with cancer pathogenesis, supporting its potential as a multi-target inhibitor in treatment strategies for triple-negative breast cancer (TNBC).

Figure 4 illustrates Mahanine's interactions with targeted proteins: MMP9 (PDB ID - 2ow2), MMP13 (PDB ID - 1xuc), EGFR (PDB ID - 1xkk), and NUDT5 (PDB ID - 2dsc, ADP-sugar pyrophosphatase). These interactions occur through van der Waals forces, hydrogen bonding, and π-based interactions. This diversity suggests that Mahanine has broad-spectrum inhibition or modulation potential, particularly against proteins linked to cancer progression and degenerative diseases. Understanding these interactions with specific residues may reveal key insights into the therapeutic potential of Mahanine as a promising agent.

Mukonicine interacts with MMP9, MMP13, EGFR, and NUDT5, as illustrated in Figure 5. This study highlights Mukonicine's effectiveness as a multi-target agent against triple-negative breast cancer (TNBC). The interactions occur through van der Waals forces, hydrogen bonds, and various π-based interactions, creating a stable binding configuration with each protein. The involvement of specific amino acid residues suggests that Mukonicine could modulate the activities of these proteins, potentially affecting cancer cell invasion, migration, proliferation, and survival. This analysis provides insight into the treatment efficacy of Mukonicine and supports further investigation into its role in targeted therapy for TNBC.

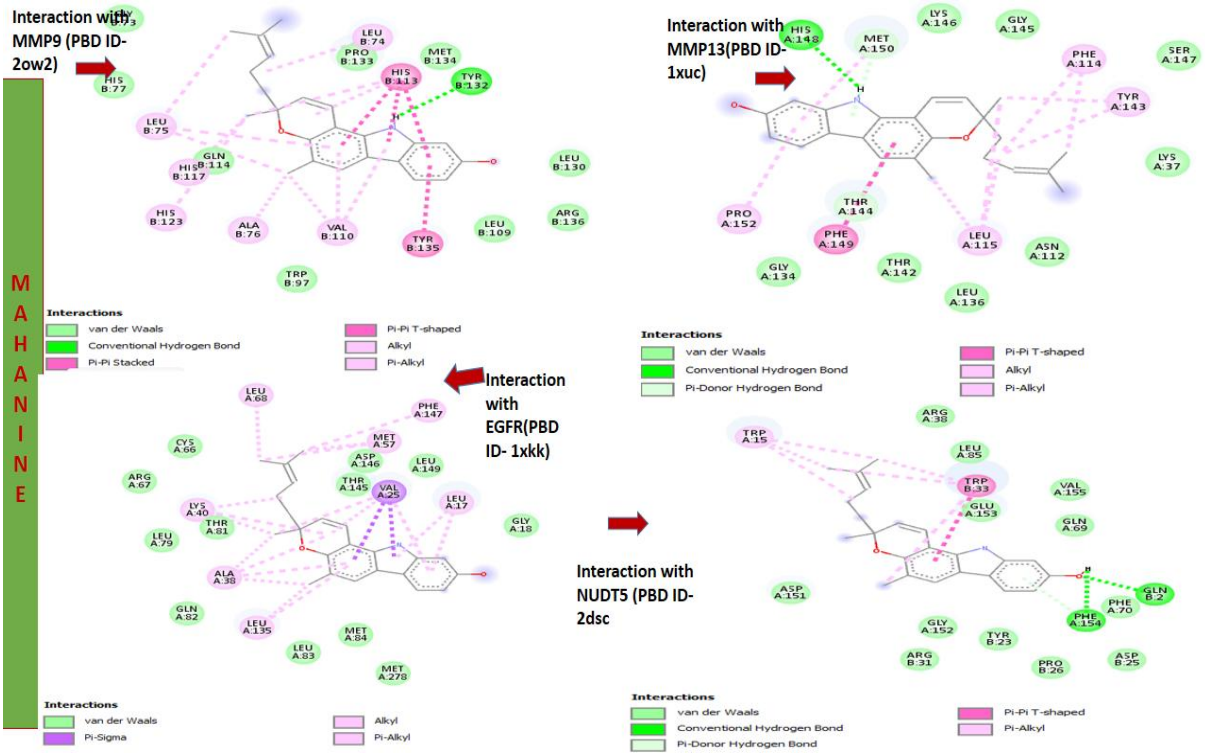


Figure 4 Mahanine interaction with MMP9 (PBD ID- 2ow2),MMP13(PBD ID- 1xuc), EGFR(PBD ID- 1xkk) and NUDT5 (PBD ID- 2dsc ADP-sugar pyrophosphatase).

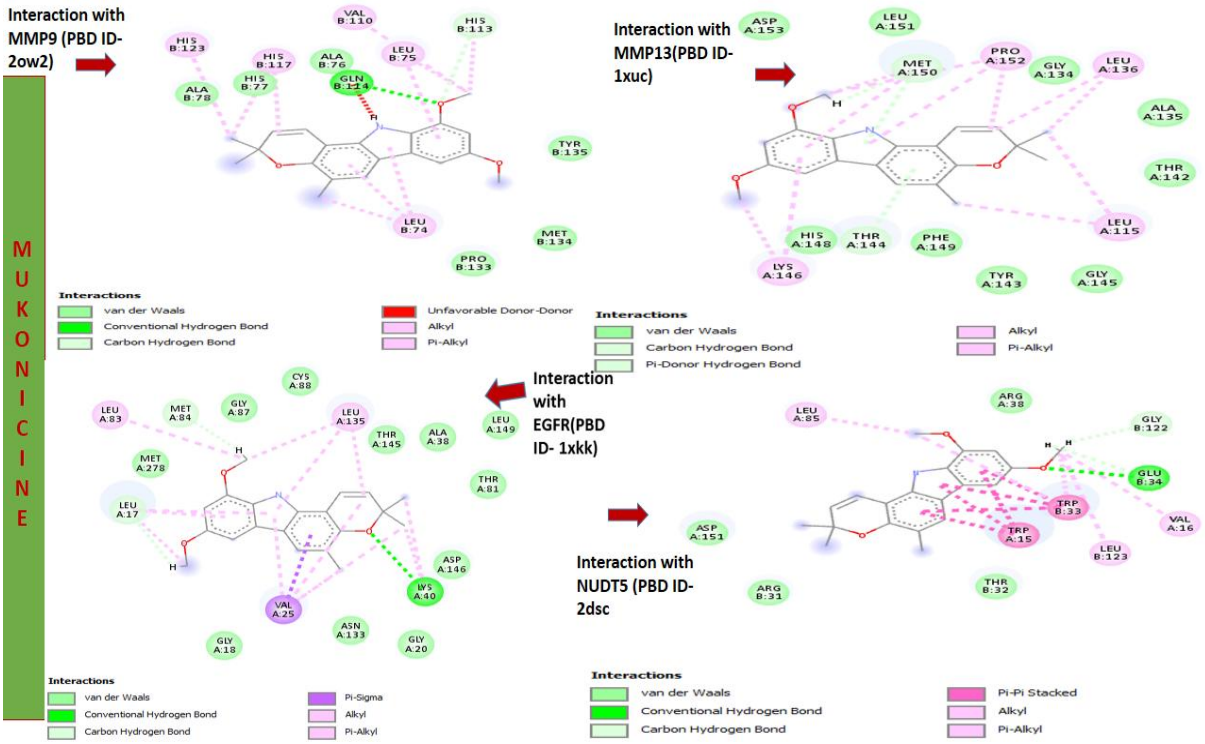


Figure 5 Mukonicine's interaction with MMP9(PBD ID- 2ow2),MMP13(PBD ID- 1xuc),EGFR(PBD ID- 1xkk),NUDT5 (PBD ID- 2dsc ADP-sugar pyrophosphatase).

Conclusion and Future Prospects

The combination of infrared spectral data and molecular docking studies suggests that the carbazole alkaloids Koenigicine, Koenigine, Mahanine, and Mukonicine may serve as effective inhibitors for triple-negative breast cancer (TNBC) due to their interactions with proteins MMP9, MMP13, NUDT5, and EGFR. The structural features of these alkaloids facilitate binding to the respective proteins, which may inhibit cancer progression by slowing down cell proliferation and metastasis. The higher binding affinities of Koenigicine, Koenigine, Mahanine, and Mukonicine indicate that they could act as promising multi-target inhibitors for TNBC treatment. Mahanine is particularly potent against NUDT5 and EGFR, suggesting it may provide the most significant inhibition of TNBC cells and could be considered a lead compound for future investigation. Koenigicine and Koenigine also demonstrate promising multi-target binding, particularly against EGFR and MMPs, which are relevant to TNBC proliferation and metastasis.

Mukonicine, while exhibiting relatively lower binding affinities, is less effective across the targets than the other ligands and may have limited therapeutic potential for TNBC. Therefore, further experimentation is necessary to confirm these inhibitory effects and their potential applications in treatment.

Additionally, these carbazole alkaloids show strong van der Waals forces, hydrogen bonds, and π-π stacking interactions with the mentioned proteins. Optimizing these interactions may enhance selectivity and potency. The interactions identified in this docking study could be crucial for improving binding specificity and affinity in drug development. Consequently, these compounds should be included in experimental studies to validate their obstructive roles and clarify their antitumor efficacy in treating TNBC by targeting cancer proliferation (EGFR and NUDT5) and metastasis (MMP9 and MMP13).

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Author contributions

AS: Conceptualization, Investigation, data collection, Writing original draft, Figure Preparation, writing review and editing; NK, SR & RC: Analysis, visualization, arranging references; MS, AAJ and HST Figure and Table preparation, reviewing and editing; AKS: Conceptualization, Supervision, Writing-Reviewing, and Editing. All authors read and approved the submitted version.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

Not applicable.

Consent to publish

All the authors consented to publish the manuscript.

Data Availability Statement

All the findings have been disclosed in the manuscript, which is supported by the data. Additional data can be made available upon subsequent request.

References

Ajay, S., Rahul, S., Sumit, G., Paras, M., Mishra, A., & Gaurav, A. (2011). Comprehensive review: *Murraya koenigii* Linn. *Asian Journal of Pharmacy and Life Science*, *1*(4), 417-425.

Almutairi, S., Kalloush, H. M., Manoon, N. A., & Bardaweel, S. K. (2023). Matrix Metalloproteinases Inhibitors in Cancer Treatment: An Updated Review (2013–2023). *Molecules*, *28*(14), 5567. https://doi.org/10.3390/molecules28145567.

Arif, M. A. A., Warsito, S. H., Lamid, M., Lokapirnasari, W. P., Khairullah, A. R., et al. (2024). Phytochemical Analysis of Curry Leaf Extract (*Murraya koenigii* L.) as a Potential Animal Feed and Medicinal Ingredient. *Pharmacognosy Journal*, *16*(2), 471–477. https://doi.org/10.5530/pj.2024.16.75

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E., Linder, T., Wawrosch, C., et al. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, *33*(8), 1582–1614. https://doi.org/10.1016/j.biotechadv.2015.08.001

Bai, X., Ni, J., Beretov, J., Graham, P., & Li, Y. (2021). Triple-negative breast cancer therapeutic resistance: Where is the Achilles' heel?. *Cancer Letters*, *497*, 100–111. https://doi.org/10.1016/j.canlet.2020.10.016.

Balakrishnan, R., Vijayraja, D., Jo, S. H., Ganesan, P., Su-Kim, I., & Choi, D. K. (2020). Medicinal profile, phytochemistry, and pharmacological activities of *Murraya koenigii* and its primary bioactive compounds. *Antioxidants*, *9*(2), 101. https://doi.org/10.3390/antiox9020568

Beć, K., Grabska, J., & Huck, C. (2020). Biomolecular and bioanalytical applications of infrared spectroscopy - A review. *Analytica Chimica Acta*, *1133*, 150–177. https://doi.org/10.1016/j.aca.2020.04.015.

Cheang, M. C., Voduc, D., Bajdik, C., Leung, S., McKinney, S., Chia, S. K., Perou, C. M., & Nielsen, T. O. (2008). Basal-Like Breast Cancer Defined by Five Biomarkers Has Superior Prognostic Value than Triple-Negative Phenotype. *Clinical Cancer Research*, *14*(5), 1368–1376. https://doi.org/10.1158/1078-0432.ccr-07-1658.

Dassault Systèmes BIOVIA (2016). Discovery Studio Modelling Environment, Release 2017; Dassault Systèmes: San Diego, CA, USA.

Dubey, A., Alanazi, A.M., Bhardwaj, R., & Ragusa, A. (2024). Identification of potential NUDT5 inhibitors from marine bacterial natural compounds via molecular dynamics and free energy landscape analysis. *Molecular Diversity*, *29* (1), 1-15, https://doi.org/10.1007/s11030-024-10950-5.

Endo, H., Owada, S., Inagaki, Y., Shida, Y., & Tatemichi, M. (2018). Glucose starvation induces LKB1-AMPK-mediated MMP-9 expression in cancer cells. *Scientific Reports 8*,10122. https://doi.org/10.1038/s41598-018-28074-w.

Gaikwad, S., Gawande, V., Mhaske, S., & Sharma, T. (2025). A review on the medicinal significance of *Murraya koenigii*. *International Journal of Pharmaceutical Sciences*, *3*(1), 166–174. https://doi.org/10.5281/zenodo.14590932.

Gangawat, R., Parashar, R., & Yadav, R. K. (2024). Hepatoprotective Potential of *Murraya koenigii* (Curry Leaves) against Xenobiotics, Heavy Metals, and Hepatotoxic Agents: A Comprehensive Review. *Current Drug Discovery Technologies*, (in press). https://doi.org/10.2174/0115701638310869240628060001.

Ghosh, S., Ghosh, S., & Wategaonkar, S. (2020). C–H•••Y (Y=N, O, π) Hydrogen Bond: A Unique Unconventional Hydrogen Bond. *Journal of the Indian Institute of Science*, *100*(1), 101–125. https://doi.org/10.1007/S41745-019-00145-5.

Gonzalez-Avila, G., Sommer, B., Mendoza-Posada, D. A., Ramos, C., Garcia-Hernandez, A. A., & Falfan-Valencia, R. (2019). Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. *Critical reviews in oncology/hematology*, *137*, 57-83.

Hansen, P.E., Vakili, M., Kamounah, F.S., & Spanget-Larsen, J. (2021). NH Stretching Frequencies of Intramolecularly Hydrogen-Bonded Systems: An Experimental and Theoretical Study. *Molecules*, *26*(24), 7651. https://doi.org/10.3390/molecules26247651.

Kar, T., Dugam, P., Shivhare, S. C., Shetty, S. R., Choudhury, S., et al. (2024). Epidermal growth factor receptor inhibition potentiates chemotherapeutics-mediated sensitization of metastatic breast cancer stem cells. *Cancer Reports*, *7*. https://doi.org/10.1002/cnr2.2049.

Leidy, J., Khan, A., & Kandil, D. (2014). Basal-Like Breast Cancer: Update on Clinicopathologic, Immunohistochemical, and Molecular Features. *Archives of Pathology & Laboratory Medicine*, *138*(1), 37–43. https://doi.org/10.5858/arpa.2012-0439-ra.

Lejeune, M., Reverté, L., Gallardo, N., Sauras, E., Bosch, R., et al. (2023). Matrix Metalloproteinase-9 Expression is Associated with the Absence of Response to Neoadjuvant Chemotherapy in Triple-Negative Breast Cancer Patients. *International Journal of Molecular Sciences*, *24*(14), 11297. https://doi.org/10.3390/ijms241411297

Lorion, C., Bardin, V., Bonnet, S., Lopez-Gaydon, A., Vogelgesang, B., & Bechetoille, N. (2023). Elastogenic potential and antisagging properties of a novel *Murraya koenigii* extract. *Journal of Cosmetic Dermatology*, *23*(3), 1036–1044. https://doi.org/10.1111/jocd.16059.

Mandel, A., Larsson, P., Sarwar, M., Semenas, J., Khaja, A. S. S., & Persson, J. L. (2018). The interplay between AR, EGF receptor and MMP-9 signaling pathways in invasive prostate cancer. *Molecular Medicine*, *24*(1) 34. https://doi.org/10.1186/s10020-018-0035-4.

Mcule (2024). Mcule: A Platform for Molecular Design and Drug Discovery. Available at mcule.com.

National Center for Biotechnology Information. (2024). Compound summary for CID -278055 (Koenigicine), 5318825 (Koenigine), 36689305 (Mahanine), 86242003 (Mukonicine). PubChem. Retrieved October 10, 2024, from https://pubchem.ncbi.nlm.nih.gov/compound/.

Nedeljković, M., & Damjanović, A. (2019). Mechanisms of Chemotherapy Resistance in Triple-Negative Breast Cancer-How We Can Rise to the Challenge. *Cells*, *8*(9), 957. doi: 10.3390/cells8090957.

Newman, D. J., & Cragg, G. M. (2020). Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. *Journal of Natural Products*, *83*(3), 770–803. https://doi.org/10.1021/acs.jnatprod.9b01285.

Ozaki, Y. (2021). Infrared spectroscopy—Mid-infrared, near-infrared, and far-infrared/terahertz spectroscopy. *Analytical Sciences*, *37*(9), 1193–1212. https://doi.org/10.2116/ANALSCI.20R008.

Patil, R., Mandlik, S., & Mandlik, D. (2023). *Murraya koenigii* (Curry Tree): A review of its Phytochemistry, Ethnomedicinal uses, and Pharmacology with Respect to Molecular Mechanisms. *Current Traditional Medicine*, *10*(5), 73-79. https://doi.org/10.2174/2215083810666230609163404.

Pinto, P., & Cojandaraj, L. (2024). Protective Effects of *Murraya koenigii*: Focus on Antihyperlipidemic Property. *Current Drug Therapy*, *20*(1), 24-32. https://doi.org/10.2174/0115748855278592240131105512.

Polik, W.F., & Schmidt, J.R. (2022). WebMO: Web-based computational chemistry calculations in education and research. *WIREs Computational Molecular Science*, *12*, e1554. https://doi.org/10.1002/wcms.1554.

Pradhan, S. P., Subedi, I., Adhikari, K., Gc, A., Pradhan, S. P., Aryal, M. R., Ghimire, G. P., & Pandey, B. P. (2024). In vitro and in silico approach for the evaluation of enzyme inhibitory potential of Kadipatta (*Murraya koenigii*) collected from western Nepal. *Deleted Journal*, *5*(3), 200161. https://doi.org/10.1016/j.ctmp.2024.200161.

Qian, J., Ma, Y., Tahaney, W. M., Moyer, C. L., Lanier, A., et al. (2024). The novel phosphatase NUDT5 is a critical regulator of triple-negative breast cancer growth. *Breast Cancer Research* : *BCR*, *26*(1), 23. https://doi.org/10.1186/s13058-024-01778-w.

Sadwal, S., Bharati, S., Dar, Z. A., & Kaur, S. (2023). Chemopreventive potential of hydroethanolic *Murraya koenigii* leaves extract against DMBA induced breast carcinogenesis: In-silico and in-vivo study. *Journal of Ethnopharmacology*, *319*, 117124. https://doi.org/10.1016/j.jep.2023.117124.

Samanta, S.K., Choudhury, P., Kandimalla, R., Aqil, F., Moholkar, D.N., et al. (2024). Mahanine mediated therapeutic inhibition of estrogen receptor-α and CDK4/6 expression, decipher the chemoprevention-signaling cascade in preclinical model of breast cancer. *Journal of Ethnopharmacology*, *319* (2), 117235. https://doi.org/10.1016/j.jep.2023.117235.

Suvarna, E., Setlur, A. S., Chandrashekar, S., & Niranjan, V. (2024). Computational molecular perspectives on novel carbazole derivative as an anticancer molecule against CDK1 of breast and colorectal cancers via gene expression studies, novel two-way docking strategies, molecular mechanics and dynamics. *Computational Biology and Chemistry*, *108*, 107979. https://doi.org/10.1016/j.compbiolchem.2023.107979.

Szczygielski, O., Dąbrowska, E., Niemyjska, S., Przylipiak, A., & Zajkowska, M. (2024). Targeting Matrix Metalloproteinases and Their Inhibitors in Melanoma. *International Journal of Molecular Sciences*, *25*(24), 13558. https://doi.org/10.3390/ijms252413558.

Thomford, N. E., Senthebane, D. A., Rowe, A., Munro, D., Seele, P., Maroyi, A., & Dzobo, K. (2018). Natural Products for Drug Discovery in the 21st Century: Innovations for Novel Drug Discovery. *International Journal of Molecular Sciences*, *19*(6), 1578. https://doi.org/10.3390/ijms19061578.

Wang, T., Tan, Y., Chen, Y., & Tan, C. (2023). Infrared spectral analysis for prediction of functional groups based on feature-aggregated deep learning. *Journal of Chemical Information and Modeling*, *63*(15): 4615-4622. https://doi.org/10.1021/acs.jcim.3c00749.

Yuan, H., Ma, Q., Ye, L., & Piao, G. (2016). The Traditional Medicine and Modern Medicine from Natural Products. *Molecules*, *21*(5), 559. https://doi.org/10.3390/molecules21050559.

Zagami, P., & Carey, L.A. (2022). Triple negative breast cancer: Pitfalls and progress. *NPJ Breast Cancer*, *8*(1), 95. https://doi.org/10.1038/s41523-022-00468-0.

Zhang, H., Qi, Y., Mao, Y., & Zheng, J. (2024). PDZK1 suppresses TNBC development and sensitizes TNBC cells to erlotinib via the EGFR pathway. *Cell Death and Disease*, *15*(3), 199. https://doi.org/10.1038/s41419-024-06502-w.

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CONSERVATION STATE OF THE NATIONAL ZOO FOREST OF ABIDJAN (COTE D'IVOIRE)

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Regeneration

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ABSTRACT

This study aimed to assess the degradation of the National Zoo of Abidjan. To achieve this, we employed two field methods: surface survey and linear survey. The surface survey involved counting all plant species within each plot, focusing on those with circumferences greater than or equal to 7.85 cm at breast height. A 100-meter-long rope was stretched horizontally just above the ground for the linear survey. Using this technique, 100 measurements were taken at regular one-meter intervals. At each contact point, we recorded the species and the distance at which each individual was encountered along the survey line. The results indicated that the forest possesses a good regeneration capacity. Analysis of the structural profiles revealed numerous openings, and it was found that the most dominant species include lianas (such as *Acacia kamerunensis*, *Adenia cissampeloides*, and Harms) and nanophanerophytes. Additionally, the observations showed that this area is a remnant of degraded semi-deciduous forests. Based on these findings, developing strategies to protect this forest and provide valuable goods and services to Abidjan is essential.

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1 Introduction

Côte d'Ivoire is home to 3,853 species of flora, which are threatened with extinction due to illegal activities such as agricultural clearing and forest over-exploitation (Aké-Assi 2002; FAO/PNUD 1981). Most of this flora is now found only in protected areas like national parks, classified forests, and nature reserves (Dao 1999). However, local populations increasingly encroach on these protected areas in their search for arable land and various plant species with different benefits, resulting in a continuous reduction of these areas. The government has implemented several measures to combat the decline of protected land and enhance the conservation potential of these biodiversity reservoirs. These include establishing a forest police force and creating animal parks. The National Zoo of Abidjan is one such park and plays a crucial role for the country, particularly for the city of Abidjan. It houses various animals, including lions, elephants, chimpanzees, and crocodiles (N'guessan et al. 2021). Additionally, the zoo is significant for wildlife education and conservation awareness, serving as a recreational site, an educational center, and a community meeting place. Nevertheless, the National Zoo of Abidjan and its remaining forest are under pressure from rapid urbanization in the city. Recently, the forest has faced increasing degradation (Cisse 2024). The loss of this forest could have significant consequences for wildlife, as some plants provide food for animals in captivity. Therefore, finding ways to preserve this forest is essential. Given its importance, studying the vegetation structure is necessary. This study aims to assess the degradation of this forest remnant and contribute to improving its management.

2 Material and methods

The study was conducted at the National Zoo of Abidjan, situated in the heart of Abidjan, Côte d'ivoire, at the intersection of the Deux Plateaux and Abobo Dokui neighborhoods, near the Abidjan Military Hospital (HMA) (Figure 1). The zoo spans approximately 20 hectares, with four hectares currently in operation. It is home to around 270 animals representing 48 species. The animals are divided into four sections: carnivores, duikers, crocodiles, and primates.

2.1 Methods

This study employed two field methods: surface survey and linear survey. The surface survey was conducted in three randomly

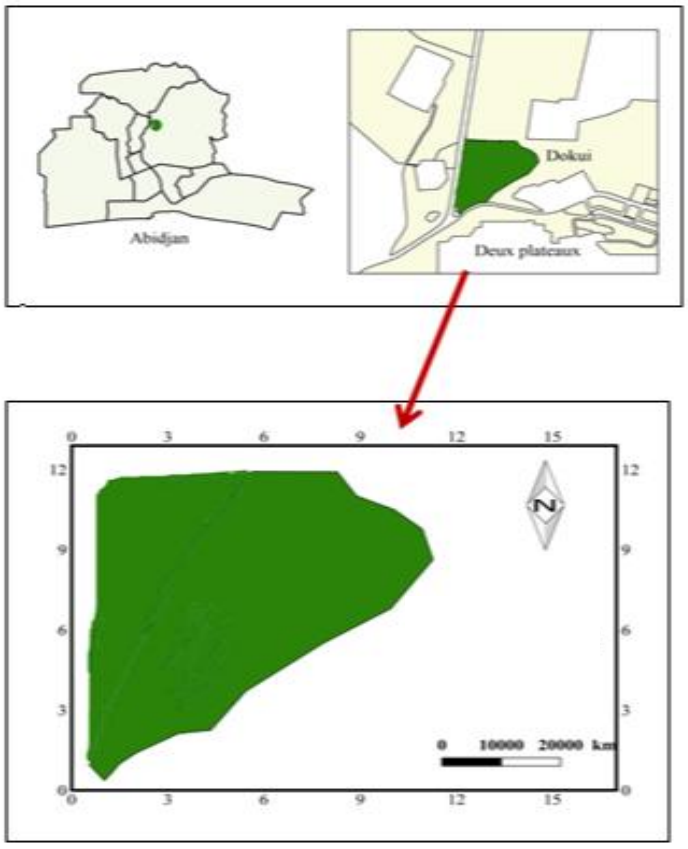

Figure 1 Location of the Forest of the National Zoo of Abidjan

selected plots within the National Zoo of Abidjan forest. In each plot, 100 m² areas (10 m x 10 m) were established along a 100 m line (Figure 3). All tree species with circumferences greater than or equal to 7.85 cm at breast height were inventoried within each plot. A 100-meter-long cord was stretched horizontally just above the ground for the linear survey. This setup allowed for 100 measurements to be taken at regular one-meter intervals. For each contact, the species and the distance from the starting point of the survey line were recorded. Additionally, each vegetation contact's minimum and maximum heights were measured using a vertically erected 4 m marker. Heights above 4 m were estimated following the method outlined by Gautier et al. (1994). A sample was taken for analysis for each contact. A total of three linear surveys were conducted in this study. The study area was divided into three zones: A, B, and C. Each survey zone was designated as Zone A, B, and C. This method enabled us to obtain the various surveys' structural profiles and vegetation cover. It has been previously used in studies by Chatelain (1996), Kouamé et al. (1998), and Bakayoko et al. (2001, 2004) in Côte d'Ivoire.

2.2 Structural data analysis

2.2.1 Diameter classes

The distribution of individuals by diameter class is commonly called "total structure" by foresters (Bouko et al. 2007). This concept provides insight into the demographic structure of wooded areas by using histograms that display the distribution of individuals across various diameter classes. To achieve this, we counted the number of individuals in each plot and then categorized them into different diameter classes. This classification enabled us to create distribution curves for the individuals.

2.2.2 Vegetation structure

We constructed structural profiles to understand how individuals are spatially distributed along the transect. This involves creating a graphical representation of all contacts recorded along the survey line. The x-axis represents the distance along the transect where these contacts were noted, while the y-axis indicates the height of the contacts. This method is useful for describing the horizontal structure of vegetation and how species are distributed along linear surveys. The survey's structural profiles highlighted abundant or characteristic species distribution within each survey (Chatelain 1996). These profiles allow us to assess the height of the vegetation in the surveys and evaluate their state of degradation (Kouamé 1998).

2.2.3 Vegetation cover

Vegetation cover refers to the area occupied by individuals of a species. According to Chatelain (1996), this is determined by projecting the crown width vertically onto the ground. In the current study, species cover is estimated as the percentage of contact points between the species and a vertically positioned milestone. Cover can be analyzed at two levels. At the species level, it is expressed by the frequency with which the species is encountered during the survey. From the perspective of vertical vegetation distribution, cover indicates the distribution of all species combined across defined height intervals, expressed as the percentage of contacts within those intervals. A cover histogram was created for each survey, and the cover was calculated using the formula developed by Menzies (2000).

$$\text{Cover (C)} = \frac{A}{B} \times 100$$

A : Number of points on the transect line on which a species is present.

B : Total number of measurement points on the transect.

In this study, cover measurement focused on noting the presence or absence of contact within specific height intervals at each point. The height intervals used were defined by Richards (1952), Kahn (1982), and Bongers et al. (1988). For each survey, we constructed a cover histogram. Following the approach of Chatelain (1996), Emberger et al. (1968), and Kouamé (1998), we categorized the height intervals as follows: $0 < \text{height} \leq 4$ m (lower tree stratum), $4 < \text{height} \leq 16$ m (upper tree stratum), $16 < \text{height} \leq 32$ m (emergent stratum), and height > 32 m (upper emergent stratum).

2.2.4 Number and width of gaps

To assess the state of vegetation degradation, we evaluated the width and number of gaps in the canopy (Kouamé 1998). The number of gaps is useful for determining the degree of openness at different height thresholds and understanding the dynamics and heterogeneity of the vegetation. According to Bakayoko (2005), the width and the number of gaps provide insights into the extent of openings observed in various surveys. Given the low canopy height in our plots, with a maximum vegetation height rarely exceeding 20 meters, we standardized our assessment by using 10 meters across all plots to ensure non-zero cover values (Bakayoko 2005). We could count the gaps by constructing a curve indicating maximum heights.

2.2.5 Vegetation State Assessment

According to Chatelain (1996), an analysis of the cover (Figure 2) by height class identified three types: A, B, and G. Each type corresponds to a specific plant formation. Type A represents less degraded environments, while Types B and G characterize degraded semi-deciduous forests, as outlined below.

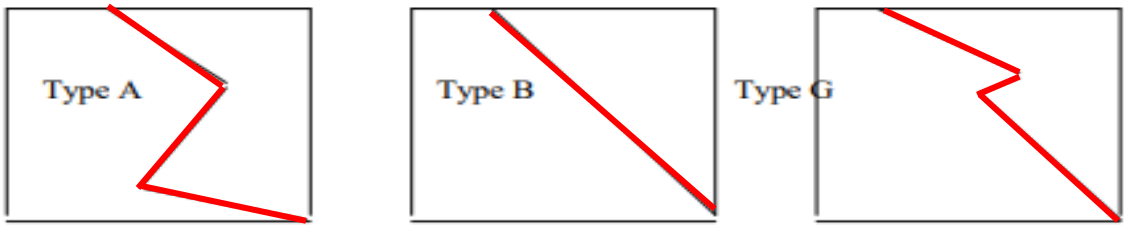

Figure 2 Vegetation condition based on cover type

2.3 Principal Component Analysis

Principal Component Analysis (PCA) is a method used to represent data in a reduced space, making it easier to analyze complex datasets. This technique is particularly common in ecology, where it helps to evaluate species distributions across multiple sites. The primary objective of PCA is to identify and highlight the main trends in the variability of these distributions. By reducing the data to lower dimensions, typically two-dimensional representations, PCA simplifies analysis and interpretation. This process allows researchers to determine which variables significantly contribute to forming the first two principal axes. Moreover, it facilitates the extraction of coordinates for the observations, particularly focusing on the variables that strongly influence those axes.

3 Results

3.1 Diameter class

The histogram illustrating the distribution of trees by diameter class reveals an inverted 'J' shape across the three studied plots (Figure 3). As the diameter increases, the number of individual trees decreases. In all three plots, the diameter class of 0-5 cm contains the highest number of individuals, representing the most abundant group of regenerating trees.

3.2 Cover and Structural profile

Figure 4 illustrates the cover and structural profiles of the three plots. The vegetation cover rate decreases progressively in a staircase manner, reaching the upper stratum at 32 meters across

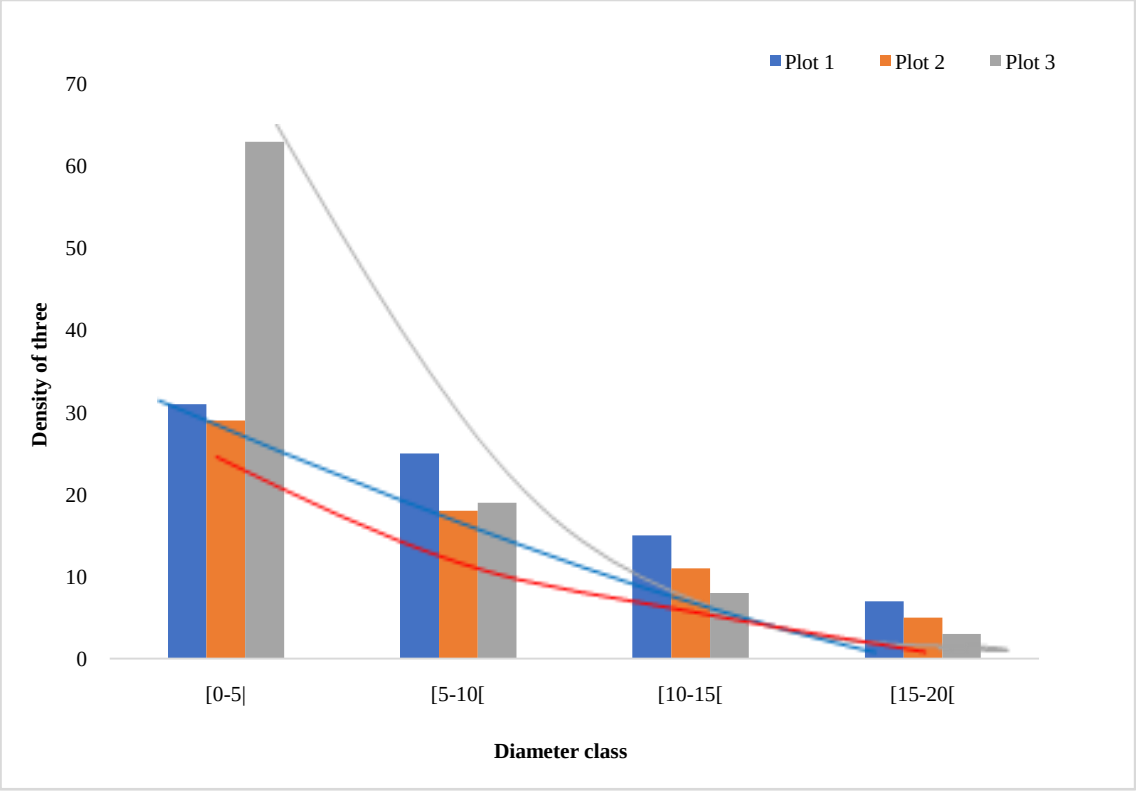

Figure 3 Distribution of stems by diameter class

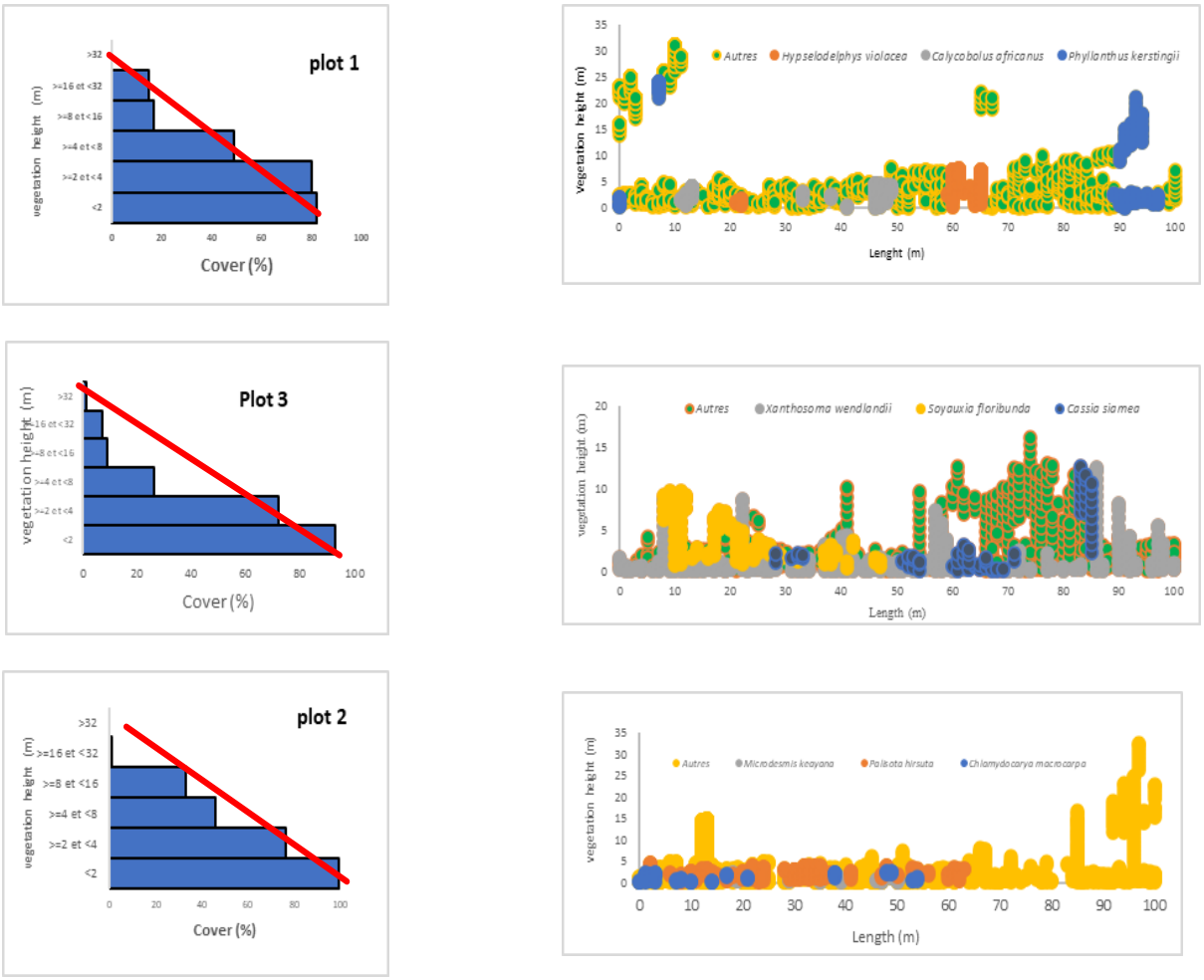

Figure 4 Cover and structural profiles of the vegetation in the three plots

all three zones. In zones A and C, recovery falls below 20% in the 16 to 32 m and 8 to 16 m strata. In contrast, zone B shows cover below 20% in the 8 to 16 m stratum but exceeds 30% in the 16 to 32 m stratum. In zones A and B, cover surpasses 40% in the 4 to 8 m stratum, whereas it remains below 40% in zone C. Overall, the spatial distribution of characteristic species varies significantly from one survey to another. Additionally, many openings and dense undergrowth are present. Regarding plot 1, the vegetation appears generally shorter and more heterogeneous, characterized by numerous openings. The structural profile of this plot is predominantly composed of species such as *Calycobolus africanus*, *Hypselodelphys violacea*, and *Phyllanthus kerstingii*. Species like *Xanthosoma wendlandii*, *Soyauxia floribunda*, and *Cassia siamea* primarily dominate plot 2. In Plot 3, the dominant species include *Chlamydocarya macrocarpa*, *Palisota hirsuta*, and *Microdesmis keayana*. Among these dominant species, *C. africanus*, *H. violacea*, and *C. macrocarpa* are categorized as lianas. *P. kerstingii*, *X. wendlandii*, and *P. hirsuta* are classified as nanophanerophytes, while *Cassia siamea* and *Microdesmis keayana* are considered microphanerophytes. Notably, *Soyauxia floribunda* is the only mesophanerophyte species observed among the recorded species.

3.3 Influence of structural parameters and canopy openness on the plots

We conducted a Principal Component Analysis (PCA) on the structural data. Figure 5 illustrates that the first two axes of the PCA account for 29.61% of the total variability. Specifically, Axis 1 explains 70.39% of this variability, with key contributing variables being the strata at depths of ≥ 16 m and < 32 m and those > 2 m. For Axis 2, the contributing variables include strata ≥ 32 m, those in the range of ≥ 2 and < 4 m, ≥ 8 and < 16 m, the width of gaps at 10 m, and the number of gaps at the height of 10 m. These variables explain 29.61% of the variability associated with this axis. Aside from the overlaps and structural profiles of plots 1 and 3, only the overlap and profile of plot 2 show a correlation with the number and width of gaps.

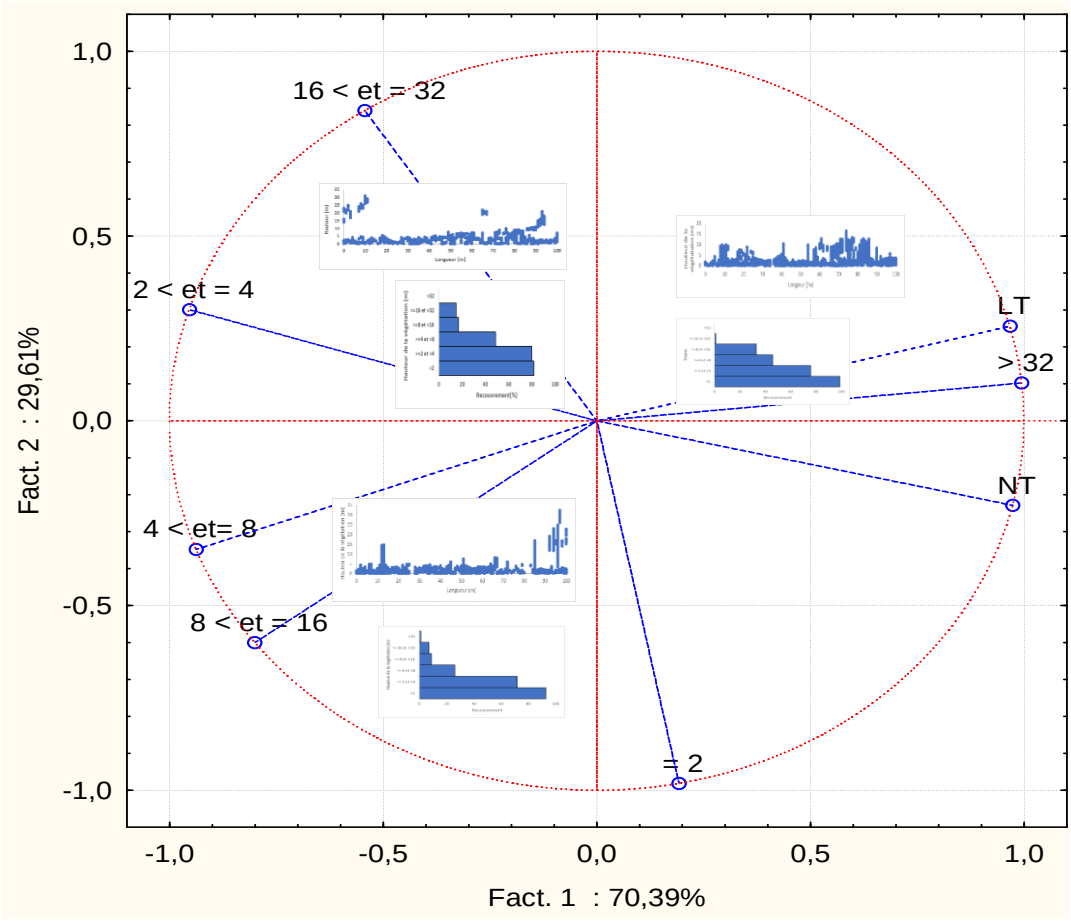

Figure 5 Principal Component Analysis of structural parameters and canopy openness for the surveys in the three plots

4 Discussion

In the forest of the National Zoo of Abidjan, the horizontal structure of the vegetation displays an inverted 'J' shape histogram for each plot. This indicates a strong representation of individuals with a diameter of 5 cm or more (Missa et al. 2023). Such a pattern suggests that in a stable natural environment, the number of individuals in a stand decreases regularly as one moves from smaller-diameter trees to larger ones. Senterre and Nguema (2001) demonstrated that this trend reflects a strong capacity for forest regeneration. Similarly, Adou-Yao et al. (2007) observed this pattern in Taï National Park, noting that the youngest and smallest-diameter trees are the most numerous among all species. This is characteristic of forests with good regeneration capacity (Nusbaumer et al. 2005). Structural profiles indicate a dominance of lianas and nanophanerophytes throughout the forest. These findings contrast with those of Bakayoko (2005), who reported a dominance of megaphanerophytes and mesophanerophytes. This discrepancy may be attributed to the different states of degradation of the study sites. The structural profiles also reveal a noticeable presence of numerous gaps, which aligns with the observations made by Kouamé (1998) regarding semi-deciduous humid dense forests. Kouamé et al. (1998) indicated that the numerous openings indicate anthropogenic activities by local communities. The percentage of cover in the lower strata is prevalent throughout the forest. Bamba (2004) and Bakayoko (2005) made similar observations in the large fragments of Banco National Park, stating that high percentages in the lower strata are indicators of degradation in these areas. These elevated percentages of cover at lower strata may stem from an abundance of understory species and the absence of individuals taller than 32 meters in the peripheral zone of the National Zoo Forest. Soro (2020) made similar observations, suggesting that the lack of large individuals may be due to delayed growth caused by anthropogenic actions affecting certain emergent species in these regions.

The examination of the forest covers indicates a similarity with Type B, as Chatelain (1996) described, which characterizes degraded semi-deciduous forests. This degradation of habitat structure may be attributed to human exploitation, which significantly impacts the forest's integrity (Missa et al. 2019). Such degradation is largely due to extensive incursions by farming

communities into this forest area for harvesting and woodcutting (Gnahore 2021). The PCA analysis enabled us to categorize the three plots based on their level of degradation. Notably, Plot 2 is the most affected by human activities. The classification of vegetation degradation was based on canopy openness measured at 10 meters and other structural parameters, which served as a reference for evaluating the effects of degradation on various structural and floristic factors. Specifically, by counting the gaps intersected by a line drawn at a height of 10 meters and measuring their total width, we found that the vegetation in Plots 1 and 3 is the densest, while Plot 2 exhibits the most open vegetation.

Conclusion

The analysis of the degradation state of the forest at the National Zoo of Abidjan indicates a good representation of trees with a diameter of 5 cm or more, which is a sign of healthy regeneration capacity. However, the classified forest in this area is significantly degraded. The presence of numerous gaps in the structural profiles can be attributed to local communities' anthropogenic activities. The loss of this forest would lead to immeasurable damage to the region's biodiversity and microclimate. To protect it, it is essential to develop conservation policies to preserve floral biodiversity for the survival of all the ecosystems within the study area.

Conflict of interest

The authors declare no conflicts of interest

References

Adou-Yao, Y. C., Dengueadhe, K.T.S., Kouame, D., & N'guessan, K. E. (2007). Diversite Et Distribution Des Ligneux Dans Le Sud Du Parc National De Taï (Pnt), Côte D'ivoire. *Agronomie Africaine, 19* (2), 113 – 123.

Aké-Assi, L. (2002). *Flore de la Côte d'Ivoire 2, catalogue systématique, biogéographique et écologie*. Conservatoire et jardin botanique Genève (Suisse), pp. 401.

Bakayoko, A., Kouamé, N'.F., Tra Bi, F. H., & Traoré, D. (2001). Quelques aspects floristiques et structuraux de la forêt classée de Bossématié, dans l'Est de la Côte d'Ivoire. *Annale des Botanique de l'Afrique de l'Ouest*, (0), 7-20.

Bakayoko, A., Martin, P., Gautier, L., Chatelain, C., Traore, D., & Spichiger, R. (2004). Etude comparative des massifs forestiers entourant la zone de Taï à Zagné (Sud-Ouest de la Côte d'Ivoire). *Candollea, 59* (2), 191-229.

Bakayoko, A. (2005). *Influence de la fragmentation forestière sur la composition floristique et la structure végétale dans le Sud-Ouest de la Côte d'Ivoire*. Thèse de Doctorat, Université de Cocody, Abidjan, Côte d'Ivoire, pp.229.

Bongers, F., Popma, J., del Castillo, J. M., & Carabias, J. (1988). Structure and floristic composition of the lowland rain forest of Los Tuxtlas, Mexico. *Vegetation, 74*, 55-80.

Bamba, I. (2004). *Étude comparative de deux grands fragments forestiers, entre Zagné et Taï, au sud-ouest de la Côte d'Ivoire*. DEA de Botanique, Université de Cocody, Abidjan, Côte d'Ivoire, pp. 57.

Bouko, B. S., Sinsin, B., & Soulé, B. G. (2007). Effets de la dynamique d'occupation du sol sur la structure et la diversité floristique des forêts claires et savanes au Bénin. *Tropicultura, 25* (4), 221-227.

Chatelain, C. (1996). *Possibilités d'application de l'imagerie satellitaire à haute résolution pour l'étude des transformations de la végétation en Côte d'Ivoire forestière*. Thèse Doctorat. Es Science Naturelle Faculté des. Sciences Université de Genève, pp.206.

Cissé, D. E. (2024). *Composition floristique et diversité de la flore du zoo national d'Abidjan*. Memoire de Master, Université Nangui Abrogoua, pp. 42.

Dao, H. (1999). *Connaissance de l'environnement et système d'informations géographiques : le cas de la déforestation en Côte d'Ivoire*. Thèse Université. Genève, pp. 231.

Emberger, L., Godron, M., & Daget, P. (1968). *Code pour le relevé méthodique de la végétation et du milieu*. Paris pp. 292.

FAO/UNEP. (1981). *Tropical forest resources assessment project (in the frameworkof GEMS)*. Vol. 1: Forest resources of tropical Asia. Vol. 2: Forest resources of tropical Africa. FAO, Rome, 2 vol., 475 + 586 pp.

Gautier, L., Châtelain, C., & Spichiger, R. (1994). Presentation of a relevé method for vegetation studies based on finc-scale satellite imagery. In: *Comptes rendus de la treizième réunion plénière de l'AETFAT, Zomba, Malawi* (pp. 1339- 1350). The National Herbarium and Botanic Gardens of Malawi.

Gnahore, E. (2021). *Dynamique de la végétation et de la flore du parc national du banco (Abidjan, Côte d'Ivoire)*. Thèse de Doctorat, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire 180 p.

Kahn, F. (1982). La reconstitution de la forêt tropicale après une culture traditionnelle. (SudOuest de la Côte d'Ivoire) Mém. Éditions de l'Office de la Recherche Scientifique et Technique Outre-Mer Collection MÉMOIRES NO 97, Paris, pp. 150.

Kouamé, F.N., Tra Bi, F.H., Ettien, D.T., & Traoré, D. (1998). Végetation et flore de la forêt classeé du Haut-Sassandra, en Côte d'Ivoire. *Revue du CAMES, 1*, 28-35.

Kouamé, N. F. G. (1998). *Influence de l'exploitation forestière sur la végétation et la flore dela Forêt Classée du Haut Sassandra (Centre Ouest de la Côte d'Ivoire)*. Thèse de 3 eme cycle, Université de Cocody, Abidjan, pp. 227.

Menzies A. (2000). *Structure et composition floristique de la forêt de la zone Ouest du Parc nationale de Tai (Côte d'Ivoire)*. Diplôme, Université de Genève, pp. 124.

Missa, K., Yao, K., Koffi,J K., & Soro, K.(2023). Diversité floristique et structure de la végétation d'une carrière de granite dans le districtf de Yamoussoukro, Centre de la Côte d'Ivoire. *International Journal of Biological and Chemical Sciences*, *17* (4). 1519-1529.

Missa, K., Soro, D., Seguena, F., & Bakayoko, A. (2019). Floristic and structural studies of the forest blocks of Nangui Abrogoua University, Côte d'Ivoire. *Journal of Biodiversity and Environmental Sciences, 14* (1), 61-71

N'guessan K. A., Boussou K. M. R., & Koffi B. J.C. (2021). Etude comparée de l'alimentation des chimpanzés ex-situ et en milieu naturel : cas du Zoo National d'Abidjan et du Parc National de Taï, Côte d'Ivoire Kouamé Antoine. *Afrique Science, 19*(4), 14 – 24.

Nusbaumer, L., Gautier, L., Châtelain, C., & Spichiger R. (2005). Structure et composition floristique de la forêt classée de Scio. *Candollea*, *60* (2), 393-443.

Richards, P.W. (1952). *The tropical rain forest, and ecological study*. Cambridge University Press, pp. 450.

Senterre, B., & Nguema, N. (2001) La diversité des ligneux dans la forêt de Nsork (Guinée équatoriale). *Systematics and Geography of Plants, 71* (2), 837-846. https://doi.org/10.2307/3668722.

Soro, Y. (2020). *Etude floristique et structurale des ils du lac de BUYO au Nord-Est du Parc National de Tai et dynamique de reconstitution après anthropisation (Côte d'Ivoire)*. Thèse de Doctorat, Université Nangui Abrogoua, Abidjan, Côte d'Ivoire, pp. 180.

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Heat Pump Drying of Nutmeg Pericarp: Engineering Properties, Drying Kinetics, and Haghi-Angiz-II Modelling for Process Optimisation

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Heat pump drying

Drying kinetics

Mathematical modelling

R studio

Haghi and Angiz-II modelling

Moisture diffusivity

ABSTRACT

This study investigates the engineering properties of nutmeg pericarp and develops a mathematical model to describe its drying behavior in a heat pump dryer. Nutmeg pericarp, an underutilized part of the nutmeg fruit, is a rich source of phytochemicals but is highly perishable, necessitating immediate postharvest drying for further processing. Drying experiments were conducted at a controlled temperature of 55°C and a relative humidity of 37%. Regression modeling was used to analyze the drying kinetics, utilizing MATLAB R2020a and R Studio software. Various statistical metrics, including the coefficient of determination (R^2), adjusted R^2, and root mean square error (RMSE), were evaluated to determine the predictive accuracy of different thin-layer drying models. Among the models assessed, the Haghi and Angiz-II model best fit the experimental data, achieving the highest R^2 value of 0.999. A scatter plot comparing the experimental and predicted moisture ratios further confirmed the reliability of this model. The effective moisture diffusivity ranged from 2.1×10^{-8} to 9.08×10^{-8} m^2/s. Additionally, the quality assessment indicates that heat pump drying positively influences the key quality attributes of nutmeg pericarp.

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1 Introduction

Nutmeg (*Myristica fragrans* Houtt.) is a single-seeded fleshy drupe from an evergreen tree native to the Banda Islands in the Moluccas, Indonesia (Van Gils and Cox 1994). The fruit consists of an outer fleshy pericarp, an inner kernel (nutmeg), and mace, the dried red aril encasing the kernel. The pericarp naturally splits open upon maturation, separating the seed and mace (Wallis 1985; Periasamy et al. 2016). The pericarp accounts for approximately 80-85% of the total fruit weight. Meanwhile, the nutmeg pericarp has limited uses in food products such as pickles, wines, jams, and jellies, which are commercial trades that predominantly focus on seed and aril. As a result, the pericarp is often considered agricultural waste with low economic value. However, it contains diverse, active phytochemicals, including monoterpene hydrocarbons, monoterpene acids, flavonoids, alkaloids, tannins, and aromatic ethers (Suwarda et al. 2021). Due to the perishable nature of nutmeg pericarp, immediate postharvest handling is essential to prevent spoilage and maintain quality.

Drying is a commonly used postharvest technique aimed at extending the shelf life of nutmeg pericarp by reducing biochemical, chemical, and microbiological degradation. Various drying technologies have been developed to enhance product quality and improve energy efficiency. Among these, heat pump drying is an advanced method characterized by lower energy consumption and superior retention of quality attributes. This system extracts heat from ambient air to warm the drying air (Tunckal and Doymaz 2020). It operates on a refrigeration principle, cooling and dehumidifying the air stream, condensing moisture, recovering latent heat, and recirculating the air. This approach is particularly advantageous for drying heat-sensitive materials, as it maintains low relative humidity and allows for precise control over drying conditions, thereby preserving the nutritional and sensory qualities of the final product (Patel and Kar 2012).

Extensive research has shown the effectiveness of heat pump drying for various agricultural products, demonstrating its ability to operate at lower temperatures and humidity levels while improving energy efficiency compared to conventional drying methods. However, studies focusing on nutmeg pericarp are limited, primarily due to its categorization as agricultural waste in many regions. Given its significant potential in various industries, immediate postharvest drying of nutmeg pericarp is crucial for minimizing losses. Consequently, using a heat pump drying system, the present study investigates nutmeg pericarp's engineering properties and drying characteristics. Empirical and theoretical mathematical models have also been developed and validated to characterize its drying kinetics. The findings of this research aim to optimize the heat pump drying conditions and predict the quality attributes of the final product, thereby facilitating its potential commercial utilization.

2 Materials and Methods

2.1 Sample Preparation

Fresh nutmeg pericarp samples were obtained from a local Tavanur, Malappuram, Kerala, India farmer. The engineering properties of the mature pericarp were evaluated to understand its behavior during handling and subsequent processing. The fruit was then sliced into uniform thicknesses of 5.4 ± 0.8 mm to facilitate the drying experiments.

2.2 Engineering properties of nutmeg pericarp

2.2.1Shape and size

The shape and size of the nutmeg pericarp are characterized by sphericity, which describes how the material's shape relates to a sphere of equal volume. It is calculated using the following formula (Sahay and Singh 2009):

$$\text{Sphericity} = \frac{(\text{lbt})^{1/3}}{\text{l}} \quad (1)$$

Where l, b, and t are the nutmeg pericarp's length, width, and thickness, respectively.

2.2.2 Bulk density, True density and Porosity

The density of samples is classified into two categories: bulk density (ρ_B) and true density. Bulk density is the ratio of the sample's weight to its bulk volume. For irregular agricultural products, such as nutmeg, bulk volume is measured using the platform scale method (Sahay and Singh 2009; Murakonda et al. 2022). It is calculated as follows:

$$\text{Bulk density}, \rho_B = \frac{\text{Weight of sample , kg}}{\text{Volume of sample , m}^3} \quad (2)$$

The volume is determined by:

$$\text{Volume, m}^3 = \frac{\text{Weight of displaced water , kg}}{\text{Weight density of water , kg/m}^3} \quad (3)$$

The true density (ρ_T) of nutmeg pericarp is the ratio of the sample's weight to its true volume, excluding pore spaces. It is determined using the toluene displacement method (Kumar et al. 2022):

$$\text{True density}, \rho_T = \frac{\text{Weight of sample ,kg}}{\text{True volume , m}^3} \quad (4)$$

The porosity of nutmeg pericarp is the amount of pore space present in the pericarp, expressed as follows (Kumar et al. 2022):

$$\text{Porosity} = \frac{\text{True density} - \text{Bulk density}}{\text{True density}} \quad (5)$$

2.3 Nutmeg pericarp drying analysis

The drying experiment was carried out using a heat pump dryer. 100 g of uniformly sliced samples were processed at a temperature of 55°C and a relative humidity of 37% for 6 hours. After drying, the samples were stored in polyethylene packaging in a dark environment for further analysis.

2.3.1 Moisture content

Moisture content was measured on a wet basis using an infrared moisture meter (SHI-AM Technologies, Gujarat, India).

2.3.2 Colour analysis

Color parameters (L*, a*, and b*) were measured using a Lovibond tintometer. The L* value indicates lightness on a scale from 0 to 100, the a* value ranges from green (-) to red (+), and the b* value ranges from blue (-) to yellow (+). For fresh samples, the tintometer was placed directly on the cut surface of the pericarp. For dried samples, thin slices were made and placed in a cuvette for measurement. The total color change (ΔE) was calculated using the method described by Nkhata (2020):

$$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2} \quad (6)$$

Where ΔL, Δa, and Δb represent the colour differences before and after drying

2.3.3 Antioxidant activity

The pericarp of nutmeg is rich in bioactive compounds that provide antioxidant activity, which helps to neutralize free radicals that can damage cells. The antioxidant properties were measured using the DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay described by Adiletta et al. (2018). This assay was conducted on fresh and dried nutmeg samples to assess how drying affects antioxidant activity. The percentage inhibition of antioxidant activity was calculated using the following equation:

% inhibition of antioxidant activity =

$$\left(\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100 \quad (7)$$

2.3.4 Rehydration ratio

The rehydration ratio, which indicates the degree of cellular and structural disruption that occurs during the drying process, was determined by soaking 5 grams of dried nutmeg pericarp samples in 200 milliliters of distilled water until a constant weight was reached (Murali et al. 2021). The weight of the samples was recorded after 30 minutes, and the rehydration ratio was calculated using the following formula:

$$\text{Rehydration ratio} = \frac{\text{Weight of rehydarted sample}}{\text{Weight of dried sample}} \quad (8)$$

2.3.5 Shrinkage

Shrinkage is measured by the change in volume during drying, expressed as the ratio of the volume after drying to before drying (Yan et al. 2008).

$$\text{Shrinkage} = \frac{\text{Volume of sample after drying}}{\text{Volume of raw sample}} \times 100 \quad (9)$$

2.3.6 Effective moisture diffusivity

The drying of agricultural produce primarily occurs during the falling rate period, where the movement of moisture is mainly driven by internal diffusion. This process can be mathematically described using Fick's second law of unsteady state diffusion, as outlined by Zeng et al. (2024):

$$\frac{\delta M}{\delta t} = D_{eff}\, M \quad (10)$$

Nutmeg pericarp is considered an infinite slab, and Crank provided the solution to Fick's law (Crank 1975). The moisture ratio was determined by following the formula:

$$MR = \frac{8}{\pi^2}\sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \exp \left(\frac{(2n+1)^2 \pi^2 D_{eff}\, t}{4L^2}\right) \quad (11)$$

Where D_{eff} represents the effective moisture diffusivity (m^2/s), t denotes the time of drying (s), L is the half thickness of the sample (m), and n is a positive integer. The above equation is simplified for longer drying periods as follows,

$$MR = \frac{8}{\pi^2}\sum_{n=0}^{\infty} \exp \exp \left(\frac{-\pi^2 D_{eff}\, t}{4L^2}\right) \quad (12)$$

Taking natural logarithm, the D_{eff} is calculated as follows,

$$\ln \ln (MR) = \ln \ln \left(\frac{8}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff}\, t}{4L^2}\right) \quad (13)$$

The plot of ln MR against time (min) was developed to determine the slope. The effective moisture diffusivity coefficient D_{eff} was then calculated from the slope of the plot using the given equation,

$$D_{eff} = \frac{-\text{slope} \times 4 \times L^2}{\pi^2} \quad (14)$$

2.3.7 Mathematical modelling of drying Kinetics

To analyze the drying behavior, various mathematical models were employed to describe the drying kinetics of nutmeg pericarp. The moisture ratio concerning drying time for the experimental data of nutmeg pericarp was fitted to six thin-layer drying models: the Lewis model, Page model, Henderson and Pabis model, Wang and Singh model, Haghi and Angiz-II model, and the Logarithmic model (Haghi and Angiz 2007; Ertekin and Firat 2015). The equations for these models are provided in Table 1.

Table 1 Mathematical models used to determine the drying kinetics of nutmeg pericarp

S. No.	Name of model	Equation
1	Lewis model	MR = exp(-kt)
2	Page	MR = exp(-kt^n)
3	Henderson and Pabis	MR = aexp(-kt)
4	Wang and Singh	MR = 1+at+bt^2
5	Haghi and Angiz -II	MR = a+bt+ct^2+dt^3
6	Logarithmic	MR = aexp(-kt)+c

The goodness of fit for these models was evaluated using several metrics: the coefficient of determination (R^2), adjusted R^2, and root mean square error (RMSE). The model with the highest R^2 and adjusted R^2 values and the lowest RMSE was the best fit for the drying behavior (Murali et al. 2021). The goodness of fit of a regression model is assessed through R^2, which ranges from 0 to 1; higher values indicate a better fit (Venu et al. 2023). RMSE is a statistical measure that quantifies the difference between predicted and actual values, focusing attention on more significant errors to evaluate the accuracy of predictive models (Venu et al. 2024). For the regression analysis of non-linear equations, MATLAB R2020a and R Studio were utilized (Venu et al. 2024).

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n} (P_i - Q_i)^2} \quad (15)$$

Where n = Number of data points, P_i = Predicted value for the i^{th} data point, Q_i = Actual value of the i^{th} data point.

The moisture ratio (MR) describes the variation in moisture content over time and is expressed as described by Murali et al. (2021):

$$\mathrm{MR} = \frac{M - M_e}{M_0 - M_e} \quad (16)$$

Where M is the moisture content at time, t and M_0 and M_e are the initial and equilibrium moisture content. The value Me is significantly smaller than Mt or M_0 during extended drying time (Tunckal 2020). Therefore, MR can be simplified to:

$$\mathrm{MR} = \frac{M}{M_0} \quad (17)$$

2.4 Statistical Analysis

The statistical analysis of the drying data offers valuable insights into moisture loss over time. The mean sample weight throughout the drying process was recorded as 31.75 g, with a standard deviation of 28.71 g, indicating significant variation in moisture content during drying. The initial weight of the pericarp was recorded at 100.6 g, which decreased progressively to a final weight of 12.4 g. The median weight was determined to be 16 g, reflecting the central tendency of the dataset. The data displayed a skewed distribution, with a rapid decrease in weight during the initial drying phase, followed by a gradual stabilization. This pattern is typical of drying kinetics, where free water evaporates quickly in the early stages, while bound moisture is removed more slowly in the later stages. This statistical evaluation enhances our understanding of drying behavior and contributes to modeling the kinetics needed to optimize drying conditions and predict equilibrium moisture content.

3 Results and Discussion

3.1 Engineering properties of nutmeg pericarp

Five nutmeg pericarp samples' shape, length, width, and thickness were analyzed. The average sphericity was found to be 0.954 ± 0.03. In a study by Yamagar and Borkar (2018), the reported average sphericity was 0.82. This observed difference may be attributed to variations in physical and geographical factors. The findings also indicated that the nutmeg pericarp's average bulk density and true density ranged from 1000 ± 0.0018 kg/m^3 to 1019 ± 0.01 kg/m^3, respectively. Additionally, the porosity was measured at 0.019 ± 0.011.

3.2 Quality characteristics of dried nutmeg pericarp

3.2.1 Colour analysis

The L* value of the dried sample (57.06±0.08) was slightly lower than that of the fresh sample (75.567±0.82), indicating a darker appearance. This darker color can be attributed to the concentration of constituents in the pericarp. Additionally, the Maillard reaction contributes to the browning, which further affects the color. Similar results were reported by Salehi and Kashaninejad (2018). The darkening observed during the drying process is associated with several factors, including pigment degradation, ascorbic acid oxidation, and non-enzymatic Maillard browning, as noted by Maskan (2001), Nadian et al. (2015), and Han and Jin (2024). In terms of color, the redness (a* value) of the pericarp significantly increased from 1.934±0.64 to 12.33±1.02, which can be attributed to pigment formation during the Maillard reaction, as reported by Tunckal and Doymaz (2020). Similarly, the yellowness (b* value) of the pericarp rose from 16.367±1.31 to 25.23±0.75, consistent with findings by Chong et al. (2013).

3.2.2 Antioxidant activity

The results showed that the antioxidant activity of the dried samples (89.72) was comparable to that of the fresh sample (89.5). This similarity indicates that the heat pump drying process effectively preserved the antioxidant properties. The slight increase in activity may be attributed to the formation of oxidized polyphenols and Maillard reaction products during the drying process (Vidinamo et al. 2022).

3.2.3 Rehydration ratio

The rehydration ratio of dried nutmeg pericarp was 2.4, indicating minimal structural damage during drying (Murali et al. 2021). This higher rehydration ratio is attributed to improved water removal from the tissues, as reported by Vadivambal and Jayas (2007).

3.2.4 Shrinkage

Shrinkage occurs when water is removed during drying, decreasing the product's volume. In this study, the sample exhibited a shrinkage of 26.2%, which indicates improved porosity and enhances its rehydration capacity (Hawlader et al. 2006). Additionally, shrinkage is affected by controlled temperature and humidity conditions. The findings suggest that effective drying was achieved without compromising the structural and nutritional quality of the product.

3.2.5 Effective moisture diffusivity

The natural logarithm of the moisture ratio, ln (MR), was plotted against drying time (min) to determine the slope. This slope was then used to calculate the effective moisture diffusivity (Figure 1). The calculation was based on Fick's second law of diffusion, which describes moisture migration during drying. The effective moisture diffusivity of nutmeg pericarp was found to be 5.5 X 10^{-8} m^2/s, and it varies with values ranging from 2.1 X 10^{-8} to 9.08 X 10^{-8} m^2/s, depending on the drying stage. The diffusivity values obtained were consistent with the typical range reported for food products, which spans from 10^{-11} to 10^{-6} (Olanipekun et al. 2014).

3.2.6 Mathematical modelling of drying

To evaluate various thin layer drying models for nutmeg pericarp, we fitted the experimentally obtained moisture ratios to six mathematical models: Lewis, Page, Henderson and Pabis, Wang and Singh, Haghi and Angiz II, and the logarithmic model. We assessed the statistical performance of each model using the values of R^2, adjusted R^2, and RMSE, as summarized in Table 2. The most suitable model was chosen based on the highest R^2, adjusted R^2 values, and the lowest RMSE. A comprehensive comparison of the statistical performance of the different moisture ratio models is presented in Figure 1.

The first three models, i.e., Lewis, Page, Henderson, and Pabis, exhibited low R^2 values of 0.28, indicating poor predictive accuracy for the drying behavior of nutmeg pericarp. In contrast, the logarithmic model showed moderate predictive performance with an R^2 value of 0.50, indicating a slight improvement. Among the six selected thin layer models, the Wang and Singh model and the Haghi and Angiz -II model displayed significantly higher R^2 values of 0.9678 and 0.998, respectively. This implies that both models could explain more than 90% of the drying data.

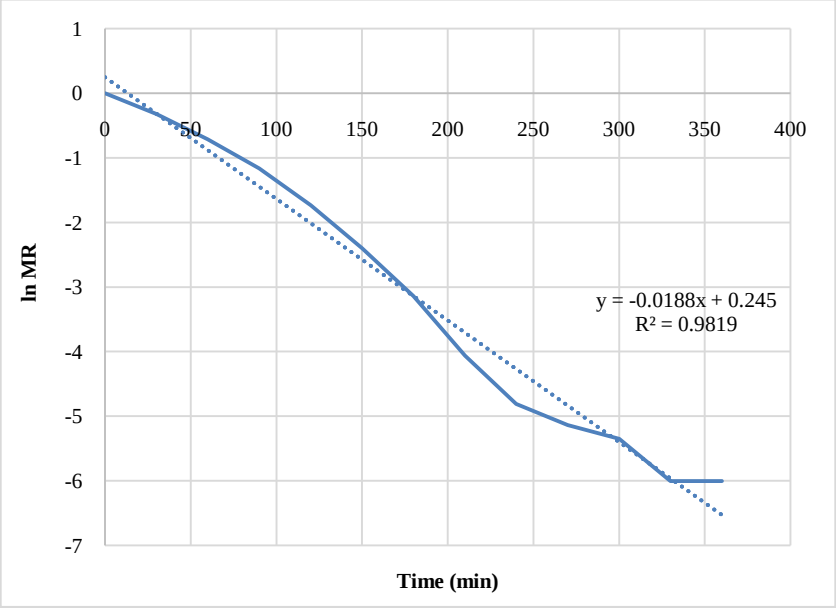

Figure 1 Plot of ln MR against drying time (min) for nutmeg pericarp drying

Table 2 Mathematical modelling of drying behaviour of nutmeg pericarp

S. No.	Name of model and equation	R^2 value	Adj. R^2 value	RMSE value	Constants
1	Lewis model MR= exp(-kt)	0.2873	0.2873	0.2741	k= 0.9706
2	Page MR= exp(-kt^n)	0.2873	0.2225	0.2863	k = 0.9706 n = 0.9572
3	Henderson and Pabis MR= aexp(-kt)	0.2873	0.2225	0.2863	a = 1 k = 0.9706
4	Wang and Singh MR= 1+at+bt^2	0.9678	0.9649	0.0609	a = -0.00825 b= 0.00001594
5	Haghi and Angiz -II MR = a+bt+ct^2+dt^3	0.9998	0.9997	0.0059	a = 1.006 b = -0.0108 c = 3.83e-05 d = -4.464e-08
6	Logarithmic MR= aexp(-kt)+c	0.5192	0.4230	0.2467	a = 0.8436 c= 0.1564 k= 0.9706

3.2.6.1 Comparative Analysis of best fitting models

To ensure a thorough comparison, we evaluated the models with higher R^2 values by plotting the observed and fitted values (Figure 2). Although both models demonstrated strong correlations, Figure 2(b) clearly shows that the Haghi and Angiz-II model outperformed the Wang and Singh model in accurately predicting drying behavior. The experimental data closely align with the 45° reference line, indicating a more substantial agreement between the observed and fitted moisture ratios. In contrast, the Wang and Singh model (Figure 2a) exhibits more data dispersion, signifying lower predictive accuracy. This comparison highlights the superior performance of the Haghi and Angiz-II model in effectively capturing the complexities of the drying process, demonstrating its effectiveness over the Wang and Singh model.

The analysis of moisture ratio models reveals significant differences in performance, as indicated by the R^2 and RMSE values. The Haghi and Angiz II model outperformed the other thin-layer models, achieving the highest R^2 value of 0.9998, an adjusted R^2 of 0.9997, and the lowest RMSE value of 0.0059. This demonstrates a robust fit of the experimental data. In contrast, the Wang and Singh model displayed an R^2 of 0.9678 and an RMSE of 0.0609, indicating more significant discrepancies from the actual

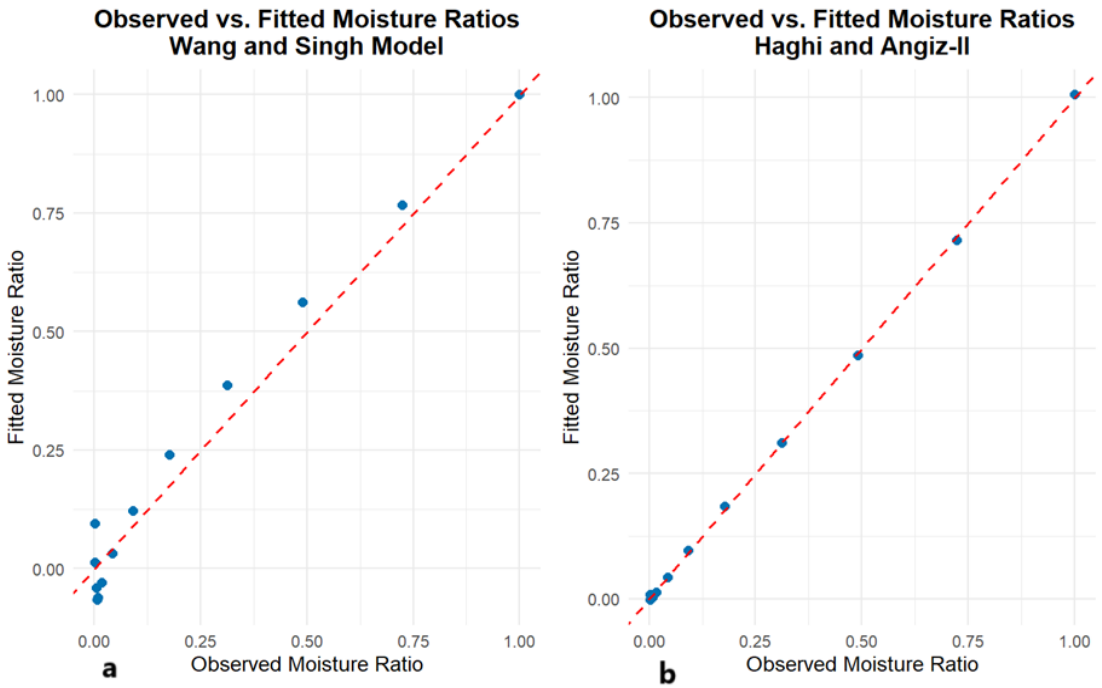

Figure 2 Plot of observed against fitted moisture ratio values of nutmeg pericarp: (a) Wang and Singh model, (b) Haghi and Angiz-II model

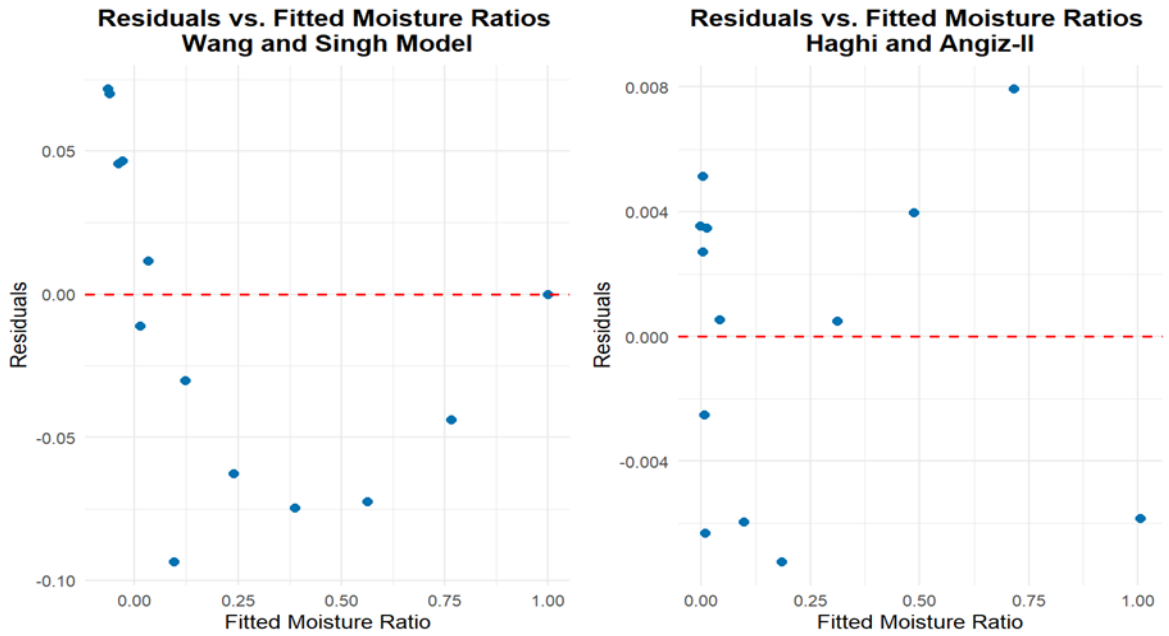

Figure 3 Plot of residuals against fitted moisture ratio values of nutmeg pericarp: (a) Wang and Singh model, (b) Haghi and Angiz-II model

moisture ratios. The observed versus fitted plot further supports these findings, showing that the data points for the Haghi and Angiz II model cluster closely around the reference line, illustrating its accuracy. These statistical results suggest that the Haghi and Angiz II model is the most reliable and suitable for accurately predicting the drying behavior of nutmeg pericarp. Additionally, the residuals against the fitted plot, as shown in Figure 3, reveal a more random distribution for the Haghi and Angiz II models, indicating minimal bias. In contrast, the Wang and Singh model exhibits a noticeable pattern in the residuals, which points to potential predictive shortcomings.

3.2.6.2 Time-moisture ratio relationship and model equation

Figure 4 shows a time versus moisture ratio plot featuring model curves. This reinforces the conclusion that the Haghi and Angiz II

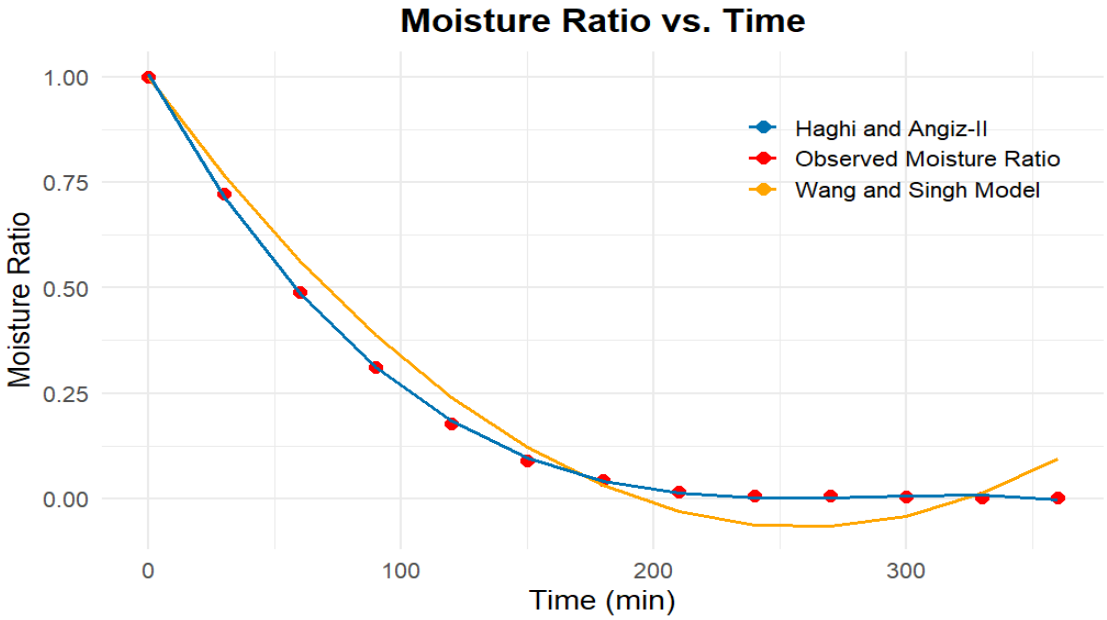

Figure 4 Plot of moisture ratio versus time of nutmeg pericarp using Observed values, Wang and Singh, Haghi and Angiz-II model

model effectively captures the dynamics of the drying process. In contrast, the Wang and Singh model does not perform as well, which justifies the preference for the former in practical applications.

The mathematical equation can express the Haghi and Angiz II model:

$$MR = 1.006 - 0.0108\,t + 3.83 \times 10^{-5}t^2 - 4.464 \times 10^{-4}t^3 \quad (18)$$

This equation underpins the model's high R^2 and low RMSE values, reinforcing its effectiveness in capturing the complexities of the drying process.

Conclusion

The study thoroughly examined the engineering properties, drying kinetics, and quality attributes of nutmeg pericarp dried using a heat pump dryer. The drying process was carried out at a consistent temperature of 55°C and a relative humidity of 37%, which reduced the moisture content from 87% (wet basis) to 3.3% (wet basis) within 6 hours. Mathematical modeling of the drying behavior indicated that the Haghi and Angiz-II models provided the most accurate predictions, demonstrating superior performance in illustrating the moisture removal kinetics of nutmeg pericarp slices. Additionally, the quality analysis showed that heat pump drying significantly improved the key quality parameters of the dried product. Overall, the findings confirm that the heat pump dryer performed exceptionally well in drying nutmeg pericarp while effectively preserving the quality of the dried product.

Conflict of Interest

The authors declare no conflict of interest.

Ethical Clearance

Not application on this study.

References

Adiletta, G., Petriccione, M., Liguori, L., Pizzolongo, F., Romano, R., & Di Matteo, M. (2018). Study of pomological traits and physico-chemical quality of pomegranate (*Punica granatum L.*) genotypes grown in Italy. *European Food Research and Technology, 244*(8), 1427-1438.

Chong, C. H., Law, C. L., Figiel, A., Wojdylo, A., & Oziembłowski, M. (2013). Colour, phenolic content and antioxidant capacity of some fruits dehydrated by a combination of different methods. *Food Chemistry, 141*, 3889-3896. http://dx.doi.org/10.1016/j.foodchem.2013.06.042.

Crank, J. (1975). *The mathematics of diffusion*. Oxford University Press. 44p.

Han, L., & Jin, T. X. (2024). Effects of combined vacuum and heat pump drying on drying characteristics and physicochemical properties of pineapple. *LWT- Food Science and Technology, 192*, 115727. https://doi.org/10.1016/j.lwt.2024.115727.

Ertekin, C., & Firat, M. Z. (2015). A Comprehensive Review of Thin Layer Drying Models Used in Agricultural Products. *Critical Reviews in Food Science and Nutrition, 57* (4), 701-717.

Haghi, A.K., & Angiz, F.Z. (2007). Heat and mass transfer in thermal drying of wool: a theoretical approach. *The Proceedings of the 5th Asia-Pasific Drying Conference*, *1*, 443-448.

Hawlader, M.N.A., Perera, C.O., & Tian, M. (2006). Comparison of the retention of 6-gingerol in drying of ginger under modified atmosphere heat pump drying and other drying methods. *Drying Technology, 24*(1), 51–56. https://doi.org/10.1080/07373930500538675.

Kumar, V., Sharma, H. K., & Kumar, N. (2022). Engineering Properties of Foods. In H. K. Sharma, & N. Kumar, (Eds). *Agro-Processing and Food Engineering: Operational and Application Aspects* (pp. 23-80). Singapore: Springer Singapore.

Maskan, M. (2001). Kinetics of colour change of kiwifruits during hot air and microwave drying. *Journal of Food Engineering, 48,*169-175.

Murakonda, S., Patel, G., & Dwivedi, M. (2022) Characterization of engineering properties and modeling mass and fruit fraction of wood apple (*Limonia acidissima*) fruit for postharvest processing. *Journal of the Saudi Society of Agricultural Sciences*, *21*(4), 267-277 https://doi.org/10.1016/j.jssas.2021.09.005

Murali, S., Delfiya, D. S. A., Kumar, K. S., Kumar, L. R. G., Nilavan, S. E., Amulya, P. R., & Samuel, M. P. (2021). Mathematical Modeling of Drying Kinetics and Quality Characteristics of Shrimps Dried under a Solar–LPG Hybrid Dryer. *Journal of Aquatic Food Product Technology*, *30*(5), 561–578. https://doi.org/10.1080/10498850.2021.1901814

Nadian, M. H., Rafiee, S., Aghbashlo, M. S., & Mohtasebi, S. S. (2015). Continuous real-time monitoring and neural network modeling of apple slices color changes during hot air drying. *Food and Bioproducts Processing, 94*, 263-274. https://doi.org/10.1016/j.fbp.2014.03.005.

Nkhata S. G. (2020). Total color change ($\Delta E*$) is a poor estimator of total carotenoids lost during postharvest storage of biofortified maize grains. *Heliyon*, *6*(10), e05173. https://doi.org/10.1016/j.heliyon.2020.e05173.

Olanipekun, B. F., Tunde-Akintunde, T. Y., Oyelade, O. J., Adebisi, M. G., & Adenaya, T. A. (2014). Mathematical modelling of thin-layer pineapple drying. *Journal of Food Processing and Preservation*, *39*, 1431-1441.doi:10.1111/jfpp.12362.

Patel K. K., & Kar, A. (2012). Heat pump assisted drying of agricultural produce-an overview. *Journal of e Food Science and Technology*, *49*(2), 142-60. doi: 10.1007/s13197-011-0334.

Periasamy, G., Karim, A., Gibrelibanos, M., Gebremedhin, G., & Gilani, A.H. (2016). *Nutmeg (Myristica fragrans Houtt.) Oils*, In: V. R. Preedy (ed.) *Essential Oils in Food Preservation, Flavor and Safety* (pp. 607–616). Elsevier. doi:10.1016/B978-0-12-416641-7.00069-9.

Sahay, K. M., & Singh, K. K. (2009). *Unit operations in Agricultural Processing*. Vikas Publishing house PVT LTD. New Delhi. pp: 7-8.

Salehi, F., & Kashaninejad, M. (2018). Modeling of moisture loss kinetics and color changes in the surface of lemon slice during the combined infrared-vacuum drying. *Information Processing in Agriculture*, *5*(4), 516-523.

Suwarda, R., Suneth, R.F., Wahid, & Sondari, D. (2021). Potential and opportunities of nutmeg pericarp as functional foods. *E3S Web of Conferences*, *306*, 04007. doi:10.1051/e3sconf/202130604007.

Tunçkal, C. (2020). Investigation of performance and drying kinetics of the closed, partially open, and open heat pump drying systems. *Journal of Food Process Engineering*, *43*(12), e13566.

Tunckal, C., & Doymaz, İ. (2020). Performance analysis and mathematical modelling of banana slices in a heat pump drying system. *Renewable Energy*, *150*, 918–923. doi:10.1016/j.renene.2020.01.040.

Vadivambal, R., & Jayas, D. S. (2007). Changes in quality of microwavetreated agricultural products-a review. *Biosystems Engineering*, *98*, 1–16. doi:10.1016/j.biosystemseng.2007.06.006.

Van Gils, C., & Cox, P.A. (1994). Ethnobotany of nutmeg in the Spice Islands. *Journal of Ethnopharmacology*, *42*, 117–124. https://doi.org/10.1016/0378-8741(94)90105-8.

Venu, V., Vipin P. R., & Prajitha N. K. (2023). A Comparative Analytical Study of Many Regression Model Approaches, Arima Model and a Hybrid Model for Forecasting Area, Production, and Productivity of Coconut in Kerala, India. *Current Journal of Applied Science and Technology*, *42*(40), 37-49.

Venu,V., Anjitha, P.K., Vipin, P.R., Ramdas, E.R., Senthilkumar, R., & Sreenath, B. (2024). Temporal Trends and Future Projections: A Deep Dive into India's Buffalo Milk Production Through Time Series Modelling. *Asian Journal of Dairy and Food Research*, *43*(3), 1-9 doi: 10.18805/ajdfr.DR-2199.

Vidinamo, F., Fawzia, S., & Karim, M.A. (2022). Effect of drying methods and storage with agro-ecological conditions on phytochemicals and antioxidant activity of fruits: a review. *Critical Reviews in Food Science and Nutrition*, *62*, 353–361. https://doi.org/10.1080/10408398.2020.1816891

Wallis, T. E. (1985). *Text book of pharmacognosy* (5th ed., p. 652). New Delhi, India: CBS Publishers and Distributors.

Yan, Z., Gallagher, M. J. S., & Oliveira, F. A. R. (2008). Shrinkage and porosity of banana, pineapple and mango slices during air-drying. *Journal of Food Engineering*, *84*, 430-440. doi:10.1016/j.jfoodeng.2007.06.004.

Yamagar, S., & Borkar, S. (2018). Comparative study of physical properties of Nutmeg (*Myristica fragrans* Houtt.) of different varieties. *International Journal of Chemical Studies*, *6*(6), 2561-2566.

Zeng, Z., Han, C., Wang, Q., Yuan, H., Zhang, X., & Li, B. (2024) Analysis of drying characteristic, effective moisture diffusivity and energy, exergy and environment performance indicators during thin layer drying of tea in a convective-hot air dryer. *Frontiers in Sustainable. Food Systems*, *8*, *1371696*. doi: 10.3389/fsufs.2024.1371696.