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THE INTERNATIONAL CONFERENCE OF PHARMACEUTICAL MATERIAL, ENGINEERING AND APPLIED SCIENCE (ICOPMES) 2020

The International Conference on Pharmaceutical Material, Engineering, and Applied Sciences (ICOPMES) 2020 is an international event held to gather scientists, professionals, and other expertise to discuss new developments in pharmaceutical sciences in general. ICOPMES 2020 is organized by the Faculty of Pharmacy, Hasanuddin University, and will be held virtually on 18-19 November 2020. We invite you to submit your research products and to exchange information and scientific expertise with the topics included as follow:

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In an effort to produce effective and safe medicinal preparations, and to keep abreast of developments in the world of health and medicine, various researches have been carried out by all scientists, including Indonesian scientists. Due to the development of various diseases and the increasing needs of the medical world, the use of conventional preparations has begun to be abandoned. Recently, many researches related to dosage formulations have focused on the development of novel drug delivery systems (NDDS) or modification of the physicochemical character of a drug to meet its therapeutic objectives.

Therefore, the Faculty of Pharmacy, Hasanuddin University provides a scientific forum entitled the International Conference on Pharmaceutical Materials, Engineering and Applied Science (ICOPMES) 2020. This scientific conference is expected to be a platform for scientists to discuss, inventory, and publish innovations in the form of research results in (but not limited to) pharmaceuticals. This scientific conference will present a theme covering all pharmaceutical sciences, including the application of knowledge from various fields, such as chemistry, biology, epidemiology, statistics, mathematics, physics, to aspects of clinical pharmacy and social pharmacy. This shows the commitment of this activity to bring together scientists, professional practitioners with various other parties to collaborate in the form of an exchange of ideas to then find solutions to world health problems. Finally, due to the current conditions of the Covid-19 pandemic, this scientific conference will be held virtually. However, the organizing committee has been determined to include several invited speakers from various countries, so that even though it is implemented virtually, it is hoped that the objective of this conference will be maximally achieved.

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Table of contents

EFFECT OF GREEN TEA EXTRACT ON MODULATING THE ANTIBACTERIAL ACTIVITY OF STANDARD ANTIBIOTICS AGAINST THE CLINICAL ISOLATES OF *Acinetobacter baumannii* *10.18006/2021.9(Spl-2-ICOPMES_2020).S202.S207*	**S202—S207**
***In silico* SCREENING OF *Ziziphus spina-christi* (L.) Desf. AND *Strychnos ligustrine* COMPOUNDS AS A PROTEASE INHIBITOR OF SARS-COV-2** 10.18006/2021.9(Spl-2-ICOPMES_2020).S208.S214	**S208—S214**
EVALUATION OF ANTIOXIDANT AND ANTIFUNGAL PROPERTIES OF PALU SHALLOT (*Allium ascalonicum* L VAR. *Aggregatum*) 10.18006/2021.9(Spl-2-ICOPMES_2020).S215.S221	**S215—S221**
ASSESSMENT OF PROBIOTIC PROPERTIES AND SAFETY OF LACTIC ACID BACTERIA ISOLATED FROM SOUTH SULAWESI ETHNIC CHEESE 10.18006/2021.9(Spl-2-ICOPMES_2020).S222.S227	**S222—S227**
A COMPARATIVE PHARMACOGNOSTIC STUDY OF THE TWO *Orthoshipon aristatus* (BLUME) MIQ. VARIETIES 10.18006/2021.9(Spl-2-ICOPMES_2020).S228.S233	**S228—S233**
DISSOLUTION IMPROVEMENT OF KETOPROFEN THROUGH POLYMER MATRIX COMPOSITE POVIDONE K-30/TiO_2 *10.18006/2021.9(Spl-2-ICOPMES_2020).S234.S240*	**S234—S240**
DEVELOPMENT OF METRONIDAZOLE MICROSPONGE INCORPORATED INTO CARBOMER-BASED VAGINAL GEL *10.18006/2021.9(Spl-2-ICOPMES_2020).S241.S247*	**S241—S247**
STUDY OF ACUTE AND SUB-ACUTE TOXICITY OF *Boehmeria virgata* (FORST) GUILL LEAF EXTRACT IN WISTAR RATS *10.18006/2021.9(Spl-2-ICOPMES_2020).S248.S258*	**S248—S258**
STABILITY OF THIOAMIDE TYPE OF PIPERINE UNDER ACIDIC AND BASIC CONDITIONS *10.18006/2021.9(Spl-2-ICOPMES_2020).S259.S263*	**S259—S263**
ANTIBACTERIAL ACTIVITY OF ROBUSTA COFFEE (*Coffea robusta* L.) PEEL EXTRACT AGAINST HUMAN PATHOGENIC BACTERIA *10.18006/2021.9(Spl-2-ICOPMES_2020).S264.S268*	**S264—S268**
COMPARISON OF α –GLUCOSIDASE INHIBITORY ACTIVITY OF *Moringa oleifera* ETHANOLIC EXTRACT *10.18006/2021.9(Spl-2-ICOPMES_2020).S269.S273*	**S269—S273**
EFFECT OF HONEY CONSUMPTION AFTER PHYSICAL EXERCISES ON ELECTROLYTES AND BLOOD GLUCOSE LEVELS *10.18006/2021.9(Spl-2-ICOPMES_2020).S274.S279*	**S274—S279**
ANTIOXIDANT ACTIVITY OF FOREST MANGGOSTEEN (*Garcinia hombroniana* Pierre) FRACTION USING DPPH AND ABTS METHOD *10.18006/2021.9(Spl-2-ICOPMES_2020).S280.S285*	**S280—S285**

Journal of Experimental Biology and Agricultural Sciences, September - 2021; Volume – 9(Spl-2-ICOPMES_2020) page S202 – S207

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EFFECT OF GREEN TEA EXTRACT ON MODULATING THE ANTIBACTERIAL ACTIVITY OF STANDARD ANTIBIOTICS AGAINST THE CLINICAL ISOLATES OF *Acinetobacter baumannii*

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KEYWORDS

Green Tea

Acinetobacter baumannii

Antibiotic resistance

Modulator

ABSTRACT

Most of the clinical isolates of *Acinetobacter baumannii* are found resistant to the β-lactam antibiotics. This research aimed to determine the ability of green tea extract in modulating the antibacterial activity of standard antibiotics amoxicillin, cefotaxime, and imipenem against the clinical isolates of *A. baumannii*. The clinical isolates used in this study were collected from the Laboratory of Clinical Pathology, Wahidin Sudiro Husodo Hospital Makassar, Indonesia. To determine whether the bacterial isolate is resistant, the experiment was carried out using disk agar diffusion and Vitek-2 methods. Further, the antibacterial activity of the green tea, selected antibiotics, and their combination was determined by using a checkerboard microdilution assay. Results of the study revealed that among the selected two clinical isolates one of the *A. baumannii* isolates was found resistant to selected standard amoxicillin-clavulanate, cefotaxime, and imipenem, while the other one was found sensitive. Further, green tea extract with a concentration of up to 1.2 mg/ml didn't have any significant effect on the inhibition of *A. baumannii* growth. Similarly, at the same concentration (1.2 mg/ml) no modulation effect of green tea extract was reported on the antibacterial activity of amoxicillin, cefotaxime, and imipenem against the *A. baumannii* isolates.

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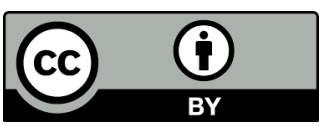

1 Introduction

Now a day's antibiotic resistance problems have emerged as a most serious threat which is challenging researchers, and health professionals in search of new treatments or push them for modification in medical intervention. Currently, inappropriate use of antibiotics, genetic mutation, and other protective mechanisms such as the formation of persister cells have been mainly linked to a large number of antibiotic resistance to pathogenic bacteria (Alnimr et al., 2020). Currently, *Acinetobacter baumannii*, a nosocomial pathogen, and gram-negative coccobacillus bacteria has been reported as a resistant strain to several β-lactam antibiotics, and occasionally it converted to multidrug or pan-drug strains (Prashanth & Badrinath, 2004; Gustawan et al., 2014; Mayasari & Siregar, 2015; Alnimr et al., 2020; Abdi et al., 2020).

In addition, the prevalence of *A.baumannii* was moderately found, but the occurrence of resistance cases is high. Gustawan et al. (2014) found that about 23 bacterial isolated from infected pediatric patients were notably classified as multidrug-resistant (MDR) to aminoglycoside, carbapenem, quinolone, cephalosporin, penicillin, β-lactamase inhibitors, and tigesicline groups. This paramount pathogen in clinical terms, particularly in hospital-acquired infections requires the search for new antibiotics. The search for new antibiotics takes a long time and high-cost demand. Instead of trying to search for a combination of antibiotic regimens, screening for the active compound from plants that can modulate the antibacterial activity of existing antibiotics could be an alternative way (Chambers et al., 2019).

According to Osterburg et al. (2009), epigallocathechin-3- gallate (EGCG) from green tea was able to inhibit *A. baumannii* with a minimum inhibitory concentration (MIC) of 78 to 625 µg/mL, while the results of Betts & Wareham (2014) suggested slightly higher MIC of EGCG against the same bacteria (128 - 1024 µg/mL). Other studies have also shown the synergistic effect of epigallocatechin gallate with carbapenem antibiotics on *A. baumannii* (Lee et al., 2017). The green tea extract in water or hydro alcohol is rich in catechins and caffeine alkaloids (Perva-Uzunalić et al., 2006; Hu et al., 2016). Therefore, green tea extract has various other natural substances which had been claimed to affect several antibiotic activities. Previous researchers found the effect of green tea extract against several microorganisms in line with its synergistic properties with antibiotics (Bazzaz et al., 2016; Lee et al., 2017). The current study was carried out to evaluate the synergistic effect of green tea water extracts with the standard antibiotics amoxicillin, cefotaxime, or imipenem against clinical isolate of *A.baumannii*.

2 Materials and Methods

2.1 Materials

A. baumannii isolates were collected in 2019 from the Laboratory of Clinical Pathology Wahidin Sudiro Husodo Hospital, Indonesia. For culturing the collected bacterial isolates Brain Heart Infusion Broth (BHIB), and Muller Hinton Agar (MHA) media (Merck) was used. In other materials, Vitek-2 compact (bioMe´rieux), green tea water extract (collection of Laboratory of Microbiology, Faculty of Pharmacy, UNHAS), 96-well plate, standard antibiotic disc (Oxoid), 0.5 Mc Farland (HiMedia), and Tetrazolium Chloride (Merck) was also used during the current study.

2.2 Methods

2.2.1 Susceptibility assay by agar diffusion method

Two clinical isolates of *A. baumannii* were collected in 2019 from the sputum of ICU patients admitted to the Wahidin Sudiro Husodo Hospital, Makassar, Indonesia. Ten microliters of *A. baumannii* bacterial suspension equivalent to 0.5 McFarland solution (10^8 CFU/ml) was inoculated on the MHA media by the spread plate method. After that, a disc of selected antibiotics were placed on the media and incubated for 24 hours at 37^{o}C. Bacterial isolates were tested for sensitivity using various classes of antibiotics, including penicillin (amoxicillin-clavulanate), cephalosporin (cefotaxime, ceftazidime, cefazoline, ceftriaxone), and carbapenem (imipenem, meripenem, doripenem).

2.2.2 Susceptibility assay by Vitek-2 method

A sterile tube containing 0.45% saline buffer was inoculated with 145 µl of *A.baumannii* isolate which is equivalent to 0.5% McFarland turbidity, AST GN/cassette was put into each of these tubes and then inserted into the Vitek-2 compact device.

2.2.3 Determination of total phenolic content of green tea water extracts using Folin-Ciocalteu method

Ten milligrams of the green tea extract was dissolved in 25 ml of analytical grade methanol. Similarly, gallic acid was also dissolved in the methanol and made a series of dilutions, 1 ml of each test solution and standard solution were added with 5 ml of Folin-ciocalteu (7.5% solution). The mixture was let stand for 8 minutes, and this was followed by adding 4 ml of 1% NaOH and incubates for 1 hour. Measurement of the absorption of each solution was conducted at a wavelength of approximately 730 mm. Test the blank was measured in the same way without the addition of test solutions. The calibration curve was prepared and the equivalent concentrations were calculated (Sartini et al., 2019).

2.2.4 Determination of epigallocatechin-3-gallate (EGCG) content by using ultra-fast liquid chromatography

The tea extract was dissolved in methanol until a concentration of 10 ppm was obtained. EGCG concentration analysis was performed by pro-liquid chromatography having Shim-pack Vp-ods column (UFLC Simadzu), and phosphate buffer (pH 5): acetonitrile (95.5% v/v) used as a mobile phase. The injection volume was 20 µl and the flow rate was 1 ml/minute (Sartini et al., 2019).

2.2.5 Synergy test of green tea extract using the checkerboard microdilution assay method

Ten microliters of antibiotic solution and 10 µl of green tea extract were inserted into sterile 96-well plates with a dilution factor of 1: 2, the concentration of green tea extract was 12 mg/ml, while in the case of standard antibiotic amoxicillin, cefotaxime, and imipenem this concentration was 2 mg/ml, 0.5 mg/ml, and 0.25 mg/ml respectively. This was followed by the addition of 178 µl of BHIB media, and 2 µl of the test bacteria to each well. The plate was incubated for 24 hours at 37^{o}C. After incubation, 10 µl of 0.1% TTC solution was added and incubated for 30 minutes at room temperature. The lowest concentration that shows the absence of bacterial growth (marked by the absence of changes in the color of the media to pink/red) is the minimum inhibitory concentration of the sample. If the color that arises is not clear, because of the color of the extract, it is etched onto the BHIB media so that it is sterile and re-incubated for 24 hours for the confirmation test.

2.3 Statistical Analysis

Two-way analysis of variance (ANOVA $p<0.05$) was applied to subjugate data in the form of Mean ± SEM (Standard Error of Mean).

3 Results and Discussion

3.1 Total phenolic and EGCG content of green tea extract (GTE)

Previous studies suggested that the green tea polyphenolic compounds contributed as antibacterial and modulator compounds in association with some antibiotics against various bacteria. Green tea contains various polyphenols catechin including Epigallocatechin-3-gallate (EGCG) which has antibiotic properties. The results of the current study showed that the green tea water extract contained a total polyphenol of 508.5 + 5.4 mg gallic acid equivalent/g extract and EGCG content of 340.7 + 20.4 mg/g extract), which is around 66.9% of the total polyphenols. The high value of total polyphenol content obtained in this study might be due to the extraction procedure in which extraction was carried out with the aid of high pressure. Xi et al. (2015) suggested that the green tea extraction carried out with the help of high pressure (450 Mpa) yielded higher total phenolic compounds than the conventional extraction methods.

3.2 Susceptibility assay of *A. baumannii* clinical isolates

The antibiotic susceptibility of both the clinical isolates collected from the Laboratory of Clinical Pathology, Wahidin Sudiro Husodo Hospital, Makassar was tested against the selected antibiotics by disc diffusion and Vitek-2 methods (Table 1 & 2). Results presented in table 1 and 2 have been shown that *A. baumannii* isolate 231/07 has been resistant to amoxicillin-clavulanate antibiotics, some cephalosporin groups, and carbapenem groups, while *A. baumannii* isolate 288/05 has a sensitivity to some carbapenem (imipenem, meropenem, doripenem) and relatively sensitive to some cephalosporins. Similar results were reported by Lin & Lan (2014), these researchers found that several isolates of *A. baumannii* were resistant to most of the antibiotics, including the carbapenem group. According to Xioa et al. (2016), the causes of carbapenem (imipenem) resistance in *A. baumannii* might be the presence of β-lactamase genes such as blaKPC, blaIMP, blaOXA-24, blaOXA -58, blaSHV, blaGIM; these genes are involved in efflux pumps and mutations in protein-coding genes. Further, Badave & Kulkarni (2015) reported that 62.5% of 72 clinical isolates produced biofilm which might be associated with the multiple drug resistance in *A.baumannii*.

Table 1 Antibiotic susceptibility of *A. baumannii* isolates against selected antibiotics by using disc diffusion method

Isolate code	Diameter of inhibitory zone (mm)						
	Penicillin	Cephalosporin				Carbapenem	
	AMC	CAZ	CRO	CTX	CZ	MEM	IMI
231/07	8 R	8 R	8 R	8 R	8 R	8 R	11 R
288/05	24 S	25 S	20 I	18 I	8 R	18 S	35 S

AMC = Amoxicillin-clavulanate (20/10 µg); CTX = Cefotaxime (30 µg); CAZ = Ceftazidime (30µg); CZ= Cefazoline (30 µg); IMI = Imipenem (10 µg); MEM = Meropenem (10 µg); S= Sensitive, I = Intermediate, R = Resistant

Table 2 Antibiotic susceptibility of *A. baumannii* isolates against selected antibiotics by using Vitek-2 method

Isolate code	MIC values (ppm) using Vitek-2 method						
	AMC	CTX	CRO	CAZ	IMI	MEM	DORI
231/07	nd	≥ 64 R	≥64 R	≥64 R	≥16 R	≥16 R	≥ 8 R
288/05	nd	8 S	16 I	4 S	≤ 0.25 S	≤ 0.25 S	≤ 0.12 S

AMC = Amoxicillin-clavulanate; CTX = Cefotaxime; CAZ = Ceftazidime ; CRO = ceftri-axone; IMI = Imipenem; MEM = Meropenem; DORI = Doripenem; nd= no detection; S= Sensitive, I = Intermediate, R = Resistant

3.3 Synergistic effect of green tea extract along with selected antibiotics

The synergistic effect of green tea extract with three antibiotics viz., amoxicillin, cefotaxime, and imipenem were tested by checker board microdilution assay and the results are presented in table 3 & figure 1.

Based on the data given in table 3, the green tea extract has the least inhibitory effect on both clinical isolates of *A. baumannii* and found minimum inhibitory levels of 1.2 mg/ml which suggested that the green tea extract is not effective in inhibiting the growth of these bacteria. Some differences are reported in the MIC value given in table 2 and 3, this might be due to the different methods used for the estimation of MIC, in table 2 results of Vitek-2 rapid method was given which is more sensitive than the microdilution method (table 2).

The MICs of amoxicillin, cefotaxime, and imipenem showed that the antibiotics have experienced resistance against the clinical isolates. In the case of synergistic effect, no modulation effect of the green tea extract was reported on the antibacterial activities of the selected antibiotics. The previous study of Jung & Park (2015) have been reported MDR *Acinetobacter* which has resistance against the penicillin, cephalosporin, fluoroquinone, and aminoglycoside groups, and no plant extracts have been found that can increase the antibiotic activity of these antibiotics against MDR *A.baumannii*. While the results of Lee et al. (2017) are contradictory to the findings of the current study and these researchers reported that the EGCG had a synergistic effect against *A. baumannii* with carbapenem antibiotics. Epicatechin is an important component of green tea extract which is responsible for the antibacterial activities of green tea but according to Betts et al. (2017), it is required in a minimum concentration of 1024 - 2048 ppm for bacterial growth inhibition. In contrast to this, Sartini et al. (2020) reported that at 75 ppm GTE the antibacterial activity of amoxicillin increased by 32 fold as compared to the 8 ppm to 25 ppm concentration. The poor synergistic effect of EGCG reported in the current study might be due to the less concentration of the green tea extract. Research by Bazzaz et al. (2016) also states that the combination of methylxanthine and some antibiotics are antagonistic to some pathogenic bacteria. Further, Bazzas et al. (2018) suggested that the type of bacterial strains also plays an important role in determining the efficacy of the methylxanthine and antibiotic gentamycin combination, because several strains of *Staphylococcus aureus* and *Escherichia coli* have different synergistic and non-synergistic effects against this combination.

Table 3 MIC (mg/ml) values of green tea extract, antibiotics, and combination of antibiotics with green tea extract

Test Sample	MIC (mg/ml)	
	A. baumannii isolate 288/05	*A. baumannii* isolate *231/07*
Green Tea Extract (GTE)	> 1.2	> 1.2
Amoxicillin	>0.2	>0.2
Cefotaxime	>0.05	0.0125
Imipenem	0.025	0.025
Amoxicillin + GTE	>0.2	>0.2
Cefotaxim +GTE	>0.05	>0.05
Imipenem +GTE	0.025	0.025

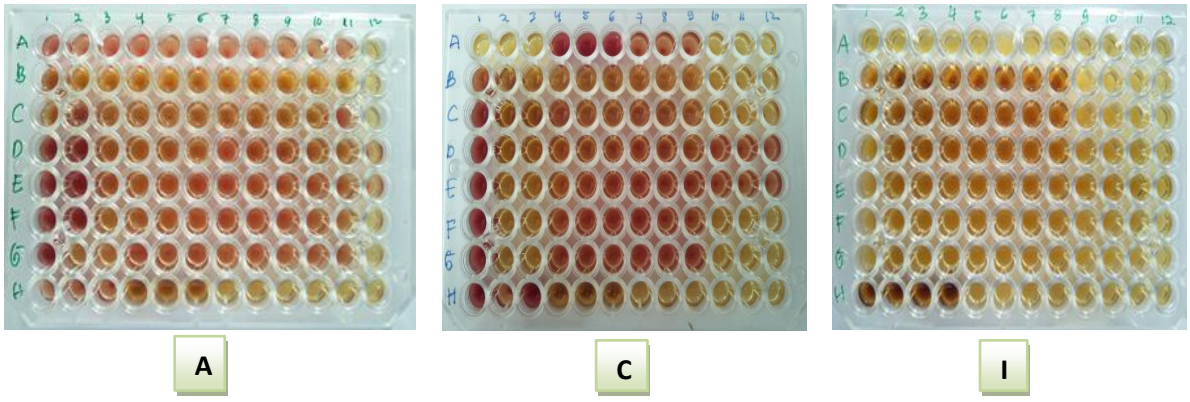

Figure 1 Evaluation of antibiotic sensitivity by checkerboard microdilution assay in 96-well microtiter plate; here Column 1 (B-G): concentration of GTE (1.2 – 0. 004) mg/ml; Line A (1-11): cocentration of amoxicillin (0.2– 0.002) mg/ml; cefotaxime (0.5 – 0.0002) mg/ml; imipenem (0.25 – 0.0003) mg/ml; plate A= amoxicillin –GTE, plate C = Cefotaxime-GTE, plate I = Imipenem-GTE

Conclusion

Results of the study can be concluded that the water extract of green tea did not have any significant effect on the increasing antibacterial activity of amoxicillin, cefotaxime, and imipenem against the selected clinical isolates of *A.baumannii*. Further research is needed to establish the required concentration and combination of green tea extract against *A. baumannii* isolates.

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Conflict of Interest

No conflict of interest in this research

Reference

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Journal of Experimental Biology and Agricultural Sciences, September - 2021; Volume – 9(Spl-2-ICOPMES_2020) page S208 – S214

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In silico SCREENING OF *Ziziphus spina-christi* (L.) Desf. AND *Strychnos ligustrine* COMPOUNDS AS A PROTEASE INHIBITOR OF SARS-COV-2

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Strychnos ligustrine

Jubanine B

ABSTRACT

Diseases caused by the coronavirus have become an important concern in early 2020. The coronavirus is a new type of virus that is included in the SARS-CoV-2 group. One of the possible mechanisms of SARS-CoV-2 inhibition involves protease receptors inhibition. This research was aimed to *in silico* screening of *Ziziphus spina-christi* (L.) Desf., and *Strychnos ligustrine* active ingredients as the main protease inhibitors of SARS-CoV-2 by assessing the ligand-binding affinity in the binding pocket of SARS-CoV-2 main protease protein. The molecular docking method is generally used to predict the inhibitory site and bonds formation. In the current study, some generally used antiviral compounds from the PDB (Protein Data Bank) were also used to compare the affinity strength of the test compound against the protease receptor (code of 5R7Y). The inhibitory activity against the main protease receptor proven by the ChemPLP score is more negative than the receptor's native ligand and the comparison compounds. Jubanine B, a compound of *Z. spina-christi* has the most robust inhibition activity on the SARS-CoV-2 protease receptor. Results of this study can be concluded that this can be used to develop as a candidate for traditional medicine against SARS-CoV-2 but still it required some more *in vitro* and *in vivo* studies.

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1 Introduction

The coronavirus disease 2019 (COVID-19) is a disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The virus is a new type of virus in the coronavirus family that can affect the respiratory system at various levels with symptoms ranging from mild to severe (Food and Drug Monitoring Agency, Republic of Indonesia 2020; Niaz, 2020). SARS-CoV-2 is transmitted through direct contact with infected patients, droplets released by patients when coughing/sneezing, and hand touching the mouth, nose, and eyes after touching objects contaminated with the virus. The World Health Organization (WHO) has also warned of the possibility of transmission of the SARS-CoV-2 through the air for medical staff (airborne precaution for medical staff) based on a study that the coronavirus can survive in the air in certain environments (Food and Drug Monitoring Agency, Republic of Indonesia 2020).

SARS-CoV-2 is a single-stranded RNA (ribonucleic acid) enveloped virus, targeting cells through the structural protein Spike (S protein) that binds with the angiotensin-converting enzyme 2 (ACE2) receptor (Elfik, 2020). After receptor binding, viral particles use the host cell's receptors and endosomes to enter the cell. Transmembrane serine protease 2 (TMPRSS2) protein facilitates cell entry through S protein (Fehr & Perlman, 2015). Once inside the cell, viral polyproteins encoding the replication transcriptase complex are synthesized. In this, the virus synthesized RNA via RNA polymerase which are dependent on the RNA. Structural proteins are synthesized towards the completion of assembly and release of viral particles (Fehr & Perlman, 2015). The steps of this viral life cycle provide a potential target for drug therapy. The targets of these drug products include ACE2, S protein and TMPRSS2 (type 2 transmembrane serine protease) as well as 3-chymotrypsin like protease (3CL) a protease inhibitor (Sanders et al., 2020). 3C-like proteinase formerly known as C30 Endopeptidase is the main protease found in coronaviruses. The main protease operates at no fewer than 11 cleavage sites on the large polyprotein 1ab (replicase 1ab, ~790 kDa); the recognition sequence at most sites is Leu-Gln↓(Ser, Ala, Gly) (↓ marks the cleavage site). Inhibiting the activity of this enzyme would block viral replication because no human proteases with similar cleavage specificity are known (Zhang et al., 2020). The COVID-19 mechanism can be inhibited by several drug compounds including chloroquine, hydroxychloroquine, darunavir, ribavirin, arbidol, remdesivir, lopinavir and camostat mesylate (Sanders et al., 2020; Santos et al., 2020). Protein-ligand docking in the drug development process predicts the complex structure of small ligands with proteins. Molecular docking yields a score proportional to the total ligand-protein binding energy. By this, comparing the scores of a compound with other compounds can explain why one compound is more potent than another. The smaller the score of a docking result means that the protein-ligand complex is more stable so that the ligand (compound) predicted is more potent (Purnomo, 2013). Through visualization, it will be seen which amino acids play an essential role in maintaining the stability of these compounds at the receptor-binding site (Purnomo, 2013; Syahputra et al., 2014; Jualiana & Amin, 2016). In the invention of new drug compounds from natural ingredients, an *in silico* molecular docking test of plant compounds against target proteins is highly recommended. Since the *in silico* test can predict the potential of the desired plant compounds, it will save research costs and help to decide what can be tested *in vitro* or *in vivo* and which compound can serve as a potential antiviral drug. Thus, an *in silico* test can save the cost of drug discovery (Purnomo, 2013).

There are several natural compounds such as hesperidin, cannabinoids, pectolinarin, epigallocatechin gallate, and rhoifolin reported from various plants and have been studied *in-silico* and have better free energy bonds with Matrix (M) and Spike (S) proteins from SARS-CoV-2 (Trina et al., 2020). It is suspected that these compounds have the potential to serve as phytochemical antivirus that can inhibit viral replication.

This research was carried out to explore new candidate compounds from *Z. spina-christi*, and *S. ligustrine* as the main protease inhibitor of SARS-CoV-2 using molecular docking. Both plants contain alkaloids, flavonoids, terpenoids, triterpenoids, steroid and phenol type compounds (Asgarpanah & Haghighat, 2015; Maulana, 2018; Negash, 2019). Among these, alkaloid is quite dominant and this compound has properties according to the already drugs used to treat coronavirus such as chloroquine, hydroxychloroquine, darunavir, ribavirin, arbidol, remdesivir, lopinavir, camostat mesylate etc (Santos et al., 2020).

2 Materials and Methods

2.1 Material

The materials used are 5R7Y (Protein) chemical structures, chemical compounds structure of Arabic lote tree (*Z. spina-christi*) and snakewood (*S. ligustrine*). The already reported compounds of the Arabic lote tree are christinine I, christinine II, christinine III, geranyl acetate, phloretin 3',5'-di-C-glucoside, betulinic acid, cyanotic acid, quercetin 3-xylosyl-(1->2)-rhamnoside, jubanine A, jubanine B, jubanine C, jubanine G, jubanine H, amphibine A, amphibine B, amphibine D, Amphibine C, amphibine E, amphibine F, amphibine G, amphibine H, amphibine I, alpha terpineol, beta-sitosterol, ziziphine F, methyl hexadecanoate, methyl octadecanoate used for in-vitro docking (Asgarpanah & Haghighat, 2015; Maulana,

2018; Negash, 2019). Furthermore, the already reported compounds of the tested snakewood are strychnine, brucine, strychnopentamine, malagashanine, 2,6-dimethoxyphenol, 10-hydroxyusambarensine, 3-ethoxyacetophenone, 2,6-dimethyl-4-nitrophenol, 2-methoxy-4-methyl phenol also used for in-vitro docking (Taek &Tukan, 2018; Novian et al., 2019). In addition, the following compounds: Tryptamine, vincoside, 3-epi-strictosidinic acid, yohimbine, reserpine, camptothecin, secologanin, 2-propanone, 1-(4-hydroxy-3-methoxyphenyl), 3-methoxyacetophenone, guaiacol, n-pentanal, 4-allyl-2,6–dimethoxyphenol, 19(S)-methoxytubotaiwine, 19(R)-methoxytubotaiwine, dihidrocapsaicin, and ervatamine which are previously reported from snakewood also tested in the current study (Gusmailina & Komarayati, 2015; Rale, 2018; Novian et al., 2019). The chloroquine, hydroxychloroquine, darunavir, ribavirin, arbidol, remdesivir, lopinavir, and camostat mesylate were used as comparison compounds.

2.2 Bioinformatics Tools

For this study YASARA, PDB, PLANTS, Marvinsketch and PYMOL software were used along with a Lenovo C9LS2ES Intel(R) Celeron(R) CPU N3060; 4 GB RAM; 1,60 GHz 64-bit operating system laptop as hardware.

2.3 Methods

2.3.1 Protein Optimization and RMSD Value Determination

Native ligands are prepared and optimized for protein crystal structure using the PLANTS program to obtain scores. The best score is selected and saved in Mol2 file format. The amount of RMSD poses of the optimization results regarding the experimental results or protein crystal structure is calculated with the YASARA program.

2.3.2 Test and comparator ligand docking against receptor

In this, docking is carried out between each test compound ligand using the PLANTS program. The docking runs by typing the script into the CMD worksheet to determine the target protein's binding-site radius and binding-site centre. The docking process runs automatically by plantconfig protocol. The molecular docking set was determined in 10 replications with 10 confirmations to locate and determine the lowest amount of energy (Tegar & Purnomo, 2013). The best score of test compound ligand is obtained from the result of docking. This value will be compared with the best score of the comparator compound ligand.

2.3.3 Visualization

Docking results file making from each test compound ligand is with the YASARA program (PDB file type). This docking result file will be visualized and interpreted to determine the interactions that occur using the PyMOL application.

3 Results and Discussion

The main protease protein's crystal structure in the docking process is a protein that was validated and complied with the RMSD requirements of less than 2Å (Rangwala & Karypis, 2008). 5R7Y met the RMSD requirement for native ligand pose and the redocking pose was 1.4829Å in the redocking process. The molecular docking with RMSD of less than 2Å meant that the position of ligand that bound to the protein's active side did not move too far since the conversion from 2 Å. The range size is equal to 0.2 nm corresponded to that of an atom's diameter which is about 0.1 nm.

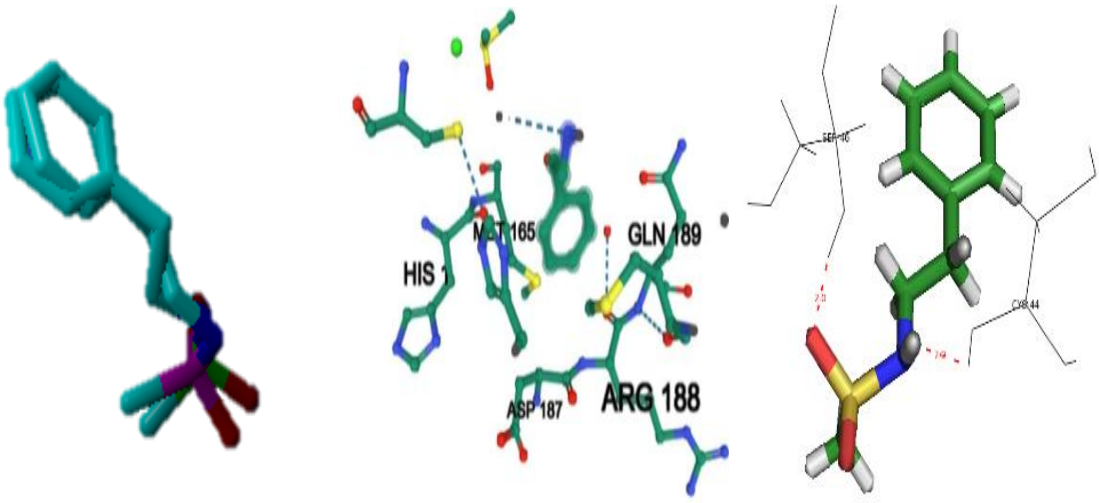

Figure 1 Superimpose of native ligand pose and the redocking native ligand pose and its residues amino acid interactions

3.1 Docking Results Analysis

There are 27 compounds of *Z. spina-christi*, and 26 compounds of *S. Ligustrine* were tested for their affinity as protease inhibitors against the 5R7Y receptor. All these compounds were tested in silico using the molecular docking method. The docking process was carried out at the binding site of the 5R7Y receptor and the ChemPLP score was calculated for each compound pose in the active site. ChemPLP score showed Gibbs free energy in which the smaller the ΔG value of the ligand-receptor interaction, the more stable it would be. The more negative ChemPLP score of the tested compound means stronger affinity at the receptor-binding site (Kurniawan, 2015). Table 1 represented the ChemPLP score of all compounds in the binding site of the receptor 5R7Y. It showed that all ligands have a more negative score than the native ligand (JFM), which means that all test compounds are predicted to have a better affinity than the native ligand. It can be explained that all the comparison compounds have inhibition activity on SARS-CoV-2 replication (in its antiviral role).

The ChemPLP score of all the test compounds of Z. spina-christi are smaller than the used native ligand. Further, the results also showed that among the tested 27 compounds of Z. spina-christi, ChemPLP score of 4 (four) alkaloid peptides compounds namely Jubanine B, Jubanine C, Amphibine E, and Amphibine H had a smaller ChemPLP value than the positive control (darunavir) Mauludiyah et al. (2020). Meanwhile, none of the 26 test compounds derived from S. ligustrine showed stronger affinity than the same comparator (darunavir). However, among these, there are 16 compounds were reported active because their ChemPLP scores were more negative than the native ligand.

Of the 53 compounds tested, none were had predicted to have a better or equal affinity to the remdesivir in inhibiting SARS-CoV-2, while the Jubanine B compound of Z. spina-christi leaves had a predicted strong activity because the ChemPLP was more negative than lopinavir in inhibiting the SARS-CoV-2 receptor. Additionally, this compound also had stronger activity in inhibiting the SARS-CoV-2 protease receptor. Based on this, it can be

Table 1 ChemPLP Score of the used compounds

S. No	Compounds	ChemPLP Score	Compounds	ChemPLP Score	Compounds	ChemPLP Score
	Comparison compounds		*Z. spina-christi*		*S. ligustrine*	
1	JFM 1001 (Native Ligand)	-64.492	Geranilasetat	-67.4666	Strychnine	-70.159
2	Klorokuin	-81.267	Christinin I	-69.3772	Brucine	-72.5403
3	Hidroksiklorokuin	-83.189	Christinin II	-72.3141	Strychnopentamine	-88.2194
4	Darunavir	-96.741	Christinin III	-75.5299	Malagashanine	-65.5535
5	Ribavirin	-66.8404	Phloretin 3',5'-Di-C-glucoside	-87.2636	2,5-Dimethoxybenzyl alcohol	-54.8569
6	Arbidol	-83.9751	Betulinic acid	-69.9086	2,6-Dimethoxyphenol	-55.4956
7	Remdesir	-113.848	Ceanothic acid	-74.2428	10-hydroxyusambarensine	-81.8363
8	Lopinavir	-110.28	Quercetin 3-xylosyl (1,2) rhamnoside-4′rhamnoside	-81.8981	2,6-Dimethyl-4-nitrophenol	-55.5903
9	Camostat mesylate	-90.2515	Jubanine A	-102.689	2-methoxy-4-methylphenol	-55.2316
10			Jubanine B	-110.367	3-Ethoxyacetophenone	-58.5119
11			Jubabine C	-105.471	Tryptamine	-57.6537
12			Jubanine G	-88.9747	Vincoside	-88.9451
13			Jubanine H	-89.2483	3-epi-Strictosidinic acid	-86.5313
14			Amphibine A	-101.716	Yohimbine	-77.8269
15			Amphipine B	-100.539	Reserpine	-65.3186
16			Amphibine C	-99.0982	Camptothecin	-67.3791
17			Amphibine D	-100.103	Secologanin	-73.5241
18			Amphibine E	-104.13	2-Propanone, 1-(4-hydroxy-3-methoxyphenyl)	-60.3211
19			Amphibine F	-90.6102	3-Methoxyacetophenone	-55.4328
20			Amphibine G	-96.5544	19(R)-methoxytubotaiwine	-67.0108
21			Amphibine H	-103.514	19(S)-Methoxytubotaiwine	-65.6651
22			Amphibine I	-89.4811	Dihidrocapsaicin	-80.3741
23			zizyphine F	-94.1967	Ervatamine	-72.5855
24			Alpa terpineol	-61.3281	Guaiacol	-52.9905
25			Beta sitosterol	-84.8232	n-Pentanal	-44.4185
26			Methyl hexadecanoate	-78.6953	4-Allyl-2,6 -dimethoxyphenol	-75.4356
27			Methyl octadecanoate	-83.5505		

suggested that *Z. spina-christi* can be used as a candidate for raw material in traditional drugs against SARS-CoV-2 antivirus. Further studies are required to standardize the *in vitro* and *in vivo* potential of *Z. spina-christi* extracts against SARS-CoV-2.

3.2 Visualization of Ligand – Protein Interaction

Compound visualization at the binding site is depicted in three-dimensional (3D) form so that it can visualize the amino acids involved in the active compound affinity with the SARS-CoV-2 protease enzyme, and determine the hydrogen bond distance in angstrom (Å) units between the candidate drug compounds and amino acids existing between the SARS-CoV-2 protease enzyme receptor (5R7Y). The visualisation results of the four protease inhibitor candidate compounds can be seen in Figure 2.

Amino acids residues obtained from 5R7Y receptor binding to compounds of *Z. spina-christi* include THR-25, GLN-189, ASN-142, GLU-166, and GLY-143. These amino acids are also found in amino acids at 5R7Y on the PDB database (Kurniawan, 2015; Berry et al., 2015). Thus, it can be concluded that the amino acids in the receptor are compatible. Amino acids that play an essential role in receptor compatibility include histidine (His), glutamine (Gln), arginine (Arg), glutamate (Glu), threonine (Thr), glycine (Gly), serine (Ser) leucine (LEU), asparagine (ASN), and cysteine (CYS). The location of the test compound bond to the amino acid at the 5R7Y receptor is more or less following the native ligand binding of the receptor amino acids, namely ARG-188, ASP-187, MET-49, MET-165, CYS-44, CYS-145, HIS-41, HIS-164, SER-46, THR-25, THR-45, GLN-189, LEU-57, LEU-50, ILE-43, ASN-51, GLU-47, PRO-52, ASP-48, GLY-143 (Fearo et al., 2020).

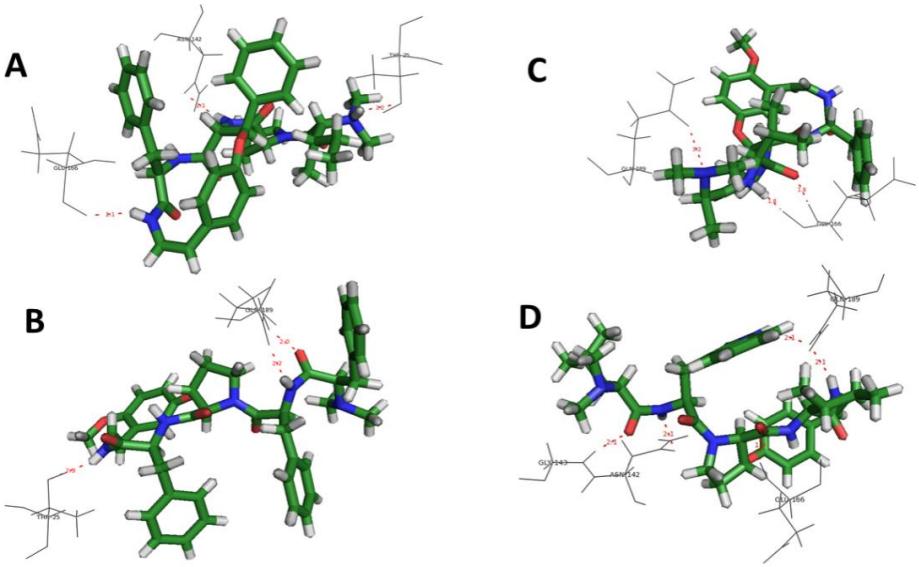

Figure 2 Visualisation result of the candidate protease inhibitor compound at 5R7Y receptor A) Jubanine C; B) Jubanine B; C) Amphibine H ; D) Amphibine E

Table 2 Amino acids residue obtained in 5R7Y receptor binding

Compound Name	Amino acid	Bond distance(Å)
Jubanine B	THR-25 GLN-189	2.1 1.9
Jubanine C	THR-25 GLU-166 ASN-142	2.2 2.1 2.1
Amphibine E	GLN-189 GLY-143 ASN-142 GLU-166	2.1 2.1 2.1 1.9
Amphibine H	GLN-189 GLU-166	3.2 1.8

Conclusion

The Jubanine B compound of *Z. spina-christi* has the most robust inhibition activity on the SARS-CoV-2 protease receptor and after some detailed study, it can be used as a candidate for traditional antiviral medicine against SARS-CoV-2.

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Conflict of Interest

The authors declare no conflict of interest.

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EVALUATION OF ANTIOXIDANT AND ANTIFUNGAL PROPERTIES OF PALU SHALLOT (*Allium ascalonicum* L VAR. *Aggregatum*)

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KEYWORDS

Total phenolics

Total flavonoids

Quercetin

DPPH

Candida albicans

Allium ascalonicum

ABSTRACT

Shallot is one of the typical plants at Palu, Central Sulawesi, Indonesia, famous by local people as fried Shallot. It is used as a cooking spice and traditional medicine for treating various diseases. This study was carried out to assess the phytochemical constituent including total phenolics, total flavonoids, and quercetin content of the Palu shallot (*Allium ascalonicum* L var. *aggregatum*), and to determine the antifungal and antioxidant properties of this plant ethanolic extract. Total phenolics/ flavonoids and quercetin concentration were determined by spectrophotometry UV-Vis and Reverse Phase - High-Performance Liquid Chromatography (RP-HPLC) methods. Antifungal activity and antioxidant capacity of the ethanolic extract was assayed by using diffusion agar and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Results of the study revealed that the total flavonoids content of the ethanolic extract was 0.3634 ± 0.018 mg QE/100 mg while total phenolics content was 0.4834 ± 0.003 mg GAE/100 mg. Meanwhile, the quercetin content was 65.46±0.0002 mg/kg. Further, ethanolic extract of Palu shallot also showed the radical scavenging activity with IC_{50} of 0.1398 mg/mL and growth inhibition on *Candida albicans* with inhibitory zone diameter range from 7.57 to 16.51 mm. This study confirms the high quality of Palu shallot as it has high total flavonoids, represented by the high quercetin concentration, and it is proposed to be a source for an antioxidant and antifungal medicinal herb.

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1 Introduction

Shallot (*Allium ascalonicum* Linn.), belonging to the family Liliaceae, is the most widely consumed vegetable in the world and widely used as a food, spice and folk medicine. The bioactive compounds and pharmacological activities such as antimicrobial (Kyung, 2012), antiviruses (Mohamed, 2010), antioxidant (Raeisi et al., 2016), antifungal (Moghim et al., 2014), haematological effects (Owoyele et al., 2004), anticancer and anti-inflammatory (Mohammadi-Motlagh et al., 2011) have been well studied so far. Meanwhile, ascalonicoside A1/A2 and ascalonicoside B (Fattorusso et al., 2002), four sulfur-containing compounds (Ogra et al., 2005), and ascalin (Wang & Ng, 2002) had also been reported from bulbs of Shallot. Besides, the flavonoid fractions of Shallot contained high amounts of free quercetin, glycoside form such as quercetin 4'-glucoside and quercetin 7-glucoside, and isorhamnetin (Fattorusso et al., 2002).

In Central Sulawesi with a dry climate, there is a type of shallot that can grow and produce well. This type of local shallot is known as Palu shallot and has been processed into a ready-to-eat product commonly called Palu fried shallot (Figure 1). Palu shallot has advantages like a dense texture, a savoury taste and an unchanging aroma even though it is stored for a long time. Besides, Palu shallot has many nutrients, including protein, fat, carbohydrates, vitamin A, vitamin E, and a small number of vitamins B (thiamine, riboflavin, niacin, pantothenic acid, and pyridoxine) (Sulfina, 2020).

Quercetin was found as one of the primary compounds in some varieties of Shallot (Sittisart et al., 2017). The antioxidant capacity is always associated with the properties of quercetin because of its ability to capture free radicals and reactive oxygen such as superoxide anions and hydroxyl radicals (Zhang et al., 2011). In addition, flavonoids compounds can also disrupt the cell proteins and shrink the cell walls causing apoptosis induction in several *candida* species (Seleem et al., 2017).

Considering the more advantage of consuming Palu shallot as daily food and the wide cultivation of this plant in Palu and its surrounding area, there is a need for extract material standardization based on phytochemical characteristics and pharmacological activity information. Therefore, this study aims to determine the total phenolics/flavonoids of extract and continuing by determination of its quercetin concentration. The antioxidant and antifungal activities of ethanol extract of bulbs of Palu shallot were also discussed.

2 Materials and methods

2.1 Materials

Fresh bulbs of Palu Shallot (*A. Ascalonicum* var. *aggregatum)* were collected from Soulove village, Sigi Regency (± 25 km from Palu City, Central Sulawesi, Indonesia). The plant identification was done in the Plant Biosystematical Laboratory, Department of Biology, Science Faculty, Tadulako University, Palu, Indonesia where a voucher specimen was deposited. Other commonly used materials are gallic acid, quercetin (≥95% purity), *1,1-difenil-2-pikrilhidrazil* (DPPH), potato dextrose agar (PDA) medium, ethanol 96%, nystatin 0.1%, NaCl 0.9% physiologic solution, sodium nitrite, sodium hydroxide, sodium carbonate, ascorbic acid sodium carbonate, aluminium chloride, Folin-Ciocalteau 50% reagent, *Dimetil Sulphoxide* (DMSO), methanol, methanol pro-HPLC, water pro-HPLC, and aqua dest. All chemicals were purchased from Sigma Aldrich.

Figure 1 Palu shallot (left), ready to eat product of Palu fried shallot (right)

2.2 Extraction

Bulbs of Palu shallot were cleaned of skin, epidermis and leaves and then chop them into smaller pieces. It was dried at room temperature protected from direct sunlight. Around 1 kg of dried bulbs was weighed and then extracted by maceration using 2.5 L of 96% ethanol. The macerator was then tightly closed and stored in a place protected from sunlight for 3 x 24 hours and concentrated using a rotary evaporator until obtained the viscous extract (64.67 g).

2.3 Total Flavonoids Determination

The measurement of total flavonoids were conducted by the aluminium chloride colorimetric method as suggested by Karagiorgou et al. (2016) and Sulastri et al. (2018,)**.** About 10 mg of sample was dissolved in 10 mL ethanol (p.a) and then diluted to 100 µg/mL. Meanwhile, quercetin as standard was also prepared by dissolving 10 mg in 10 mL ethanol p.a (1000 µg/mL) and then diluted to 5, 10, 20 and 40 µg/mL. Sample and each concentration of standard solutions (1:1) were mixed with 0.2 mL 1 M potassium acetate, 0.2 mL 10% aluminium chloride, 3 mL 96% ethanol, and 5.6 mL distilled water. Then, the mixture was incubated at room temperature for 10 minutes. The absorbance was measured by using spectrophotometer UV-Vis Cecil CE7410 at 447 nm, along with a blank solution. Total flavonoid was calculated and resulted in milligrams of quercetin equivalent (QE) per 100 mg extract. The experiment was performed in triplicates.

2.4 Total Phenolics Determination

The measurement of total phenolics was conducted by using Folin–Ciocalteu method as described by Hossain & Rahman (2011) and Sulastri et al. (2018). For this, 10 mg gallic acid as standard was dissolved in 10 mL ethanol p.a (1000 µg/mL). This solution was diluted to obtain standard solutions with the concentration series of 5, 10, 20 and 40 µg/mL. Similarly, 10 mg plant sample was also dissolved in 10 mL ethanol p.a. About 0.5 mL standards and sample solutions were mixed with 50% Folin–Ciocalteu and distilled water (1:1) and added 2 mL of sodium carbonate (7.5%, w/v) after incubation for 5 min. The mixture was then shaken and incubated for 15 min at room temperature. The absorbance of standard and sample solutions were measured by using spectrophotometer UV-Vis Cecil CE7410 at 755 nm. Total phenolic content was calculated and resulted in milligrams of gallic acid equivalents (GAE) per 100 mg extract. The experiment was performed triplicates.

2.5 Quercetin Concentration Determination

The concentration of quercetin on ethanol extract was determined by RP-HPLC. 20 mg dried ethanol extract was dissolved in 10 mL methanol and sonicated for 15 minutes. Similarly, 10 mg quercetin as standard was dissolved in 10 ml methanol and then diluted to obtain the concentration series of 1.2, 2.4, 4.8, 9.6 and 19.2 µg/mL. Samples and standard solutions were filtered through a 0.45 µm millipore filter and then injected into the column (C18 size 250 mm × 4.6 mm) on HPLC Cecil CE4201 with UV visible detector. Methanol: water (90:10, v/v) with parameters: injection volume 20 µL, the flow rate of 1 mL/min, and detection wavelength at 370 nm was used to obtain the optimum efficiency of separation. Quercetin concentration was calculated by linear regression analysis using SPSS 17.0 (SPSS. Inc, Chicago IL, USA).

2.6 Antioxidant Activity Determination

The antioxidant activity of Palu shallot ethanol extracts was determined by using DPPH radical method (Karimi & Moradi, 2015). About 3 mL of both 0.1 mM DPPH solution and ethanol solutions of extracts (concentration series of 50 – 150 µg/mL) were mixed and incubated for 30 min at the darkroom. The decreasing absorbance of the mixture was monitored at 515 nm. Blank sample and vitamin C (concentration series of 1, 2, 4, 6, and 8 µg/mL) as positive control were also prepared and measured at the same wavelength. The experiment was carried out in triplicate. The calculation of the percentage of inhibition was done by the following formula:

$$\%\text{inhibition} = \left(\frac{\text{Absorbance of blank solution} - \text{Absorbance of sample solution}}{\text{Absorbance of blank solution}}\right) x\ 100\%$$

Meanwhile, the 50% inhibitory concentration (IC_{50}) was calculated by probit analysis correlating the extract concentrations against their inhibition percentage.

2.7 Determination of antifungal activity

2.7.1 Test microorganisms

Candida albicans, isolated from candidiasis patient at Palu Health Laboratory Office, were cultured on potato dextrose agar. Incubation was performed at 37°C for 18-24 h.

2.7.2 Disc diffusion assay

The antifungal activity of ethanol extracts of Palu shallot was performed by the well-diffusion method (NCCLS, 2012). *C. albicans* fungi were cultured overnight at 37°C on Potato Dextrose broth. Inoculums consisting of 0.5 McFarland was prepared in physiologic saline. Fungi inoculums in potato dextrose agar medium (2000 µL) was poured in Petri dishes with a Potato Dextrose Agar solid medium (1000 uL) as basis layer. The sample was prepared by dissolving 1 g extract on 1 mL DMSO (1000 mg/mL) and then dilute to 250, 500 and 750 mg/mL. Sterile wells (6 mm diameter) were deposited on medium and impregnated with 50 µL of extract solutions. The plates were inverted and incubated

for 3 x 24 h at 37°C. The negative control was performed with discs containing 50 µL of DMSO and the positive control was nystatin 1 mg/mL. Each experiment was performed in triplicate. The diameters of the clear zone of growth inhibition around each disc were measured and recorded. The scale of measurement (disc diameter included) is as follow: < 12 mm is no inhibitory activity, < 20-12 mm is moderate inhibitory activity; and ≥ 20 mm is strong inhibitory activity (Espina et al., 2011).

3 Results and Discussion

In the Province of Central Sulawesi, particularly in Soulove village, Sigi Regency, there is a local commodity of superior shallots, which is already well known as a source of typical fried onion ingredients with more distinctive taste compared to other shallots in the country. It is known as local Palu shallot or better known as Palu fried shallot. Palu shallot varieties in Soulove village are cultivated traditionally by farmers. Shallot farming has been started for decades, especially around the Soulove village where shallot can adapt well to lowland areas with dry climates (Yusuf et al., 2016).

In this study, phytochemical analysis was performed to measure the amount of total phenolics/flavonoids and quercetin concentration on an ethanol extract of Palu shallot bulbs. The results can be seen in Table 1. Total phenolics was calculated according to equation from gallic acid graph with y = 0.016x + 0.048 (R^2 = 0.998). The total phenolic, expressed as gallic acid equivalents (GA), was found of 0.4834 ± 0.003 mg GA/100 mg of dry extract. Meanwhile, the total flavonoid, expressed as quercetin equivalents (QE), was 0.3634 ± 0.018 mg/100 mg of dry extract obtained from the equation of quercetin graph, y = 0.011x + 0.024 (R^2 = 0.996). *A. ascalonicum* was reported to contain more total flavonoids than other types of onion varieties (Fattorusso et al., 2002). The total flavonoid of ethanol extract of onion (*Allium cepa*) and garlic (*A. sativum*) was reported in the range of 0.015 – 0.02 mg CE/100 mg and the total phenolic of ethanol extract of garlic (*A. sativum*) was 0.6 mg GAE/ 100 mg (Priecina & Karlina, 2013).

Table 1 Phytochemical analysis of ethanol extract of Palu Shallot

Analysis	Ethanol extract
Total phenolics (mg/100 mg) in GAE	0.4834±0.003
Total flavonoids (mg/100 mg) in QE	0.3634±0.018
Quercetin concentration (mg/kg)	65.46±0,0002

Shallot was characterized by the presence of flavonoid compounds of quercetin. Therefore, quercetin concentration in ethanol extract of Palu shallot bulbs was conducted by using RP-HPLC. The quercetin concentration on the extract was quantified based on the obtained quercetin standard calibration curve, y = 9.368x + 11.82 (R^2 = 0.993). HPLC chromatogram showed that a peak for quercetin on the ethanol extract can be comparable with the peak of quercetin standard with the retention time (RT) of 2.53 (Figure 2). Using calibration curve plotting between concentration and peak area, Palu shallot was found to contain quercetin of 65.46±0.0002 mg/kg.

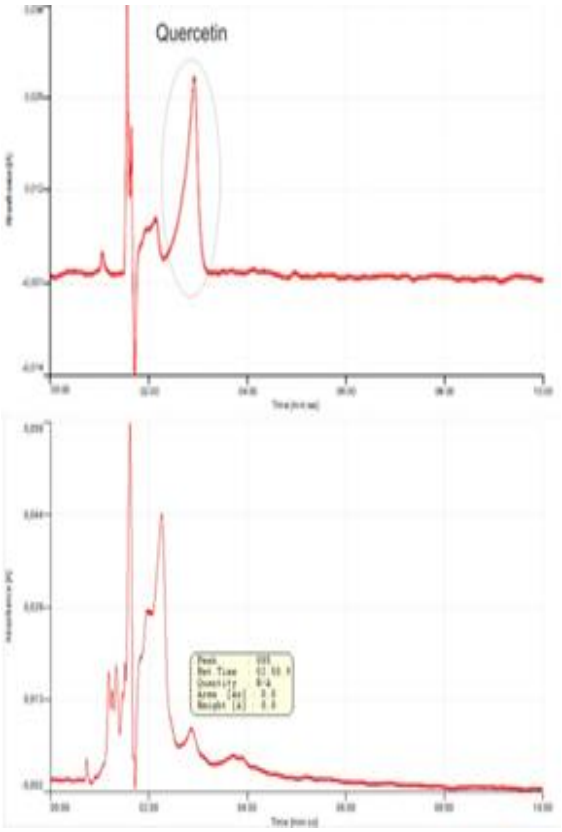

Figure 2 HPLC chromatogram of ethanol extract of Palu shallot bulbs.

This is the first report of the concentration of quercetin on Palu shallot. Pobłocka-olech et al. (2016) had studied the quercetin level on Onion (*A. cepa*) and Shallot (*A. ascalonicum*) from Polland and found that the edible part of onion only contains 11, 17, and 24 mg/kg for gold onion type red baron, amstrong, and exhibition, respectively and for shallot with types of ambition and matador did not contain quercetin (Pobłocka-olech et al., 2016). The quercetin level of red onion was reported as 30.0±0.00 mg/kg (Kwak et al., 2017). This study supports the high quality of Palu Shallot according to quercetin content on ethanol extract.

Further study was continued to assay the antioxidant and antifungal activities. The DPPH antioxidant activity test of ethanol extract showed the percentage of inhibition of 33.50%, 44.27%, and 54.45% at concentration series of 100, 125, and 150 µg/mL, respectively (Figure 3). Moreover, it also inhibited the growth of *C. albicans* at concentrations of 250, 500, 750, and 1000 mg/mL with the inhibitory zone diameter of 7.57 mm, 9.88 mm, 14.39 mm, and 16.51 mm, respectively (Figure 4). Acheampong and colleagues (2016) have reported that total phenolics have a correlation with the antioxidant activity of several vegetables, including *A. ascalonicum*. The higher phenolic content will affect the higher antioxidant capacity. The methanol extract of *A. ascalonicum* was reported to contain a total phenolic of 0.124 mg TAE/DW, and DPPH scavenging activity was 2.2708 mg/mL (Acheampong et al., 2016). Comparing this study, the ethanol extract of Palu shallot also showed high antioxidant activity where the IC_{50} was 0.1398 mg/mL (Table 2).

Table 2 IC_{50} of ethanol extract of Palu Shallot

Sample	IC_{50} (mg/mL)
Ethanol extract	0.1398
Vitamin C	0.0037

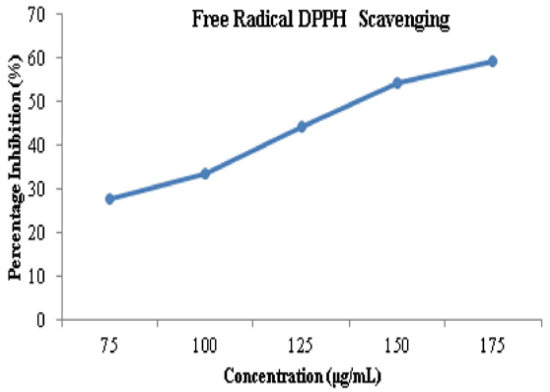

Figure 3 Antioxidant activity of ethanol extract of Palu shallot

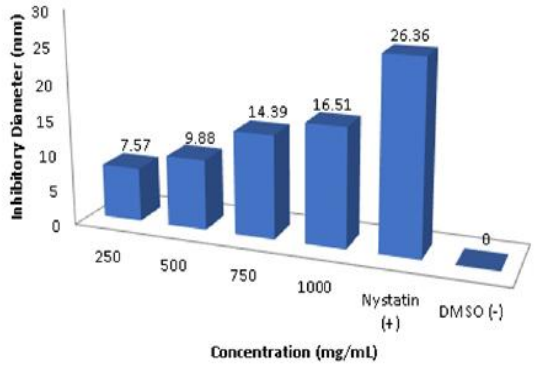

Figure 4 Inhibitory zone diameter (mm) of ethanol extract of Palu shallot against *C. albicans*

Shallot was reported to have the highest antifungal activity against the *C. albicans*. The minimum fungicidal concentration (MFC) of its ethanol extract on *C. albicans* was 20 mg/ml (Moghim et al., 2014). It is also found that shallot has more effect on saphrophyte than *C. albicans* (Mahmoudabadi & Naser, 2009). In this research, Palu shallot showed antifungal inhibition on all concentrations used with the range of inhibitory zone diameter range from 7.57 to 16.51 mm. The result is following the previous reports regarding the antifungal activity of Shallot. Wang & Ng (2002) reported ascalin as the main compound that responsible for the inhibition of mycelial growth in several fungi.

This study supports the application of Palu shallot as a potential natural antioxidant and antifungal agent based on total phenolics, total flavonoids, and quercetin content. Although it has been used as a food flavor in daily seasoning, it has also been widely applied as a raw material in the food industry (snacks production and cooking seasoning) (Sun et al., 2019). It is suggested to develop Palu shallot as raw material for drug formulation or as functional food products.

Conclusion

Palu shallot was analyzed for total phenolics, total flavonoids, and quercetin content with the amount of 0.4834±0.003 mg/100 mg GAE, 0.3634±0.018 mg/100 mg QE, and 65.46±0.0002mg/kg, respectively. Examination of DPPH scavenging activity and agar diffusion methods showed antioxidant activity with the IC_{50} of 0.1398 mg/mL and growth inhibition of *C. albicans* at all concentrations. This study supports the potency of local Palu shallot to be an antioxidant and antifungal medicinal herb.

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Conflict of Interest

The authors declare no conflict of interest

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ASSESSMENT OF PROBIOTIC PROPERTIES AND SAFETY OF LACTIC ACID BACTERIA ISOLATED FROM SOUTH SULAWESI ETHNIC CHEESE

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KEYWORDS

Lactic acid bacteria

Probiotic properties

Safety

South Sulawesi

Dangke

ABSTRACT

Probiotic bacteria have been used widely as a functional food and health supplements. The functionality and safety of probiotics are the prerequisites given by WHO (World Health Organization) and FAO (Food Agricultural Organization) before utilizing probiotics. This study focuses on assessing probiotic properties and the safety of the lactic acid bacteria (LAB) isolated from dangke—traditional cheese of South Sulawesi. In the current study, the assessment of probiotic properties was carried by assessing its tolerance against low pH and bile salts. Safety assessments were divided into two assays viz., susceptibility testing and hemolytic activity. LAB from dangke demonstrated tolerance against low pH, bile salt and susceptibility against four types of antibiotics (ampicillin, cefotaxime, erythromycin, and tetracycline). Unfortunately, resistance towards gentamycin and an α-hemolytic activity was observed. This bacterium met the functional criteria from probiotics but failed to meet the safety criteria for probiotic safety.

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1 Introduction

Probiotic incorporation in food and health supplement remains a trend ever since its first commercialization in 1903. Some probiotics used in the market today mainly originated from fermented foods or the human body and animal microflora (Zommiti et al., 2020). To ensure its beneficial action and its safety in our body, probiotic candidates must fulfil some requirements. The minimum criteria for probiotic candidates were emphasized in the International Scientific Association of Probiotics and Prebiotics (ISAPP) 2018 discussions based on joint FAO/WHO 2002 guidelines: 'a *probiotic candidate must meet functionality and safety criteria, also be easy to use in technical terms'* (FAO/WHO 2002; ISAPP 2018; Zommiti et al., 2020).

After being consumed, probiotic will be exposed to low pH and bile salts in the gastrointestinal tract; it must maintain specific viability to provide health benefits when consumed (Markowiak & Śliżewska 2017; Stasiak-Różańska et al., 2021). Though probiotics are usually encapsulated to protect their sustainability, some probiotic products suggest the protective capsule be removed before consumed by toddlers, which may affect its survival and efficacy. Furthermore, the varying incapability of several commercial probiotic supplements against the extreme condition in the stomach was reported in many studies (Zommiti et al., 2020; Stasiak-Różańska et al., 2021). Regarding its safety, the origin of the strain, its hemolysis activity, and resistance pattern to antibiotics become the requirements of a probiotic candidate must meet during the selection (Markowiak & Śliżewska 2017; Zommiti et al., 2020).

ISAPP suggested a wide distribution of mechanisms of probiotic health benefits in large taxonomic groups. Moreover, it was frequently found at the species level (Hill et al., 2014). Though not all health benefit effects might be observed in a single strain, multiple mechanisms including the production of strain-specific bioactive compounds might be expected (Hill et al., 2014). Each strain might possess different characteristics; therefore, assessments of its probiotic properties and safety to ensure the quality, functionality, and safety of a new probiotic candidate for commercial products are needed.

Dangke, an ethnic cheese of South Sulawesi, Indonesia, has been studied as the source of some potential probiotic candidates (Nur, 2012; Burhan et al., 2017). This staple food is made from buffalo or cow milk added with papaya sap to coagulate the protein then ripened; the compounds produced during the ripening process support the growth of LAB (Surono, 2015; Burhan et al., 2017; Djide et al., 2020). The current study managed to isolate and identify bacteriocin-producing lactic acid culture—*Lactobacillus fermentum* strain NBRC 15885—from Dangke; however, its probiotic properties and safety are yet to be determined (Djide et al., 2020). Hence, this study focused on assessing probiotic properties and the safety of the strain to ensure its functionality and safety as a new commercial probiotic candidate.

2 Materials and Methods

To carry out this study, *L. fermentum* strain NBRC 15885 isolated from dangke was cultured in de Man Rogosa & Sharpe (MRS) Agar (Merck®) 24 hours before the test.

2.1 Assessment of Probiotic Properties

2.1.1 Tolerance Against low pH

Tolerance against low pH was assessed following Sujaya et al. (2008) with some modification. LAB was cultured on MRS broth (Merck®) in aerobic condition at 37°C, for 48 hours. One ml of the media was transferred into 1.5 ml Eppendorf® tubes and centrifuged for 15 minutes at 3000 rpm. The filtrate was discarded, followed by adding a 500 µl saline solution (Otsuka®) into the tube. A total of 50 µl of the suspension was pipette into a new tube contained 1000 µl MRS Broth (Merck®) at pH 2 followed by incubation for 2.5 hours at 37°C. After 2.5 hours, 100µl of cell suspension was pipette into a new tube followed by10-fold dilution using saline solution (Otsuka®). Fifty microliters of the dilution were pipetted into 5 ml broth MRS Broth (Merck®) (pH 6.5) then incubated at 37°C for 48 hours. The optical density of the media was measured at a wavelength of 660 nm using Spectronic-20 (Thermo-scientific®).

2.1.2 Tolerance Against Bile Salt (0.3%)

Tolerance against low pH was assessed by using the same method mentioned above. MRS broth supplemented 0.3% bile was used in this test.

2.2 Safety Assessment of the Strain

2.2.1 Hemolytic Activity

Hemolytic activity was assessed by inoculating LAB culture in Blood Agar (Merck®) added with 5% sheep blood followed by 24 hours incubation at 37°C. The change in media colour was observed (Singh et al. 2014).

2.2.2 Antimicrobial Resistance

MRS Agar media was spread (Merck®) with previously diluted LAB culture—adjusted to the standard McFarland 0.5 (equivalent to 1.5 x 10^8cfu/ml). Antibiotic discs (ampicillin, erythromycin, tetracycline, and gentamicin (Oxoid®) were aseptically placed on the media. The plates were incubated 24 hours at 37°C. The diameter of the inhibitory zone was measured. CLSI M100-S22 (Clinical Laboratory Standard Institute 2012) was used as a reference to determine the susceptibility of test antibiotics.

3 Results

3.1 Assessment of Probiotic Properties

According to Sujaya et al. (2008), if LAB shows an OD660 > 0.01 after being grown at a low pH of 0.3% bile salt, the bacteria might survive in the gastrointestinal tract, and it can be able to withstand the extreme condition of the gastrointestinal tract. The OD660 value of dangke LAB after being grown in MRS Broth at pH 2 and MRS Broth added with 0.3% bile salts were shown in Table 1.

3.2 Safety Assessment of the Strain

Safety assessments of dangke LAB were divided into two assays viz., hemolytic activity and antimicrobial resistance. Hemolysis activity was studied on blood agar media. Cultures of dangke LAB exhibit α-hemolysis activity characterized by forming a greenish zone with a clear outer area around the streak (Figure 1).

Assessment of antimicrobial resistance was carried out against the five antibiotics i.e. ampicillin, cefotaxime, erythromycin, gentamycin, and tetracycline. According to the CLSI standard (Clinical Laboratory Standard Institute 2012), there are three categories of resistance in assessment: >14 mm inhibition zone is considered resistant (R), inhibition zone diameter ranges from 15 - 19 mm is deemed to be intermediate (I), and a diameter < 20 mm is considered to be susceptible (S). The results showed LAB were sensitive to ampicillin, cefotaxime, erythromycin, tetracycline while showing resistance to gentamicin antibiotics (Table 2).

Table 1 The OD660 value of dangke LAB grown in extreme GIT condition

Condition	OD_{660}
pH 2	1.620 ± 0.053
Bile salts 0.3%	1.627 ± 0.012

R = resistant, I = intermediate, S = susceptible

Table 2 Antibiogram Profile of *L. fermentum* NBRC 15885

Antibiotic(μg)	Diameter of inhibition zone (mm)	Interpretation
Ampicillin (10)	41.33 ± 1.61	S
Cefotaxime (30)	26.00 ± 1.80	S
Erythromycin (15)	29.56 ± 3.96	S
Gentamycin (10)	7.78 ± 0.42	R
Tetracycline (30)	21.89 ± 1.83	S

R = resistant, I = intermediate, S = susceptible

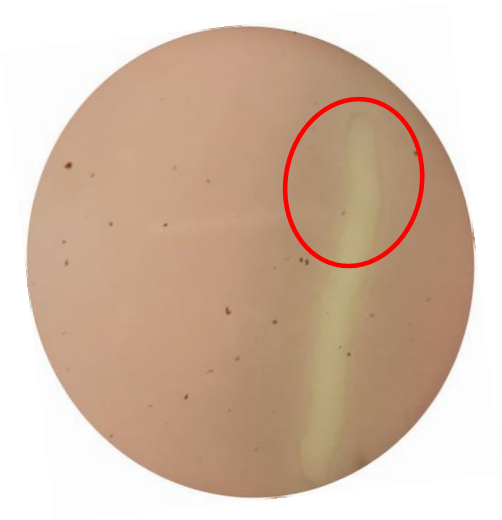

Figure 1 Hemolytic Activity of *L. fermentum* NBRC 15885 Isolated from Dangke.

4 Discussion

4.1 Assessment of Probiotic Properties

WHO/FAO defines probiotics as living microbes that provide a health benefit when given in adequate amounts (FAO/WHO 2002). Based on these definitions, probiotics must maintain specific viability to benefit our health, despite being formulated into an encapsulated product (Papadimitriou et al., 2016; Zommiti et al., 2020). The gastrointestinal tract creates various challenges for probiotic cultures, including acidity (pH 2-4), digestive enzymes (Papadimitriou et al., 2016), and bile salts (Ruiz et al., 2013), thus, resistance against these conditions becomes an essential requirement in the screening of new probiotics where a minimum decrease in viability under these critical conditions (Papadimitriou et al., 2016; Wang et al., 2018). Furthermore, acid resistance is also an important technological aspect since the accumulation of acid products from fermentation may lead to cell death (Papadimitriou et al., 2016). The acidic conditions of G.I. not only damage the cell membrane and cell walls, but also affect membrane potential, protein denaturation, and damage jeans (Papadimitriou et al., 2016), while bile salt can cause cell death through damaged lipid packaging or cytoplasm acidification (Ruiz et al., 2013). LAB tolerance against low pH can occur through several mechanisms, including production of alkali compounds that neutralize pH, biofilm formation, protection or repair of cell macromolecules, or the presence of F_0F_1-ATPase proton pump system (Jin et al. 1998; Wang et al., 2018). The mechanism of resistance of BAL against bile salts can occur through hydrolysis of bile salts, alterations in the components of cell walls and membranes, and the active efflux of bile acid and its salts (Ruiz et al., 2013). Based on the results, *L. fermentum* NBRC 15885 bacteria meet the functional criteria of probiotics.

4.2 Safety Assessment of the Strain

Haemolysis is one of the virulence factors. Haemolysis is a phenomenon that indicates the release of haemoglobin from red blood cells, it can be classified into three types: alpha (α), beta (β), and gamma (γ, non-hemolysis) types. Among those three, γ-hemolysis is considered safe. FAO/WHO requires testing of hemolysis activity for a probiotic strain if the same species shows potential for hemolysis (FAO/WHO 2002). Owusu-Kwarteng et al. (2015) reported α-hemolysis activity by *L. fermentum* strains of millet dough; therefore, hemolytic activity needs to be assessed. LAB culture of dangke shows α-hemolysis activity. The first phenomenon of α-hemolysis with true outer hemolysis was observed by Davis & Rogers in 1939, with hydrogen peroxide production as the possible mechanism. Several years later, another mechanism, such as the production of hemolysin or biosurfactants, was reported and eventually used to screen biosurfactant-producing bacteria (Morán et al., 2002). The amphiphilic parts of surfactant molecules can disrupt cell membrane integrity through modification of lipid compounds and integral proteins, which leads to lysis of red blood cell membranes (Manaargadoo-Catin et al., 2016). Several strains of *Lactobacillus* spp. are also reported to produce biosurfactants and hydrogen peroxide (Sharma & Saharan, 2014; Sharma & Saharan, 2016; Cornea et al., 2016; Mann et al., 2021).

Another safety requirement of probiotic strains by FAO/WHO is the determination of antibiotic resistance patterns (FAO/WHO 2002). Based on the test result, LAB dangke shows resistance against aminoglycoside antibiotic gentamicin which has already been reported by previous researchers (Georgieva et al., 2015; Dec et al., 2017; Zhang et al., 2018; Dong et al., 2019). The mechanism of LAB resistance against the aminoglycoside group was first reported in 1998 as a loss of aminoglycoside uptake mediated by cytochrome-mediated electoral transport (Charteris et al., 1998). Several years later, intrinsic gene *aph*(3')-IIIa, *aac*(6')-*aph*(2''), and *ant*(6) were reported as the gene responsible for aminoglycosides resistance in Lactobacillus (Wong et al., 2015). Recently, asp23 was reported to regulate the gentamicin resistance mechanism in *L. casei* (Zhang et al., 2018).

Although probiotics with resistant genes are not harmful, there is a potential to transfer the resistance gene from probiotics to indigenous microorganisms in the gut, or even opportunistic pathogens living in the gut that might cause severe infections (Zheng et al., 2017; Wang et al., 2020). Besides, several cases of difficult-to-treat bacteremia or endocarditis caused by *Lactobacillus* species which are used in probiotic products have been reported (Ashraf & Shah, 2011; Zheng et al., 2017). On the other hand, this resistance can be helpful in combination with antibiotic therapy because probiotics must survive in the influence of these antibiotics (Imperial & Ibana, 2016). The study of 43 commercial probiotic strains used as a probiotic-antibiotic combination in Japan shows that the amount used clinically with antibiotics is quite limited (Hammad & Shimamoto, 2010). These findings turn the usage of probiotics in the health field into a double-edged sword. As reviewed by Tong (2021), though some probiotic strains show resistant activity towards antibiotics, but these activities are sometimes intrinsic and have restricted transference to other genera (Tong 2021).

Conclusion

In conclusion, *L. fermentum* strain NBRC 15885 isolated from dangke exhibits probiotic potential as it can tolerate bile salt and extreme acidity. However, it didn't meet the safety criteria of FAO/WHO. It is recommended to expand the study in various research fields to uncover more potentials of this strain.

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Conflict of interest

The authors declare no conflict of interest in this study.

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A COMPARATIVE PHARMACOGNOSTIC STUDY OF THE TWO *Orthoshipon aristatus* (BLUME) MIQ. VARIETIES

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KEYWORDS

Orthosiphon aristatus

Purple variety

White variety

Crude drugs characteristic

ABSTRACT

The use of a plant as an ingredient in traditional medicine requires scientific evidence to determine its properties. Cat's whiskers (*Orthosiphon aristatus*) is one of the widely used traditional medicinal plants in various Asian and European countries. This study aimed to determine the pharmacognostic properties of purple and white varieties of *O. aristatus*. Aqueous and ethanolic extract of the stem and leaves of purple and white varieties of *O. aristatus* was prepared and investigated for the presence of active ingredients. The $AlCl_3$ colorimetric method was used for the estimation of flavones and flavonols. The level of flavonoid was reported 13.06 ± 0.13 mg QE / 1g and 6.17 ± 0.049 mg QE / 1g for the leaves and stem extracts of purple varieties respectively while this value was reported 9.76 ± 0.15 mg QE / 1g and 3.79 ± 0.03 mg QE / 1g for the white variety. From the results of the study, it can be concluded that the purple variety has a significantly higher amount of flavonoid then the white variety.

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1 Introduction

The plants are used as an active ingredient in traditional medicine from the ancient time but scientific evidence in favour of these uses are in scanty. Detailed investigation of the active ingredients available in medicinal plants also help in justifies the use of the particular plant in the traditional medicinal system (Nuari et al., 2017). *O. aristatus* is a medicinal plant which widely used as ingredients in various traditional medicine, cosmetics, and food supplements in various Asian and European countries. Some important pharmacological activities of *O. aristatus* are antidiabetic (Mohamed et al., 2011), antiviral (Ripim et al., 2018; Faramayuda et al., 2021a), antioxidants (Alshawsh et al., 2012), antiepileptic (Kar et al., 2018), and antimicrobial (Chen, 1989; Hossain et al., 2007; Ho et al., 2010). Further, in traditional medicine, this plant is extensively used for the prevention and treatment of various remedies such as cancer (Halim et al., 2017; Pauzi et al., 2018), rheumatoid arthritis (Adawiyah et al., 2018), gastric disorders (Yuniarto et al., 2017), cardiac dysfunction (Abraika et al., 2012), and improving memory (George et al., 2015). Cai et al. (2018) also suggested that *O. Stamineus* protect intestinal cells from oxidative stress and the stem have high antioxidant activity (Ameer et al., 2012; Xue et al., 2016).

Previous studies reported the presence of various secondary metabolites such as polyphenols (lipophilic flavonoids and phenolic acids), terpenoids (diterpenes and triterpenes), and sterols (Tezuka et al., 2000) from the *O. aristatus*. Olah et al. (2017) also reported the presence of caffeic acid (rosmarinic acid, cycoric acid) and polymoxylated flavonoids (sinensetin, eupatorin) derivatives from this plant. Further, the presence of the caffeine acid derivatives, triterpene saponins, diterpenes esters, essential oils and flavonoids was also reported from the stem of *O.aristatus* (Tezuka et al., 2000).

Based on the colour of the petals of flowers *O. aristatus* can be classified into three varieties, namely white, white-purple, and purple (Lai Keng & Poay Siong, 2006; Febjislami et al., 2019; Faramayuda et al., 2020; Faramayuda et al., 2021b; Faramayuda et al., 2021c). While, the stem colour of the purple variety is light purple, and for the white variety it is green-brown. Information regarding the pharmacognostic characteristics and the level of flavonoids of these *O. aristatus* varieties are limited. Therefore, this study was carried out to compare the levels of flavonoids in the leaves and stems of white and purple varieties of the *O. aristatus*.

2 Materials and Methods

2.1 Chemicals and reagents

The chemicals used in this study are analytical (pro analysis, p.a) grade ethanol 96 %, toluene, quercetin, aluminum chloride 10% and potassium acetate.

2.2 Collection of plants

The leaves and stems of purple and white varieties of *O. aristatus* were collected from the Manoko experimental garden, West Bandung, Indonesia. Collected plants were taxonomically identified by a qualified taxonomist and a type specimen was deposited at the herbarium of School of Life Science and Technology, Institut Teknologi Bandung. The collected plant samples were air-dried and powdered. The powder crude drug is then sieved using a sieving machine with a mesh sieve of 20/40 and 40/60 to obtain a powder of a suitable and uniform size.

2.3 Characterization of Plant Material

2.3.1 Water and Ethanol Soluble Contents

For the estimation of water-soluble contents, 5 grams of leaves and stem powder was macerated for 24 hours with 100 mL of water in a separating funnel. This apparatus was manually shacked many times for the first 6 hours and later on left stable for the next 18 hours. Filtrate (20 mL) was evaporated to dryness under oven at 105^{o} C in a tapered evaporator cup until a constant weight was gained. A similar procedure was used for the estimation of ethanol-soluble content, in this, instead of water, 100 mL of ethanol was used in a closed flask for separation.

2.3.2 Determination of Water Content (Distillation Method)

In this study, the toluene distillation method was used to determine the water content (Hermawan et al., 2016). An appropriate amount of the crude sample was weighed and mixed with 200 mL of toluene in a flask. The cooling and receiving tubes that have been cleaned and dried were connected with the flask and this distillation apparatus was heated for 15 minutes. When the toluene started boiling the temperature was adjusted in such a manner that it preceded the distillation at the rate of 2 drops per second until the water has been completely distilled.

2.4 Extraction of Plant Material

A total of 100 g of leaves and stems two varieties of *O. aristatus* mixed with 300 mL 96% ethanol solvent and stored for maceration. The apparatus was manually shacked many times for the first 6 hours and later on left stable for the next 18 hours (Utami et al., 2016). The extraction process is repeated twice with the same type and amount of solvent to gain the maximum concentration of extract. All macerate was collected and concentrated by evaporating using a rotary evaporator, and this was followed by water bath evaporation till the thick extract is obtained.

2.4.1 Determination of Specific Gravity

Determination of specific gravity was based on a 5% dilution of the extract in an ethanol solvent by using a pycnometer. Before determining the specific gravity, the pycnometer was properly cleaned and dried. The pycnometer was calibrated by weighing its weight and the weight of water at 25ºC. Similarly, the specific gravity of the extract was determined by a pycnometer at 25ºC. The extract's specific gravity is determined by dividing the weight of extract by the weight of water in a pycnometer at 25ºC.

2.4.2 Determination of Flavonoid Levels

2.4.2.1 Sample preparation for Quercetin Calibration Curve

Quercetin (25 mg) was taken into a 50 mL volumetric flask and dissolved in 25 mL pro-analysis ethanol (1000 µg/mL stock solution). This was followed by the making of a standard solution of quercetin. For this, 0.5 mL of the standard solution was pipette out, and in this 1.5 mL pro-analysis ethanol, 0.1 mL 10% aluminum chloride, 0.1 mL potassium acetate and 2.8 mL distilled water was added. After that, this mixture was incubated at room temperature for 30 minutes; and the mixture's absorbance was measured by a UV-Visible spectrophotometer.

2.4.2.2 Preparation of Sample Solution

The ethanol extract of the leaves and stems of purple and white varieties of cat's whiskers was prepared by Azizah et al. (2014) method, for this, 1.0 g of each sample was taken and dissolved in the 10 mL of the ethanol. This mixture was stirred for 24 hours using a stirrer at a 200 rpm speed, then filtered it, and the obtained filtrate was added to pro-analysis ethanol up to 10 mL.

2.4.2.3 Determination of Flavonoid Levels

The level of flavonoid was determined by the $AlCl_3$ colorimetric method that uses aluminum chloride as a reagent as described by Chang et al. (2002), and Azizah et al. (2014). 1.0 mL of ethanol extract was added to 10 mL of ethanol in a volumetric flask. From this, 0.5 mL of the solution was pipetted out, and mixed with a solution of 1.5 mL of pro-analysis ethanol, 0.1 mL of 10% aluminum chloride ($AlCl_3$), 0.1 mL of potassium acetate, and 2.8 mL of distilled water. In measuring flavonoid levels, the addition of potassium acetate is intended to maintain the wavelength in the visible area and detect the 7-hydroxyl group's presence. Simultaneously, the 30-minute incubation treatment is carried out before the measurement is intended so that the reaction runs perfectly to provide the maximum colour intensity. The absorbance of the mixture was measured by a UV-Visible spectrophotometer (Chang et al., 2002). The determination of the maximum wavelength was carried out in the range of 300-600 nm. The maximum wavelength produced was 433 nm at a concentration of 60 µg / mL. The level of flavonoids had been calculated by using the following formula:

$$F\ (mg/QE) = \frac{c\ x\ v}{m\ x\ f\ x\ 10^{-6}}\ x\ 100\%$$

F: the number of flavonoids in the AlCl3 method; c : quercetin equivalence (µg / mL); V: total extract volume (mL); m : sample weight (g); f : dilution factor

2.5 Statistical Analysis

Flavonoid levels were statistically analyzed using SPSS version 25 software using non-parametric statistical tests using the Kruskal-Wallis Test method (Fitria et al., 2017).

3 Results

A detailed analytical study was carried out to characterize the leaf and stem crude extract of purple and white varieties of cat's whiskers plant. Various characteristics of crude extract such as water-soluble contents, ethanol-soluble contents and crude drug water content have been studied and the obtained values were compared with the standard reference values obtained from the Indonesian Herbal Pharmacopoeia II Edition 2017 (Table 1). In the case of observed parameters, the results of the current study revealed a non-significant difference between the stem and leaves crude extract of purple and white varieties of *O. aristatus*. Overall, the purple variety of *O. aristatus* has higher water-soluble content, Ethanol soluble content and crude drugs water content. While both varieties of *O. aristatus* have significantly higher values than the standard reference values proposed by the Indonesian Herbal Pharmacopoeia II Edition (2017).

Extraction yield with ethanol solvent was also measured for both the cat's whiskers varieties and found that leaves extract of both varieties produced maximum yield, while at the species level, maximum extract yield has been given by the white varieties. Further, determination of specific gravity was carried out by pycnometer and reported that the specific gravity of both varieties was adjacent and less than one (Table 2). The level of flavonoids was measured by the $AlCl_3$ colorimetric method and reported that the level of flavonoids in the leaves (13.06mg QE/1g) and stems (6.17 mg QE/1g) of the purple variety were greater than those of the white variety. Further, the leaves of both *O. aristatus* varieties have higher flavonoid content than the stem (Table 2). Results of the Kruskal-Wallis test showed that the levels of flavonoids in leaves and stems of purple and white varieties had significant differences with a probability value of 0.15 ($p < 0.05$).

Table 1 The characteristics of the crude extract of two *O. aristatus* varieties

Observed Parameters	Purple stem (% w/w)	White stem (% w/w)	Purple leaves (% w/w)	White leaves (% w/w)	Standard Value*
Water soluble content	12.93 ± 2.16	12.55 ± 0.16	28.17 ± 1.17	13.18 ± 0.47	> 10.2 %
Ethanol soluble content	3.92 ± 0.21	3.45 ± 0.18	3.13 ± 0.14	2.55 ± 0.02	>7.2 %
Crude drugs water content	2.74 ± 0.34	1.99 ± 0.70	2.00 ± 0.71	2.50 ± 0.04	< 10 %

*Standard reference values were obtained from the Indonesian Herbal Pharmacopoeia II Edition (2017)

Table 2 The yield, specific gravity and level of flavonoids in the crude extract of two *O. aristatus* varieties

Sample Variety	Extract yield (% w / w)	Yield Specific Gravity (g / mL)	Levels of Flavonoids (mg QE / 1 g extract)*
Purple Stems	8.82	0.82 ± 0.00	6.17 ± 0.049[a]
White Stems	8.39	0.82 ± 0.00	3.79 ± 0.03[b]
Purple Leaves	13.43	0.83 ± 0.00	13.06 ± 0.13[c]
White Leaves	14.79	0.83 ± 0.00	9.76 ± 0.15[d]

*The significant difference with a probability value of 0.15 ($p < 0.05$)

4 Discussion

Crude extract characteristics evaluation has always been carried out to determine the general criteria of the used materials, these evaluations including both specific (determination of water-soluble content, ethanol-soluble content and flavonoid compounds) and non-specific parameters (determination of water content, and specific gravity). Results of the study revealed that the analysis of crude extract characteristics including water content, water-soluble extract content and ethanol-soluble extract content of both *O. aristatus* varieties met the requirements of the Indonesian Herbal Pharmacopoeia Edition (Anonymous, 2017). The determination of water content was aimed to provide a limit of water in the plant extracts. In this study also, obtained results of water content met the requirements of the Indonesian Herbal Pharmacopoeia Edition (<10% v / b).

In this study, a cold extraction method with 96% ethanol solvent was used to protect the degradation of the active substance present in the extract. Due to universal solvent properties and capability of dissolving both polar and non-polar active ingredients ethanol was used as a solvent in the current study (Utami et al., 2016). The obtained extract was concentrated by using a rotary evaporator vacuum pump, followed by evaporation of the extract over a water bath. The crude drugs used for extraction were as much as 100g and produced the total yield (Table 2). Extract yield helps determine the levels of secondary metabolites in the extract (Ahmad et al., 2015). The determination of specific gravity aims to provide an overview of the dissolved chemical content in an extract. The purpose of determining specific gravity is to know the description of the limit of the mass per unit volume between the liquid extract and the thick extract that can be poured. Specific gravity can also provide an idea of the purity and contamination of the material (Anonymous, 2017).

Determining secondary metabolite compounds' levels is one of the specific parameters of standardizing the traditional medicinal ingredients. In the current study, the level of flavonoid content in leaves and stems of purple and white varieties of *O. aristatus* was carried out by the aluminum chloride colorimetric method. The principle of the aluminum chloride colorimetric method is the formation of complexes between aluminum chloride with ketone groups on C-4 atoms and hydroxyl groups on C-3 or C-5 atoms of the flavonoids including flavone and flavonol (Chang et al., 2002). Previous studies have reported a higher level of flavonoids including sinensetin in the purple variety than the white variety; in this manner, these results are consistent with the results of the present study where the purple variety has higher flavonoids than the white variety (Lee, 2004).

Flavonols are known as markers for the presence of flavonoids because of their widespread presence in plants. In the current study, the maximum wavelength produced was 433 nm at a concentration of 60 µg / mL, this measurement of the calibration curve aimed to determine the equation of the linear line. The predefined wavelengths, namely complex wavelengths of 15 standards with aluminum chloride, indicated that the complexes formed by C-3 and C-5 hydroxyl groups of flavonol such as galangine, morine, and kaempferol, also have extraorto-dihydroxyl groups such as routine, quercetin, and myricetin, the maximum absorbance was at 415-440 nm (Chang et al., 2002). Furthermore, the absorbance of each standard series solution was measured and based on the measurement results of the quercetin standard series, the equation obtained is $y = 0.006x + 0.0697$ with a correlation coefficient (r) = 0.9992. Ho et al. (2010) suggested that the levels of flavonoids in *O. aristatus* leaf extract with ethanol and water extract was 0.82% and 1.49% respectively. This amount was lesser than the value reported in the present study, this difference in

flavonoid content might be due to the difference in the experimental conditions or plant materials. Cai et al. (2018) reported 170 µg value of the total flavonoids in the water-ethanol extract of *O. aristatus*.

Conclusion

Pharmacognosy characteristics of the two varieties of *O. aristatus* can be a basis for selecting varieties and maintaining consistency in traditional medicines' quality. After some more detailed studies, the purple variety of *O. aristatus* can be used for the development of medicine because it has higher flavonoids and it copes up with the standard reference values of Indonesian Herbal Pharmacopoeia II Edition.

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Conflict of Interest Nil

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DISSOLUTION IMPROVEMENT OF KETOPROFEN THROUGH POLYMER MATRIX COMPOSITE POVIDONE K-30/TiO_2

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Ketoprofen

Polymer matrix composite

Povidone K30

TiO_2

ABSTRACT

Ketoprofen is a nonsteroidal anti-inflammatory drug that has been classified into Class II as per the Biopharmaceutics Classification System. This substance has low solubility i.e. only 51 mg/L at 22°C. Therefore, a study of ketoprofen modification prepared in Polymer Matrix Composite (PMC) using Povidone K-30, coated with TiO2 as an effort to improve its dissolution profile, had been conducted. Composite particles were prepared through a dissolving method with a ratio of ketoprofen: Povidone K30 i.e. 1: 1 (F1), 1: 2 (F2), and 1: 4 (F3). They were evaluated and continued on the coating process using TiO2 30%, 40%, and 50%. Results of the study revealed that the PMC provides an absence of chemical intervention against ketoprofen. All the used combination ratios improved the dissolution profile by using phosphate buffer media pH 7.5 for 60 minutes. After coating, a release profile was obtained by running it on phosphate buffer media at pH 6.8 for 8 hours, this showing a sustained release profile.

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1 Introduction

Ketoprofen is a propionic acid derivative, non-steroidal anti-inflammatory drug (NSAID), which is used as an analgesic, antipyretic and anti-inflammatory. This active pharmaceutical ingredient of the drug has been categorized as Class II, in the Biopharmaceutics Classification System (BCS), which has low solubility (51 mg/L at 22°C), and high permeability (Grimling et al., 2014; Himawan et al., 2019). It has been claimed that this character may affect the low dissolution rate of ketoprofen, leading to imperfect absorption and poor bioavailability (Rençber et al., 2009; Grimling et al., 2014). In addition, ketoprofen also has gastrointestinal side effects which causing gastric irritation, especially during repeated administration. To overcome this, researchers have been studying to find a simple system that could increase the solubility of the drug (Saffoon et al., 2011), and possibly reduce the side effects of NSAID like ketoprofen. Drug release modification of ketoprofen into a sustained-release system is achievable, which can lead to reducing its dose as once-daily (Schumacher Jr, 1994; Ibrahim et al., 2010). As in this study, the development of formulas containing NSAIDs using ketoprofen as a model can be achieved through the simple method, a composite particle system.

Composite particles using a polymer as known Polymer Matrix Composite (PMC) is one simple technique completed with a coating process to control drug release (Wang et al., 2011; Dang et al., 2012; Kamari & Ghiaci, 2016). The PMC combines two or more components that have different characteristics and convert them into a new material with specific characteristics. The solubility of active pharmaceutical ingredients (API) that are difficult to dissolve could be increased through this system, which directly affecting the API's dissolution rate. In general, the composite particle may appear as three types, namely ceramic matrix composite (CMC), metal matrix composite (MMC) and polymer matrix composite (PMC) (Teti, 2002; Wang et al., 2011).

PMC is the most commonly used modification because it is a simple, reliable, and less expensive technique. PMC is formed from reinforcing material and its matrix. The matrix consists of polymeric materials, which will cause the other components to be dispersed in the polymer used. Various studies have been applying to polymer matrices to improve dissolution rates and drug release (Teti, 2002; Bazzo et al, 2009; Wang et al., 2011). Povidone or polyvinyl pyrrolidone is an inert polymer made from the monomer N-vinylpyrrolidone. This polymer has the characteristics of being soluble in water and other organic solvents and provides the ability to increase drug dissolution and release by forming pores (Kazarian & Martirosyan, 2002; Franco & De Marco, 2020).

Povidone has been categorized as safe by the FDA and is widely used as a drug carrier to increase drug solubility and control drug crystallization (Frizon et al., 2013). Thus, in this study povidone has been used to improve the solubility of ketoprofen in the composite matrix. In addition, the use of a biocompatible coating of TiO_2 played an important role in particle surface modification. Titanium dioxide could offer proper dispersion of the polymer matrix and provide optimum product yields. Furthermore, it formed a physical bridge API to the polymer matrix preventing drug degradation (Sabzi et al., 2009). Therefore, using TiO_2 as a coating matrix for composite particle ketoprofen had been also studied. Thus, proper dispersion of the polymer matrix is achievable, leading to the formation of physical interactions with the polymer matrix (Sabzi et al., 2009). The proportion of active pharmaceutical ingredients to the polymer-like povidone K-30, combined with TiO2 coatings is a vital step for composite particle formulation. Therefore, in this study povidone K-30 as a polymer matrix was used to improve the dissolution profile of ketoprofen. The opportunity of controlling the ketoprofen release had also been evaluated by using TiO_2 as a coating to form a ketoprofen composite.

2 Materials and Methods

Ketoprofen (*Pharmaceutical grade*), povidone K-30 (Dwilab mandiri), N-hexane, phosphate buffer pH 6.8, phosphate buffer pH 7.5, ethanol 96%, HCl pro-analysis, sodium dihydrogen phosphate, disodium hydrogen phosphate, and titanium dioxide, were provided by Pharmaceutic laboratory, Faculty of Pharmacy, Hasanuddin University.

2.1 Preparation of composite particle ketoprofen – povidone K-30 (CPKP)

The composite was prepared by dissolving method, with the proportion of ketoprofen : povidone K-30 in formula CPKP F1 (1:1), F2 (1:2), and F3 (1:4). Each proportion of ketoprofen and povidone K-30 were put in a and completely dissolved with 96% ethanol. The solution was then poured into a petri dish and dried in a granule dryer at a temperature of ± 40°C until a composite particle was obtained. The solid formed was also sieved with a sieve number 20 (Bazzo et al., 2009; Frizon et al., 2013).

2.2 The intervention test between ketoprofen as active pharmaceutical ingredients and adjuvants

Ketoprofen and composite product were dissolved in phosphate buffer pH 7.5 with a concentration of 10 ppm. The absorption was then measured in the 200 - 400 nm wavelength range using a UV-Vis spectrophotometer. The presence or absence of a shift in the maximum absorption wavelength in the composite particle was observed.

2.3 The dissolution test of the composite particle ketoprofen : povidone K-30 (CPKP) products

The dissolution test product was carried out using the paddle method (USP-NF, 2018). A composite particle which is equivalent

to 100 mg of ketoprofen was inserted into the capsule shell. Then, 900 ml of pH 7.5 phosphate buffer as dissolution media place at 37±0.5°C in a chamber and stirred at a speed of 50 rpm. The solution in the flask was sustainably sampled using 5 ml Millipore at the period of 5, 10, 15, 30, 45 and 60 minutes. The taken solution as a sample was measured for its absorption at the λ_{max} of ketoprofen using a UV spectrophotometer.

2.4 The coating of CPKP product with TiO_2

The CPKP product which had the optimum dissolution profile was taken equivalently to 100 mg of ketoprofen and dispersed into 20 ml of n-hexane, stirred at a speed of 800 rpm, at room temperature. TiO_2 at 30%, 40% and 50% was respectively mixed into 5 ml of n-hexane and added dropwise into the stirred phase of CPKP. Furthermore, the mixture was left for 24 hours, this was followed by the filtration and drying to form composite particles having ketoprofen : povidone K-30/TiO_2 coating (Sabzi et al., 2009).

2.5 The determination of dissolved ketoprofen in the coated products

Each composite particle ketoprofen : povidone K-30/TiO_2 was weighed as 10 mg, and dissolved in each 0.1 N HCL, and phosphate buffer pH 6.8 to 10 ml. The solution was piped 0.1 ml, and the volume was sufficient to 5 ml. Dissolved ketoprofen was determined by measuring the absorption using a UV-Vis spectrophotometer at the λ_{max} of ketoprofen with previously having a standard curve.

2.6 The dissolution test of the coated products in phosphate buffer pH 6.8

The dissolution test of the coated products was carried out as described in section 2.3. However, for this product, the test had been conducted using two dissolution media, namely 0.1 N HCl, and phosphate buffer pH 6.8. The sample solution was taken at 15, 30, 45, 60, 90 and 120 minutes intervals for the acid media, and at 1, 2, 3, 4, 6 and 8 hours for the phosphate buffer media.

2.7 The kinetics model of ketoprofen released from coated composite particles

The drug release kinetics was determined according to zero-order, first-order, and Higuchi kinetics (Mircioiu et al., 2019). The zero-order kinetics model is obtained by plotting the cumulative drug dissolved (%) against time. The first-order kinetics model is obtained by plotting the curve between the logarithms of a cumulative drug (%) remaining against time, and the Higuchi kinetics model is obtained by plotting the curve of a cumulative drug (%) dissolved against the root of time. The best-suit drug release kinetics was determined through the correlation coefficient (r) value obtained in each kinetics model. The highest correlation coefficient (closest to 1) can be assumed to be a model of formula release kinetics (Holowka & Bhatia, 2014; Mohammadian et al., 2018).

3 Results and Discussion

Composite particle ketoprofen : povidone K-30 (CPKP) is formulated through Polymer Matrix Composite (PMC) using povidone K-30 as polymer, resulting products were hygroscopic, and having small crystals-like shards form (Figure 1).

Following the process, the observation of chemical intervention among the compositions was subsequently performed. The possibility of chemical interactions occurred between ketoprofen as the active pharmaceutical ingredient, and adjuvants were evaluated using a UV-Vis spectrophotometer at the λ = 200 - 400 nm, by observing at the maximum wavelength of each product compared to unmodified ketoprofen. As depicted in Figure 2, each product CPKP, compared to ketoprofen have the similarity for the maximum wavelength (259.8 – 260.6 nm), in a slight change in respective absorbance values. This trend implies the absence of chemical interaction, and the existence of physical interaction between ketoprofen to the polymer, which is also confirming the dynamic organoleptic appearance previously described in Figure 1.

In terms of dissolution profile, the evaluation was conducted as described in the monograph, Indonesian Pharmacopoeia ed. V and <711> Dissolution, USP. The dissolved ketoprofen was determined using a UV-Vis spectrophotometer at the ketoprofenλ_{maks}, through paddle method in phosphate buffer (pH 7.5) for 60 minutes.

As presented in Figure 3, there is an increasing trend in the dissolution rate of each product (F1 to F3) compared to ketoprofen. The dissolved ketoprofen at F1, F2 and F3 at 45 minutes were 46.95 ± 0.23%, 56.89 ± 0.16% and 82.31 ± 0.38%, respectively. This shows that F3 fulfils the monograph requirements for ketoprofen dissolution set by the Indonesian Pharmacopoeia, which should be greater than 70%. In the case of F1, and F2, the dissolution rate was also higher than standard but having unmet requirements. The greater concentration of povidone-K30 in the F3 clearly describes the important effect of the hydrophilic polymer in improving the dissolution rate of hydrophobic NSAID like ketoprofen.

Furthermore, to study the opportunity in improving the drug release of the CPKP, F3 as the optimum formula met the standard requirement, was then charted to the coating process. The F3 was subsequently processed to the coating step with three variant proportions (30% (FT1), 40% (FT2), and 50% (FT3)) of TiO2 as coating material. The dissolution profile of those coated composite particles was determined in both acid and base media through the paddle method, both in HCl 0.1 N, 120 minutes (Figure 4), and in phosphate buffer pH 6.8 for 8 hours (Figure 5).

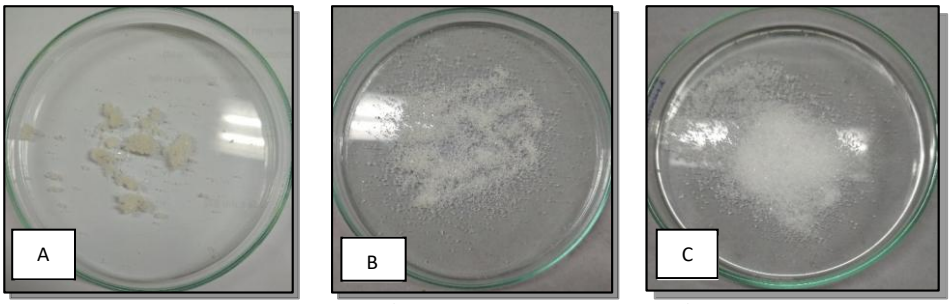

Figure 1 The organoleptic appearances of composite particle A. F1 ketoprofen : povidone K-30 (1:1); B. F2 ketoprofen : povidone K-30 (1:2); C. F3 ketoprofen : povidone K-30 (1:4)

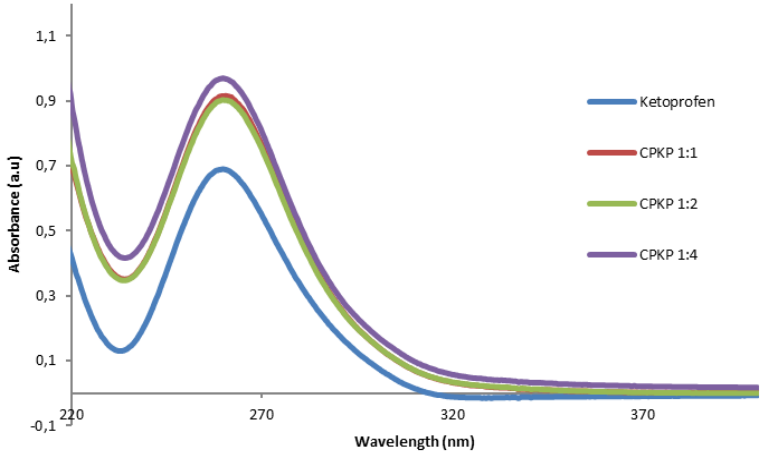

Figure 2 The UV-Vis spectra of ketoprofen compared to each product CPKP F1 ketoprofen : povidone K-30 (1:1), CPKP F2 ketoprofen : povidone K-30 (1:2), and CPKP F3 ketoprofen : povidone K-30 (1:4)

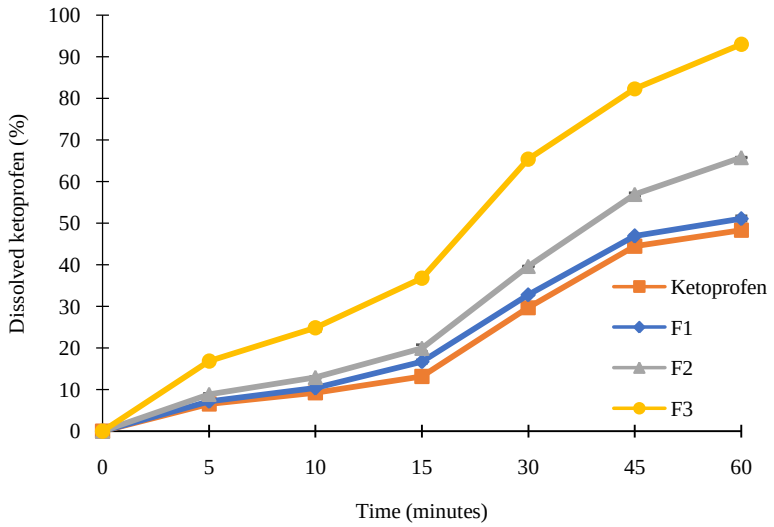

Figure 3. The dissolution profile of ketoprofen compared to each products F1 ketoprofen : povidone K-30 (1:1), F2 ketoprofen : povidone K-30 (1:2), and F3 ketoprofen : povidone K-30 (1:4).

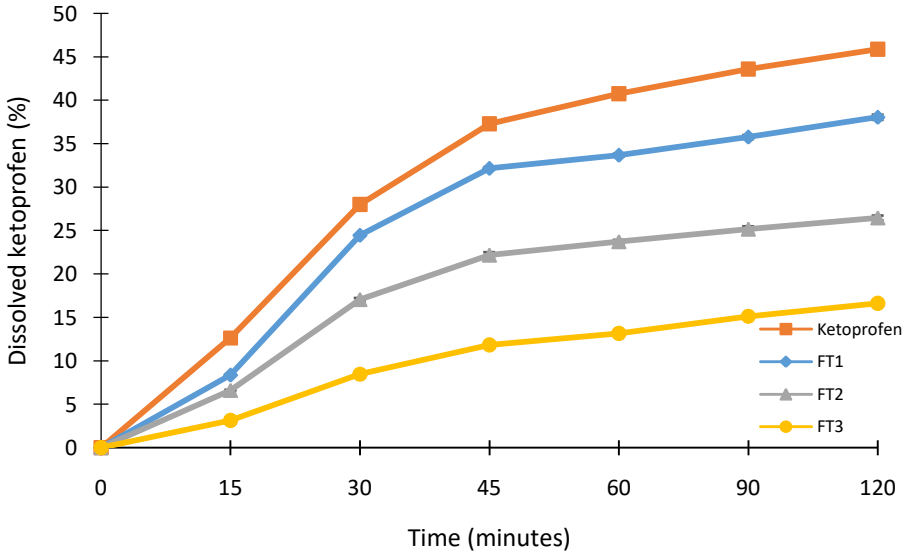

Figure 4 The dissolution profile of ketoprofen compared to each products FT1 ketoprofen : povidone K-30 (1:4)/ 30% TiO_2, FT2 ketoprofen : povidone K-30 (1:4)/ 40% TiO_2, and FT3 ketoprofen : povidone K-30 (1:4)/ 50% TiO_2 in 0.1 N HCl media.

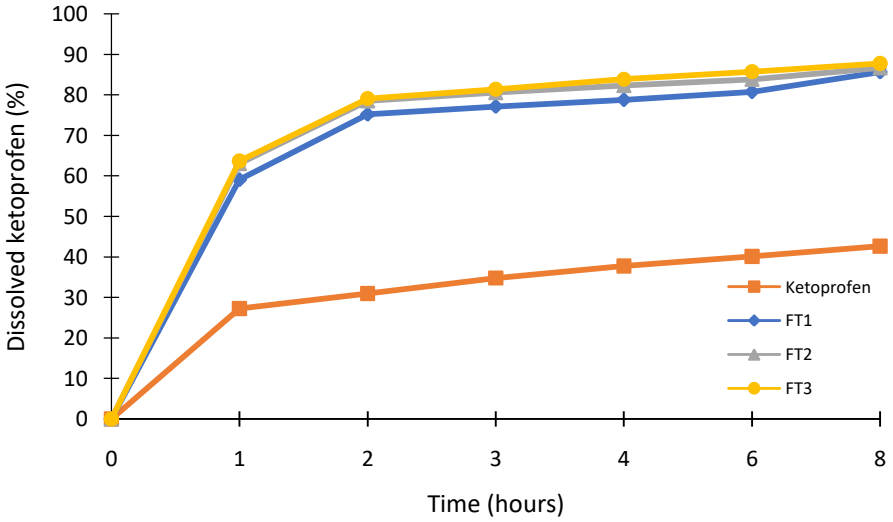

Figure 5 The dissolution profile of ketoprofen compared to each products FT1 ketoprofen : povidone K-30 (1:4)/ 30% TiO_2, FT2 ketoprofen : povidone K-30 (1:4)/ 40% TiO_2, and FT3 ketoprofen : povidone K-30 (1:4)/ 50% TiO_2 in phosphate buffer media.

Table 1 The kinetics correlation coefficient of coated composite particle F3

Formula	Correlation coefficient (r)		
	Zero-order	First-order	Higuchi
FT3	0.8122	0.9101	0.8810

As presented in figure 4, the coated composite products (FT1 to FT3) in 0.1 N HCl media having a decreasing pattern in dissolution rate compared to ketoprofen. As expected, TiO2 in this formulation would reduce the rate of drug release. In the first 15 minutes, all formulas have provided slightly ketoprofen been released. It less than 10% dissolve ketoprofen, increase in gradual pattern until 120 minutes, where FT1 released the highest ketoprofen (38.06%) compared to other products, which was controllable below the concentration of unmodified ketoprofen (45.88%). The great slower dissolution rate in FT3 might be due to

the use of 50% TiO2 that may have strong hydrogen bonds between titanium dioxide to povidone K-30 (Kamari & Ghiaci, 2016).

In contrast to the dissolution profile depicted in figure 5, each product in buffer phosphate has an increasing trend compared to unmodified ketoprofen. In the first hour, all formulas have released ketoprofen more than 50%, followed by a gradual decrease. As stated in the monograph, within 8 hours, ketoprofen has to be released more than 80%. Thus, all formulas suit this requirement, where FT1 released 85.66% ketoprofen, FT2; 86,69%, and FT3 released the highest concentration at 87.77% compared to unmodified ketoprofen which only stood at 42.7%. The results analysis of the variety of ketoprofen dissolved in phosphate buffer media pH 6.8 for 8 hours in three formulas (FT1, FT2, FT3) found to be a very significant difference (Significant level <1%). Furthermore, using the Tukey test, the highest dissolved ketoprofen results were obtained at FT3 (87.77%), which was followed by FT2 (86.69%) and FT1 (85.66%), respectively.

Considering FT3 has provided the lowest release in acid media, and the highest release in alkali media, a kinetic study of ketoprofen in this formula is further studied. The drug, ketoprofen release data were then plotted based on the zero-order, first-order and Higuchi equations (Shohin et al., 2012; Mircioiu et al., 2019).

The determination of ketoprofen released from coated composite particles was carried out by comparing the correlation coefficient of each drug release kinetic model (zero-order, first-order, and Higuchi). As presented in table 1, the correlation coefficient value that was closest to 1, (0.9101) was determined to be the best kinetics model describing the ketoprofen release out of the matrix in phosphate buffer media. This implied that FT3 product has released ketoprofen according to the first-order kinetics, in which the product has sustained release kinetics.

Conclusion

Composite particle ketoprofen : povidone K-30, especially in the proportion 1:4, had improved the dissolution profile (92.74%) of ketoprofen without involving chemical intervention. Furthermore, TiO_2 provide a positive impact on ketoprofen released in the polymer matrix composite to be a sustained-release follows the first-order kinetics.

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Conflict of interest

All the authors declare that there is no conflict of interest.

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DEVELOPMENT OF METRONIDAZOLE MICROSPONGE INCORPORATED INTO CARBOMER-BASED VAGINAL GEL

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KEYWORDS

Bacterial Vaginosis (BV)

Metronidazole

Microsponge

Gel

Vaginal delivery system

ABSTRACT

Bacterial vaginosis (BV) is a vaginal infection caused by excessive bacterial growth, thus disrupting the natural balance of bacteria inside the vagina. Metronidazole becomes a drug of choice and a widely prescribed drug for the treatment of BV. However, when applied topically, metronidazole has a low vaginal residence time because of the natural washing mechanism of the vagina. This study aimed to improve the retention time of metronidazole inside the vagina and control its release profile. This study was prepared 4 formulas of gel for metronidazole microsponges with some concentration ratio of carbomer and triethanolamine. The evaluations carried out to test the efficacy of the developed formulation included organoleptic, pH measurement, spreadability, viscosity, mucoadhesive properties, permeation test using Franz diffusion cell and retention test. The results showed that the gel appearance was white, odourless and homogenous. The characteristics of all prepared gel for pH, viscosity, spreadability, and mucoadhesive ability were appropriate to the required standard for vaginal delivery. The permeation and retention test showed that F3 with the carbomer and triethanolamine concentration of 1.25%: 1.75% was able to retain and controlled the drug release locally in the vaginal mucosa. This study provides an alternative strategy in drug formulation for the treatment of BV.

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1 Introduction

Bacterial vaginosis (BV) is a common vaginal infection that disrupts the natural balance of bacteria in the vagina (Larsson & Carlsson, 2002). BV is characterized by an overgrowth of vaginal anaerobic bacteria and depletion of the normal *Lactobacillus* population (Kumar et al., 2011). BV can cause several complications, which include fishy vaginal discharge, malodours, stomach pain, high HIV risk, preterm birth and urinary tract infection (Kumar et al., 2011). For the treatment of BV, a lot of antimicrobial agents have been used. Metronidazole is the widely prescribed drug for the treatment of BV.

Metronidazole has been reported to be effective in oral and topical applications (locally) for the treatment of BV(van de Wijgert et al., 2020). However, the oral preparation of metronidazole shows some side effects in the gastrointestinal tract (Hernández et al., 2019). Therefore, locally administered metronidazoles are generally preferable. The intravaginal delivery of metronidazole showed a better result but because of the low vaginal residence time due to the natural-washing mechanism of the vagina, it took a longer period of treatment (Kumar et al., 2011). The mucoadhesive vaginal gel can be used to keep the drug in the vagina for a certain period but it is releasing the drug slowly. Besides that, it is important to prolong the retention time of the drug inside the vagina to gain optimal drug absorption by controlling the drug release. The use of microsponge in the mucoadhesive vaginal gel can be an alternative strategy in drug administration for the treatment of vaginal infections (Sangeetha & Karki, 2015; Kalita et al., 2017). Microsponges is a controlled drug release system consists of small porous microspheres which then slowly release the encapsulated materials (active substances)(Mahant et al., 2020). The microsponge can be formulated as a mucoadhesive gel by using a suitable gelling agent for ease of use (Nagula & Wairkar, 2020). Controlling the release of drugs locally will improve the efficacy of formula with a decrease in dosing frequency and fewer side effects. The mucoadhesive gel can attach drugs to the target area so that it can overcome the problem of drugs that could easily be eliminated by the vagina (Andrade et al., 2014).

Carbomer is the most commonly used gelling agent in the manufacture of gels and is particularly meant for the application on mucous membranes. Carbomer has a high molecular weight, containing some carboxyl groups, allowing them to dissolve easily in the body fluids. The hydrogen bonding between the carbomer and mucosal fluids can facilitate the adhesion properties in the mucosa vaginal (Andrade et al., 2014). Several previous studies have used carbomers as polymers in microsponge gel formulations and the results showed that carbomers can stick to the mucosa and the swelling process occurs in the water while releasing the drug into the vagina (Shaikh et al., 2011; Salah et al., 2018). The use of carbomers in mucoadhesive vaginal gel preparations has shown a good distribution. In addition, the use of carbomers is safe, available in large quantities, effective over a wide pH range, and can maintain physico-chemical properties at extreme temperatures.

This study aimed to develop a mucoadhesive vaginal gel containing metronidazole's microsponges with various carbomer concentrations as a gelling agent. This formulation can improve the retention time of metronidazole inside the vaginal and control the profile. In this study, metronidazole's microsponge was incorporated into the carbomer-based vaginal gel. Then the prepared formulations were characterized for pH, spreadability, viscosity, mucoadhesive properties, permeation ability and retention profile.

2 Materials and Methods

2.1 Material

Aquadest, Carbomer (Merck, UK), Ethyl Celulose (Sigma, UK), Dichloromethane (Merck, UK), DMDM Hydantoin (Merck, UK), Metronidazole (Sigma, UK), Microsponge Metronidazole, Polivinilalkohol (PVA) (Sigma, UK), Glycerin, Triethanolamine (Merck, UK), NaCl (Merck, UK), KOH (Merck, UK), albumin serum (Merck, UK), lactic acid (Merck, UK), acetic acid (Merck, UK), urea (Merck, UK), and glucose (Merck, UK). All other chemicals and solvents used are of analytical grade.

2.2 Preparation of mucoadhesive gel

The mucoadhesive gel is prepared by using the formula given in table 1 with a slight modification (Kalita et al., 2017). Briefly, carbopol gel was prepared by dispersing in a sufficient quantity of water according to the concentration in each formula and allowed to hydrate overnight. Then, adjusting its pH by dropwise adding the aqueous solution of triethanolamine until a clear gel was formed. Finally, DMDM Hydantoin, glycerin and metronidazole-microsponge were added with continuous stirring until homogeneous (Sangeetha & Karki, 2015).

Table 1 Formulation of Mucoadhesive Gel

Composition (%b/b)	F1	F2	F3	F4
Microsponge (Metronidazole 0.75%)	1	1	1	1
Triethanolamine	1	1.5	1.75	2
DMDM Hydantoin	0.1	0.1	0.1	0.1
Glycerine	15	15	15	15
Carbomer	0.75	1	1.25	1.5
Aquadest	82.15	81.4	80.9	80.4

2.3 Determination of the pH

pH of Metronidazole microspongegel's was measured by using a pH-meter by inserting the electrode tip into the gel and after 2 minutes the results were recorded (Dineshmohan & Gupta, 2017).

2.4 Determination of the viscosity

The viscosity of Metronidazole microsponge gel was evaluated using a Brookfield Viscometer with 7-spindle operating at 50 rpm (Manna et al., 2016; Dineshmohan & Gupta, 2017).

2.5 Determination of spreadability

Spreadability was accessed by placing 1 gram of gel on a glass plate then covered with another glass plate, and then a pressure of 125-525 g was applied for 5 minutes. The diameter of the spreading circle is measured using a calliper (Dineshmohan & Gupta, 2017).

2.6 Determination of the mucoadhesive properties

These mucoadhesive properties of the prepared formulation were evaluated by using the rotating cylinder method with a slightly modified dissolution apparatus (Shaikh et al., 2011). The temperature is maintained at 37^{o}C, rotation speed was about 100 rpm. The bovine vaginal mucosa is clamped and attached to the paddle side. After that, the gel formulation was applied approximately 500 mg to the entire bovine vaginal mucosa and immersed in an artificial vaginal fluid (pH 4.2). The retention time was determined by the length of time the gel formulation takes to be released from the bovine vaginal mucosa.

2.7 Permeation study

In vitro drug release study was investigated using the modified Franz diffusion cell with the bovine vaginal mucosa. The prepared bovine vaginal mucosa was placed between the donor and receptor compartment. The receptor compartment was filled with a capacity of 28 ml of artificial vaginal fluid at pH 4.2 operating at 100 rpm on a magnetic stirrer. The temperature was maintained at 37 ± 1^{o}C. About 1 mL sample of each formula was spread on the donor compartment. At defined time intervals of 15, 30, 45, 60, 120, 180, 240, 300, 360, 420, and 480 min 1.5 mL of sample was withdrawn and replaced with an equal volume of receptor medium. Aliquots were diluted and analyzed using UV-spectrophotometer at 320 nm (Machado et al., 2015). In this study, a control is needed to evaluate and compare the permeation ability of the mucoadhesive gel formulations. A gel containing pure metronidazole was used as a control.

2.8 Vaginal retention study

Vaginal retention study was performed to determine the content of the drug on the skin. After the in vitro drug release study, the bovine vaginal mucosa was washed with 30 mL artificial vaginal fluid (pH 4.2) using a homogenizer at 1000 rpm for 15 minutes. Then centrifugation for 30 minutes at a speed of 5000 rpm and analyzed using UV-spectrophotometer at 320 nm.

2.9 Preparation of simulated vaginal fluid

A total of 5 grams of glucose, 3.51 grams of NaCl, 2 grams of lactic acid, 1.4 grams of KOH, 1 gram of acetic acid, 0.4 grams of urea, 0.222 grams of Ca $(OH)_2$, 0.018 grams of serum albumin, and 0.016 grams of glycerin, weighed then dissolved in 1 L of deionized water and adjust pH 4.2 by the addition of 0.1 N HCl (Sanz et al., 2018)

2.10 Statistical analysis

All data were collected and analyzed using a statistical approach with the One Way ANOVA method (Andrade et al., 2014).

3 Results and Discussion

3.1 Preparation of mucoadhesive gel

In this study, a microsponge mucoadhesive gel was formulated to control the release of metronidazole locally and increase the residence time of the preparation in the vaginal mucosa. Carbomer was used as a gelling agent and mucoadhesive material. The concentration of carbomer was a range between 0.5% - 2%. Carbomer requires a neutralizing agent to form a transparent gel. The addition of triethanolamine (TEA) could neutralize the carbomer. An excessive neutralization (optimal pH 5-10) can reduce the viscosity of the formed gel (Rowe et al., 2009). The concentration of TEA that neutralizes carbomer is 1.5: 1. In this research, all formulas were developed with various concentrations of TEA and Carbomer to gain suitable properties of the mucoadhesive gel. The gel formula was made using a homogenizer with a concentration ratio of carbomer and triethanolamine. The ratio for each formula was F1 (0.75 : 1) ; F2 (1 : 1.5) ; F3 (1.25 : 1.75) ; and F4 (1.5 : 2). Physical appearances of all gel formulations were visually inspected figure 1 showed that all formulated gels were white in colour and odourless.

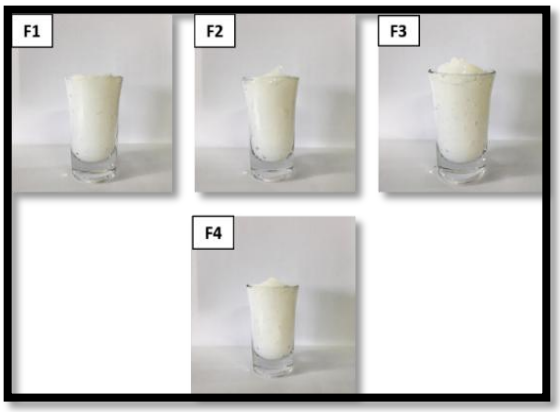

Figure 1 Mucoadhesive gel of all formulations
(a) F1, (b) F2, (c) F3, (d) F4

3.2 Determination of the pH

The pH of all developed formulations was measured by using a pH meter. The normal vaginal pH was ranged between 3.5-4.5, however, the vaginal pH will increase with the occurrence of bacterial vaginosis. The vaginal pH with VB reached 6.5-8 (Borges et al., 2016). Figure 2 showed that the pH of the various formulation of microsponge gel viz., F1, F2, F3, F4 was 6.87 ± 0.02; 6.99 ± 0.02; 7.03 ± 0.02; 7.09 ± 0.02, respectively. According to the statistical analysis results, the ratio of the concentrations between carbomer and triethanolamine for F2 and F3 did not significantly affect the pH ($p < 0.05$). It can be concluded that the pH of all formulas was still within the range of vaginal pH affected by BV. Assessment of pH has a very important role because if the pH is too acidic (<4), it causes the growth of *Lactobacillus vaginalis* bacteria and vaginal discharge, whereas a pH that is too alkaline (>8), it can causes the growth of *cocci* bacteria that causes Vaginosis. So that it becomes very important to keep the pH following VB conditions.

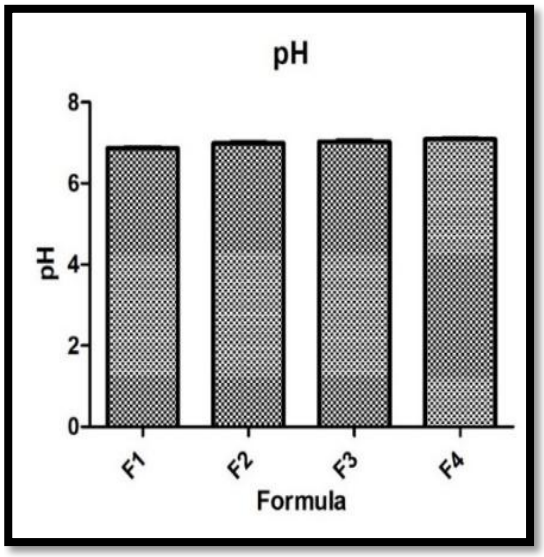

Figure 2 pH of all gel formulations

3.3 Determination of spreadability

The spreadability test is one of the important characteristics of topical formulations. Measurement of the spreadability was carried out using callipers to determine the ability of the preparation to spread when applied to the vagina. The gel formula for the vagina should have good spreadability and ease of application. Figure 3 showed the spreadability of microsponge gel range between 3.22-3.98cm/s. There was a slight decrease in spreading diameters. This variation might be due to variation in polymer concentration in each gel formula.

Viscosity affects the width of the spreading area, where the smaller the viscosity value, the smaller the resistance of the gel preparation to spread, resulting in a large spreadability value. Conversely, if the viscosity value is greater, the resistance of the gel preparation to spread is also greater, thus reducing the spreadability value make the gel thicker. Based on the previous study of Garg et al. (2002), the diameter of semi-solid preparations that are suitable for topical use is in the 3-5 cm range or it can be stated that the area of spreadability is between 7.605-19.625 cm^2. Based on statistical results, all the preparations were significantly different ($p < 0.05$). According to the research of Kalita et al. (2017), the spreadability of microspheres gel of metronidazole preparations was ranging from 3.5-5.5 g.cm/sec. From the data presented in table 1, it can be concluded that the spreadability value for F3 and F4 were in the appropriate range.

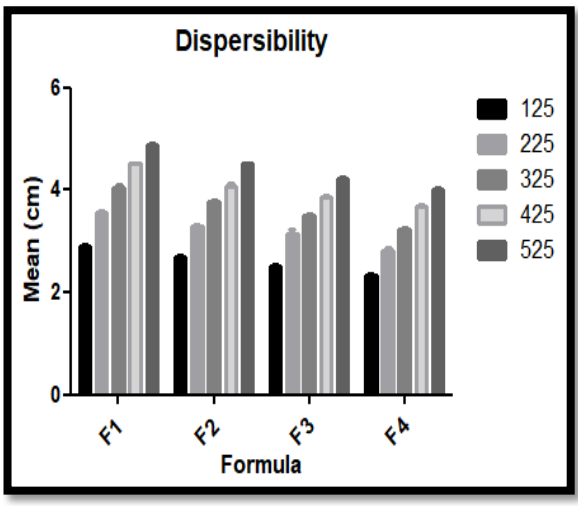

Figure 3 Spreadability of all gel formulations

3.4 Determination of viscosity

Viscosity measurement was carried out by immersing the spindle into the gel preparation. The viscosity value of microsponge gel for F1, F2, F3 and F4 was reported 25800 ± 1053.57 cPs; 32766.67 ± 650.64 cPs; 38133.33 ± 1514.38 cPs; and 47666.67 ± 2722.74 cPs, respectively (Figure 4).

The data shows that there is a significant increase in viscosity of all four formulas ($p < 0.05$). Based on the literature, the viscosity value for gels preparation using carbomeric polymers must be in the range of 10000-60000 cPs (Rowe et al., 2009). By increasing the concentration of the polymer used, the viscosity of the preparation will also increase. According to Dineshmohan & Gupta (2017), the viscosity value of microsponge gel obtained was in the range of 34,480-91,350 cPs. Therefore, the viscosity values of F3 and F4 were in the appropriate ranges.

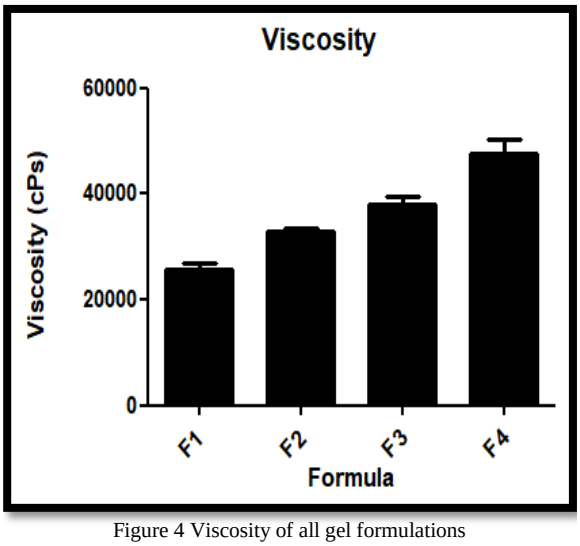

Figure 4 Viscosity of all gel formulations

3.5 Determination of mucoadhesive properties

Measurement of the strength of the mucoadhesive gel was carried out using the Rotating Cylinder Method. The microsponge gel had a mucoadhesive time of F1 2.5 ± 0.1 hours, F2 4.3 ± 0.2 hours, F3 6.4 ± 0.2 hours, and F4 6.4 ± 0.1 hours. Gel preparations for the vagina must have good bioadhesive strength because the strength of the bioadhesive affects the contact time of the preparation with the mucosa in the vagina. The high bioadhesive strength will increase the contact time of the preparation in the vagina, the use of carbomeric polymers can increase the contact time of the preparation with the mucosa, from 1-2 hours to 3-4 days, prolonged contact with mucosa accompanied by slow release of the drug at the target location. Based on the statistical results, it shows that there is a significant effect (p> 0.05) between the concentration of the carbomer polymer on the viscosity so that it can affect the bioadhesive strength of the preparation. According to Sanz et al. (2018), 6 hours of the trial is considered sufficient to assess the drug can be released on the mucosa. Thus, the F3 and F4 preparations have the highest bioadhesive strength and it can be said that this formula is suitable for bioadhesive gel preparations. A preparation that can provide a long contact time at the site of administration is needed so that drug release can be controlled locally (Shaikh et al., 2011**)**.

3.6 Permeation study

The determination of the metronidazole content was carried out using spectrophotometric analysis. In spectrophotometric analysis, it was found that the wavelength of metronidazole was 320 nm. The amount of permeated metronidazole was shown in figure 6. For F1, F2, F3, and F4 the value was reported 0.51 ± 0.02mg; 0.42 ± 0.01mg; 0.28 ± 0.01mg; and 0.16 ± 0.02mg ($p < 0.05$), respectively. The amount of drug permeated for F1 to F4 tends to decrease due to the viscosity value and the properties of the mucoadhesive gel. The greater viscosity represented the smaller the amount of drug released from the gel preparation (Shaikh et al., 2011). A gel containing pure metronidazole was used as a control in this study. From the obtained data, the number of permeated from the control formula was greater than the number of permeated from the four formulas, namely 1.14 ± 0.02mg. This is because metronidazole in the control formula does not have a retention time so that it can be released immediately. One of the aims of the microsponge gel formulation is to release the drug in a controlled release for local delivery, if the amount of permeated is high, the drug can directly penetrate or pass through the membrane (Patel et al., 2016). From the given figure 6, it was observed that microsponge gels of F1, F2, F3, and F4 show a small permeation value compared to the control, therefore the level of metronidazole released can be controlled and the desired local effect can be achieved.

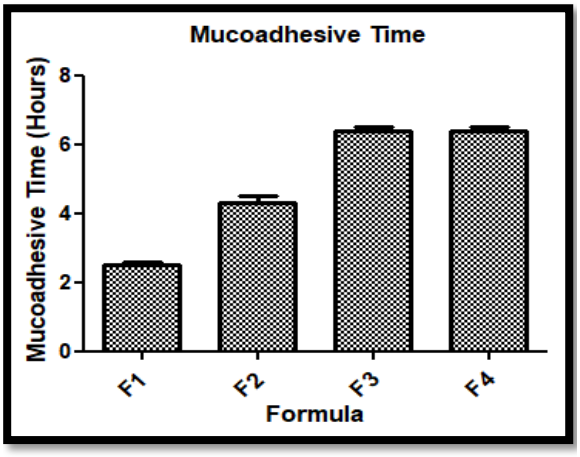

Figure 5 Mucoadhesive time of all gel formulations

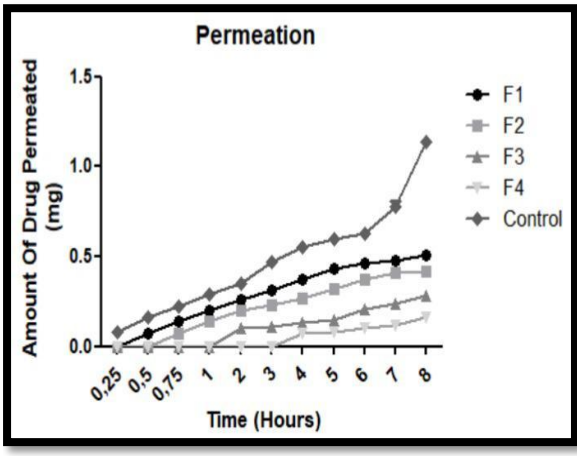

Figure 6 Permeation Study

3.7 Vaginal retention study

The permeation data does not fully support the desired local effect because the permeation test is carried out to determine the amount of drug that can pass through or penetrate the membrane. Meanwhile, to ensure that the preparation can work locally, the amount of metronidazole that can be left on the membrane must be known, therefore it is necessary to carry out a retention test for the preparation. In addition, the retention test aimed to determine the ability to control drug release from the dosage form.

Figure 7 shows the percentage of metronidazole deposited after 8 hours. One of the importance of the microsponge gel system is that it can be used to increase the residence time of the drug in the vagina. Based on the figure 7, the amount of metronidazole that was deposited after 8 hours was mostly at F3 (1.95 ± 0.04mg), compared to the amount of metronidazole at F1 (0.51 ± 0.02mg); F2 (0.73 ± 0.03mg) and F4 (0.08 ± 0.01mg), the obtained data was significantly different ($p < 0.05$). This shows that F3 has the best retention time, so it can control the release of metronidazole locally in the vagina. For microsponge gel preparation at F4, the amount of deposited metronidazole was low, however, it also had a low amount of permeated metronidazole and was significantly different ($p < 0.05$). This suggests that the higher carbomer concentration can control the permeates well but does not have a high retention time in the vagina, so it tends to release the drug and reach to the systemic circulation without providing the desired local effect. This is might be due to an excessive concentration of carbomer used in the F4 which resulting in a very viscous gel preparation. In the above histogram, the control formula which consists of pure metronidazole, shows that the amount of metronidazole deposited is less than the metronidazole microsponge gel formula, so it is known that the control formula has a greater amount to release immediately to reach the systemic circulation compared to the amount left in the membrane vagina. This is consistent with previous research (Patel et al., 2016) which states that when the release of metronidazole is not controlled, it can directly reach the systemic circulation.

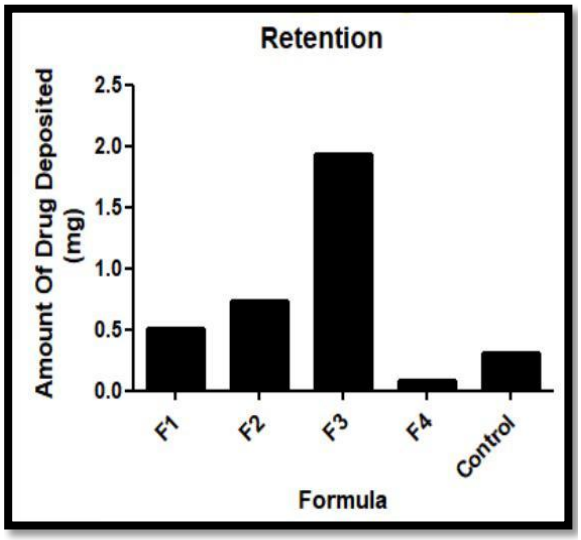

Figure 7 Retention profile of all gel formulations and control

Conclusion

The prepared gel of all formulas was white, odourless, and homogenous. The ratio of carbomer concentration showed a significant effect on pH and permeation study but not significant on the dispersion, viscosity, mucoadhesive time and retention study. Furthermore, the results showed that F3 is the best formula which has a low amount of permeation and a high amount of retention.

Conflict of Interest

The authors declare no conflict of interest.

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STUDY OF ACUTE AND SUB-ACUTE TOXICITY OF *Boehmeria virgata* (FORST) GUILL LEAF EXTRACT IN WISTAR RATS

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KEYWORDS

Boehmeria virgate

Leaf extract

Acute toxicity

Sub-acute toxicity

Hematology parameters

Biochemical parameters

Histopathological parameters

ABSTRACT

This study was carried out to evaluate the acute and sub-acute toxicity of the standardized *Boehmeria virgata* leaf extract (BLVE) in Wistar rats. *B. virgata* is a traditional herb utilized by the people of Makassar, Indonesia to cure inflammation and cancer. In the current study, thirty (30) Wistar rats were divided into 6 groups (3 groups of males and 3 groups of females) for acute treatments. Similarly, for investigating sub-acute treatment forty (40) Wistar rats were split into 8 groups (4 groups of males and 4 groups of females). For acute toxicity treatment, selected rats received 2000 mg and 5000 mg/kg of BVLE by oral administration for 14 days while for the estimation of sub-acute toxicity, experimental rats were treated with 250, 500, and 1000 mg/kg BVLE for 28 days. After 14 days of treatment, the rats were monitored for any form of changes in behavior, weight, food, water intake, and histopathology. The treated animals underwent hematological, biochemical, histopathological, and organ weight analysis after 28 days. Results of the study revealed no significant differences in body and organ weight, intake of food, and water after acute BVLE treatment in rats compared with those in the control group. The histopathological study suggested a general hydropic degeneration of the liver after acute treatment with 5000 mg/kg; such degeneration did not occur in the kidneys and kidney glomerulus of BVLE

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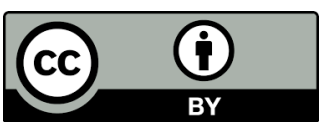

treated rats. Further, no significant toxicity was shown in hematological, biochemical, organ weight, and histopathological data in the sub-acute BVLE group after comparing treated rats with the control group. Results of the study can be concluded that BVLE is not toxic at doses of up to 1000 mg/kg. Therefore *B. virgate* fulfilled a preclinical criterion that is necessary for its further establishment as a clinically useful extract.

1 Introduction

Boehmeria virgata (Forst) Guill belongs to the family Urticaceae and has been utilized by the people of Makassar, Indonesia as a traditional medicine to cure inflammation and cancer (Manggau et al., 2011). Ethanolic extract of *B. virgata* along with *Eupatorium odoratum*, *Acalypha indica*, and *Acanthus ilicifolius* are traditionally used by the Makassar ethnic group as anticancer and anti-inflammation agents and have been well-studied for their activity against HeLa cervical cancer cells (Manggau et al., 2013). In addition, Manggau et al. (2018) reported the anti-proliferative activity of BV103 compound on HeLa cell lines and suggested that this might be due to the inhibition of p53 and Caspase-3. Similarly, Wardihan et al. (2013) also suggested the selective cytotoxicity of BVLE on some cancer cell lines, namely HeLa, T47D, WiDr, Vero cells with IC_{50} of 8.991±0.234, 12.732±0.945, 18.925±1.277, and 16.022±0.663 µg/ml respectively. Moreover, the selectivity index of the extract against HeLa, T47D, WiDr, Vero cells were reported 0.844, 1.258, 0.847, and 1.000 respectively. The BVI03 compound (10-6,6-dihydroxy-hexyl-2,3,6-trimethoxy-phenanthrene-9-carboxylic acid amide) of the *B. virgata* leaf extract (BVLE) is an alkaloid compound. Other studies also showed that *B. virgata* compound in the forms of BVI03, NBB (nano-encapsulated BVI03), and NBVG (nano-encapsulated vaginal bioadhesive gels) had anti-proliferation activities and the IC_{50} value of these forms was reported 2.88, 59.26, and 725.46 µg/ml respectively (Lukman et al., 2014). Without extensive studies, there is limited toxicological information available regarding the safety of BLVE owing to its vulnerability. Indonesian authorities are currently concerned with the safety and toxicity of medical and consumable plants (BPOM, 2014).

Presently, no research work confirms whether BLVE creates any toxicological reaction or not. Hence, this study aimed to perform an astute toxicological study with sub-sections to establish a preclinical benchmark necessary for additional establishment by evaluating biochemical, hematological, and histopathological analysis of Wistar rats treated with BVLE. Further, the effect of BVLE on the changes in behavior, skin, body, and organ weight, daily water, and food consumption levels were also taken into consideration.

2 Materials and Methods

2.1 Extraction process of the plants

B. virgata is a native plant to Malino Town, Gowa City, South Sulawesi Province, Indonesia. Plant leaves samples were collected and a specimen of the plant was identified and deposit at the Indonesian Institute of Sciences (LIPI), Jakarta, Indonesia. After being collected, the leaves were dried and chopped into small pieces. For the ethanol extract preparation, about 150g of leaf were macerated for 4 days using 70% ethanol as a solvent.

2.2 Characterization of the active isolate as a marker

The BVI03 was isolated from BVLE as per the method given by Manggau et al (2013). The active isolate of BVLE (BVI03) as a marker was characterized by various physicochemical parameters including UV of wavelength (254 and 366 nm), and IR spectra.

2.3 Animals

Both genders of adult Wistar rats with an average weight of 150 ± 10 g was used as experimental animals in the current study. These were purchased from Central Rodent Cultivation Centre, Airlangga University, Surabaya. The test animals were randomly divided into two parts, first part used for the acute toxic study has 6 groups (3 males and 3 females) while the second part has 8 groups (4 males and 4 females) for sub-acute toxicity tests, each group has five Wistar rats. Polypropylene cages were used to house the rats with the range of temperature and relative humidity of 24 - 26°C and 45–55% respectively. Water and food pellets having major food nutrients were given to the animals and the day-night time cycle was 12:12 h. They were also given a week to adapt to the new environment conditions before being used for the experiments. The experimental protocol of the animals was approved by the Indonesian committee and it was conducted according to guidelines on animal care from the National Institute of Health.

2.4 Experimental Design

For the acute toxicity tests BVLE was administered orally once a day to the rats using a gavage needle and they were evaluated daily for 14 days while for the sub-acute toxicity tests, the rats were given BVLE daily for 28 days; these rats were evaluated on the 14^{th} and 28^{th} day, by following the recommendations of the Indonesian National Agency of Drug and Food Control (Sparingga, 2014). Six groups for acute treatments (3 groups male and female Wistar rats and each group have five organisms) were treated with 2,000 and 5,000 mg/kg per day dose of BVLE. Meanwhile, eight groups undergoing sub-acute treatments (4 groups male and female Wistar rats and each group have five

organisms) were treated with 250, 500 , and 1,000 mg/kg of BVLE, respectively (Sparingga, 2014). The solvent was given to the control group for both acute and sub-acute treatments. Observations were carried out every day to ascertain the cause of death and behavioral changes. Weekly body weight analysis was also carried out and the various doses administered to the rats were adjusted weekly to enable the body weight control for the target dose level for all rats. Furthermore, there was a daily examination of the detailed preclinical analysis and measurement of the food and water consumed.

2.5 Hematology analysis

On the last day of the sub-acute study, chloroform was used to anesthetize the animals. Plastic test tubes containing EDTA (an anticoagulant) were used to collect the samples. Hematology analyses were carried out using a Hematology Analyzer (Sysmex XS-8000i®, Med Wrench US) with the flow cytometric method. This was used to determine the mean corpuscular hemoglobin (MCH), erythrocyte count (RBC), white blood cells (WBC), the concentration of MCH (MCHC), distribution width of red blood cells (RDW), the standard deviation of RDW (RDW-SD), coefficient of variation of RDW (RDW-CV), mean corpuscular volume (MCV), hemoglobin, hematocrit, neutrophil, lymphocyte, eosinophil, monocytes, and platelet counts.

2.6 Biochemical Analysis

Following the sub-acute toxicity analysis, chloroform was used to anesthetize all the animals used in the study. They subsequently bled through an inferior vena cava. After this, all specimens were gathered in sample tubes, they were placed in a standing position for outright clotting. A total of 15 minutes was used to centrifuge the clotted blood clot at 3000rpm. After that, the serum specimen was kept frozen at -80^{o} Celsius. Samples of serum were also used to analyze and determine the total creatinine, serum, Alanine-Aminotransferase (ALT), and Aspartate-Aminotransferase (AST) concentration using a Humalyzer 3500® (Human Germany).

2.7 Histopathological Observations

Kidney cells, liver cells, and kidney glomerulus cells were tested with the aid of a microscope after that each one of them had undergone eosin and hematoxylin staining. Dissection of the liver, kidney glomerulus, and kidney was performed to test for signs of toxicity. Any inherent irregularities found on the slides were tagged “mild”, “attention” and “definite” in a manner described by Jain et al (2008) and Chi et al. (2014).

2.8 Statistical analysis

Kruskal–Wallis one-way and two-way analysis of variance (ANOVA) was applied to subjugate data in the form of Mean ± SEM (Standard Error of Mean). A Mann–Whitney-U-test (two-tailed) was applied to make inter-group comparisons for responses that produced huge treatment effects in the ANOVA test with $p<0.05$.

3 Results

3.1 Characterization of the active isolate as a marker

Characterizations of ethanolic extract were performed with an ultraviolet spectrophotometer by using dichloromethane. The solvent displayed maximum absorption for BVI03 isolate at wavelengths 262, 287, and 369 nm. These wavelengths enable us to detect the presence of conjugated dienes. In addition, the UV spectrum also showed maximum absorption at a wavelength of 285 nm. The absorption peak at wavelength 285 nm indicates a transition to the state π^* at the solitary n electrons in N atoms occurring at wavelengths greater than 270 nm (Figure 1).

The infra-red spectrum data of BVI03 isolates at a wavelength of 3372 cm^{-1} indicates the presence of OH bound and an amine group. The medium absorption at a wavelength of 927 cm^{-1} and strong absorption at a wavelength of 1556 cm^{-1} indicates the possibility of primary amine. Moreover, the absorption at a wavenumber of 2926 cm^{-1} indicates the presence of an aliphatic CH group. The presence of a band that absorbs strongly and sharply at a wavelength of 1413 cm^{-1} indicates the presence of an aromatic group. Strong absorption at 647 cm^{-1}, 620 cm$^{-1,}$ and weak for 1129 cm^{-1} indicates the presence of alkenes (Figure 2).

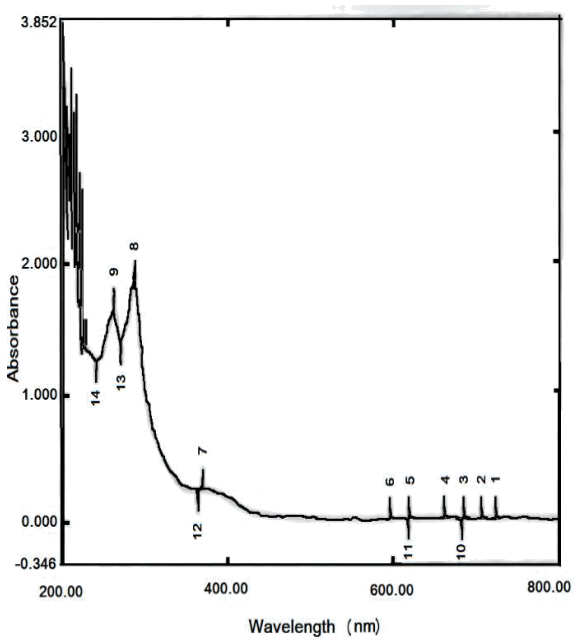

Figure 1 UV spectra data of BVI03 isolate as a marker of BVLE

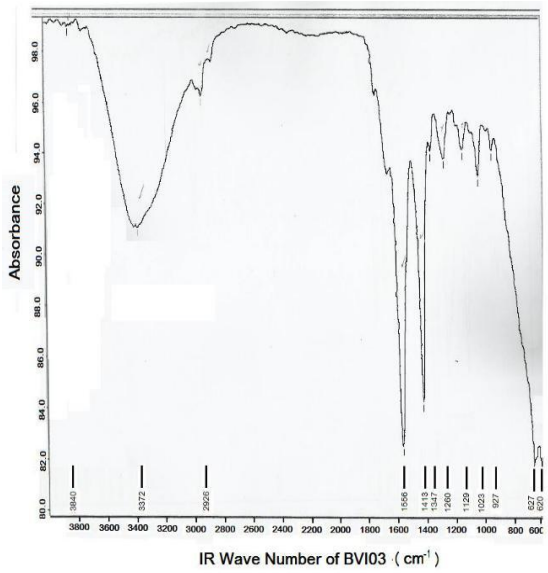

Figure 2 IR spectra data of BVI03 isolate as a marker of BVLE

3.2 Acute toxicity study

3.2.1 Animal observation

The oral administration of 2000 and 5000 mg/kg of BVLE did not produce any changes in skin color, behavior, hair loss, defecation, postural abnormalities, breathing, urination, interruption in water or food intake. When assessing their sense of touch and hearing, it was found that the rats were highly sensitive with a lower activity level. Furthermore, no death was observed in any group of BVLE treatment.

3.2.2 Bodyweight measurement

The average weight of the rats was measured for 14 successive days. The control group displayed an increase in body weight as compared to the BVLE administered group as displayed in Table 1. Minimum (not significant) changes in body weight were observed in rats which have been administered by 2000 mg/kg of BVLE.

Table 1 Body weights of rats following acute treatment with BVLE. All treatments are at par and are not significantly different

	Body Weight (g)		
	Day 0	Day 7	Day 14
Female			
Control	170 ± 2.57	171 ± 1.63	171 ± 2.74
2000 mg/kg	171 ± 1.50	173 ± 0.76	174 ± 0.47
5000 mg/kg	169 ± 4.47	170 ± 2.73	170 ± 2.18
Male			
Control	182 ± 1.43	184 ± 0.56	186 ± 4.65
2000 mg/kg	183 ± 4.65	186 ± 3.07	1.85 ± 3.61
5000 mg/kg	182 ± 4.18	184 ± 3.46	185 ± 2.54

Data indicate mean ± SEM, *n* = 5; there was no statistically significant deviation in these data compared with those in the control group, p>0.05

3.2.3 Intake of food and water

Calculation of the quantity of water and food intake was measured daily; it can be defined as the difference between the initial quantity, and the total quantity left sub-sequential to 24 hours. The obtained calculated values were depended on the total quantity of food consumed and the average physical critical weight. The obtained data indicated no statistically meaningful difference (CMC; p > 0.05). The results showed that the extract didn't affect the body weight (Table 2).

Table 2 Food intake of rats in acute toxicity treatment of BVLE

		Food Intake (grams)		
	Sex	Day 0	Day 7	Day 14
Control	Male	57.56 ± 2.27	69.38 ± 0.53	67.42 ± 0.09
	Female	45.65 ± 2.16	53.26 ± 1.26	56.37 ± 3.73
2000 mg/kg	Male	58.73 ± 1.83	64.27 ± 0.64	73.65±1.67
	Female	51.15 ± 3.34	59.43 ± 2.74	63.24 ± 1.87
5000 mg/kg	Male	62.54 ± 2.35	70.36 ± 0.29	77.54 ± 0.04
	Female	46.23 ± 2.32	54.12 ± 1.89	64.35 ± 2.67

Data indicate mean ± SEM, n = 5; There was no statistically significant deviation in these data compared with those in the control group, p>0.05

3.2.4 Organ weight

On the 14th day of treatment with BVLE, the livers and kidneys were weighed on an electronic balance and relative organ weights of the treated groups were compared to the negative control. Using Kruskal–Wallis analysis, the nonsignificant difference (p> 0.05) was reported in BVLE treated females when compared with the untreated control (Table 3). Increasing the dose of BVLE affected the degree of hydropic degeneration in the female liver and renal cells. For the liver cells of the 2000 mg/kg treatment group, the average percentage of hydropic degeneration was 39.93%, which is categorized as light damage (0-49%) while 5000 mg/kg treatment (52.39%) is categorized as moderate (50%-74%), while hydropic degeneration in the male liver and renal cells didn't occur after treatment with BVLE. Table 4 showed non-significant enlargement of mice renal glomerular after BVLE treatment dose of 2000 and 5000 mg/kg.

3.2.5 Histology of liver, renal and renal glomerular after treatment of BVLE

Histological tests of the organs indicated that acute BVLE treatment by an oral administration of up to 2000 mg/kg yields mild hydropic degeneration. At a dose of 5000 mg/kg, moderate hydropic degeneration appeared as compared with the control group (Figure 3 and 4, Table 3). Hydropic degeneration is characterized by water-containing vacuoles in the cytoplasm, cloudy, pale, and the nucleus

is pushed to the edge. Increasing the dose of BVLE affected the degree of hydropic degeneration in the female liver and renal cells. For renal cells in the 2000 mg/kg treatment group, the average percent of hydropic degeneration was 39.93%, characterized by water containing vacuoles in the cytoplasm, so that the renal cells looked paler than the normal renal cells, which is categorized as light damage (hydropic degeneration <50%). Whereas, the treatment of 5000 mg/kg (52.39%) BVLE showed water-containing vacuoles in the cytoplasm, cloudy, pale and the nucleus is pushed to the edge, which is categorized as moderate (hydropic degeneration <75%). In contrast, the hydropic degeneration in male renal cells didn't occur after treatment with BVLE.

3.3 Sub-acute toxicity study

3.3.1 Body weights and food intake

The physical weights and food intake of the rats were not altered by the sub-acute doses of BVLE administration. As shown in table 5 and 6, no significant changes were reported in both parameters i.e. body weights and food intake of both genders of Wistar rats before and after treatment with BVLE even after the 28th day of treatment. The obtained data indicated no statistically meaningful difference, $p > 0.05$. The results showed that the extract didn't affect the body weight and food intake.

Table 3 Histology and weight of female and male Wistar rat liver and renal cells after the acute treatment of BVLE

Treatment	Liver			Renal		
	Weight	Hydropic Degeneration (%)	Degree	Weight	Hydropic Degeneration (%)	Degree
Control (female)	3.51 ± 0.03	20.72 ± 3.54	-	0.61 ± 0.01	19.65 ± 0.50	-
2000 mg/kg	3.65 ± 0.02	39.97 ± 5.89	+	0.69 ± 0.002	34.02 ± 3.94	+
5000 mg/kg	3.84 ± 0.02	52.39 ± 8.28	++	0.73 ± 0.002	47.13 ± 10.90	++
Control (male)	3.56 ± 0.01	21.64 ± 4.40	-	0.63 ± 0.003	20.86 ± 3.34	-
2000 mg/kg	3.75 ± 0.02	22.97 ± 3.89	-	0.65 ± 0.006	21.02 ± 3.54	-
5000 mg/kg	3.79 ± 0.05	29.39 ± 6.29	+	0.78 ± 0.007	28.15 ± 10.90	+

Values are expressed as mean ± SEM; n = 5; significantly different from control group; p>0.05; normal (0-25%); + - mild (25-49%); ++ - moderate (50-74%); +++ - severe (75-100%) as per Ghufron (2011)

Table 4 Histology of renal glomerular after single-dose treatment of BVLE and CMC as control

No.	Treatment	Enlargement of renal glomerular (%)	Degree
1	Control	22.8667 ± 3.33933	-
2	2000 mg/kg	22.3867 ± 6.20238	-
3	5000 mg/kg	28.2133 ± 4.68740	+

Data indicates mean ± SEM; normal (0-25%); + - mild (25-49%); ++ - moderate (50-74%); +++ - severe (75-100%) as per Ghufron (2011)

Table 5 Body weights of rats following sub-acute treatment with BVLE

Treatment	Bodyweight (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Female					
Control	162 ± 4.47	165 ± 3.54	167 ± 2.74	167 ± 2.86	171 ± 2.23
250 mg/kg	162 ± 3.56	167 ± 2.73	162 ± 4.47	167 ± 2.73	172 ± 2.74
500 mg/kg	162 ± 4.47	167 ± 3.47	166 ± 4.18	168 ± 2.74	171 ± 2.24
1000 mg/kg	163 ± 4.47	168 ± 2.73	166 ± 4.18	167 ± 2.74	172 ± 2.74
Male					
Control	186 ± 3.53	188 ± 4.56	192 ± 3.71	197 ± 2.74	198 ± 4.16
250 mg/kg	187 ± 4.37	190 ± 3.07	194 ± 4.61	197 ± 5.58	202 ± 5.70
500 mg/kg	186 ± 4.18	189 ± 6.52	195 ± 3.54	193 ± 5.71	199 ± 5.41
1000 mg/kg	190 ± 5.09	193 ± 4.70	195 ± 4.18	193 ± 2.24	200 ± 4.18

Data indicate mean ± SEM; n = 5; There was no statistically significant deviation in these data compared with those in the control group, p>0.05

Table 6 Food intake for single rat following oral administration of the ethanol extract from BVLE

Treatment	Sex	Food Intake (g) Day 7	Day 14	Day 21	Day 28
Control	Male	64.12 ± 1.25	74.21±0.899	83.14±1.07	82.12 ± 1.74
	Female	49.41 ±1.13	62.71±1.18	62.03±1.17	67.85 ±0.73
250 mg/Kg	Male	64.71± 1.08	74.42±0.64	81.28±0.96	83.71 ± 0.68
	Female	49.14 ±1.03	63 ± 1.02	58.28 ± 0.68	68.42 ±0.57
500 mg/Kg	Male	64.71 ±1.01	73.42±1.23	80.36 ± 1.24	83.57 ± 0.78
	Female	49.14 ±1.01	60.01±2.34	60.15 ± 3.14	68.42 ± 0.52
1000mg/Kg	Male	65.85 ±1.10	74.85±1.42	80.24 ± 3.27	81.42± 1.42
	Female	49.57 ±0.64	60.34±3.24	60.17 ± 2.43	68.42 ± 0.36

Data indicate mean ± SEM; n = 5; There was no statistically significant deviation in these data compared with those in the control group, p>0.05

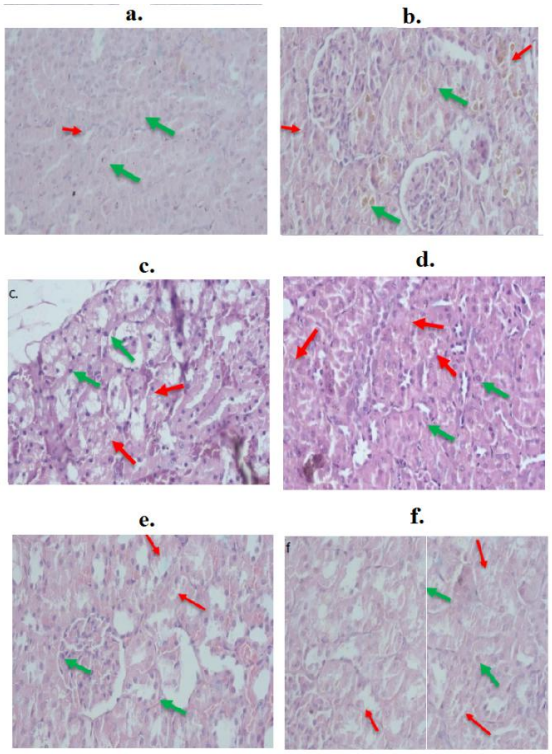

Figure 3 Renal histology of male and female in white rats control group (CMC) in males (a); and females (b); after a single (acute) dose of BVLE of 2000 mg/kg for males (c) and females (d); after administration of 5000 mg/kg for males in (e) and females in (f) (HE. 400x). Description: green arrow: normal cells, red arrow: hydropic degeneration

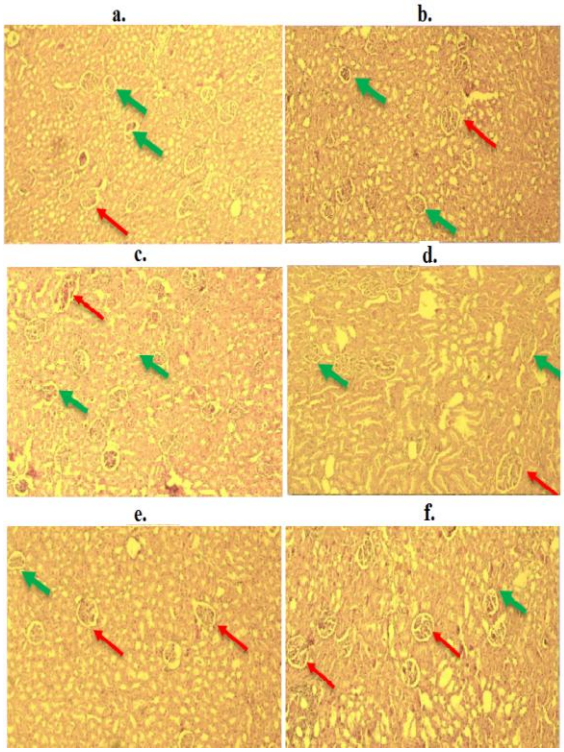

Figure 4 Renal glomerular histology of male and female white rat in control group (CMC) males (a) and females (b): after a single (acute) dose of BVLE of 2000 mg/kg for males (c) and females (d); after administration of 5000 mg/kg for males in (e) and females in (f) (HE. 400x). Description: green arrow: normal cells, red arrow: hydropic degeneration

3.3.2 Biochemical Analysis

After the administration of BVLE for the pre-treatment of AST (Figure 5), ALT (Figure 6), ureum (Figure 7), and creatinine levels (Figure 8) no significant changes were found after the 28th day of treatment compared to the control group.

3.3.3 Hematological Analysis

Results presented in tables 7-10 revealed that the hematological profiles of the control and BVLE subacute treated groups are not significantly different. This suggested that BVLE treatment didn't have any toxic effect on the hematological parameters.

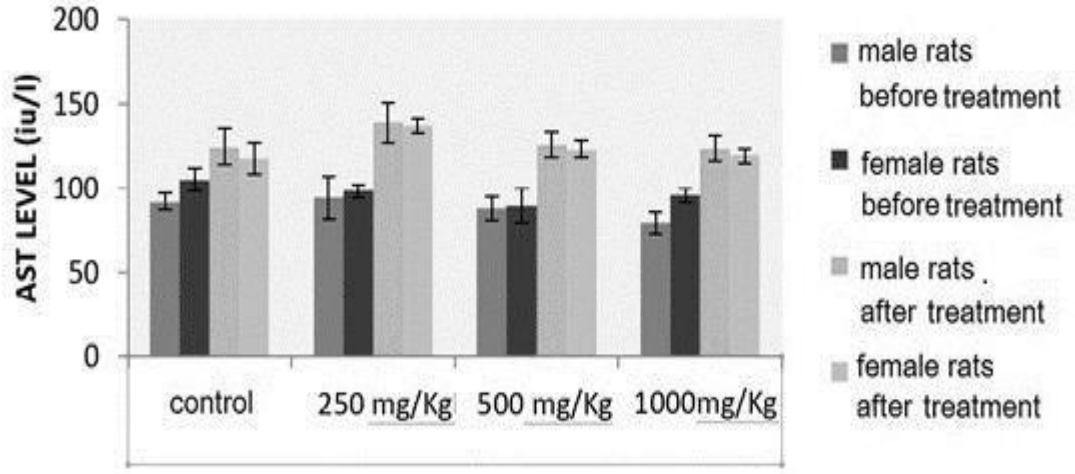

Figure 5 AST levels before and after sub-acute administration of BVLE for 28 days. Data: mean ± SEM; n = 5 (5 males, 5 females), p>0.05.

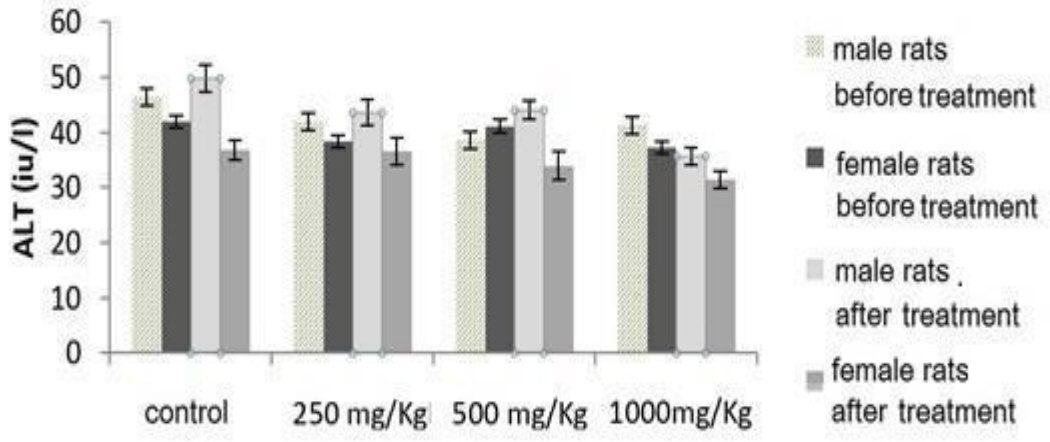

Figure 6 ALT levels before and after sub-acute administration of BVLE for 28 days. Data: mean ± SEM; n = 5 (5 males, 5 females), p>0.05.

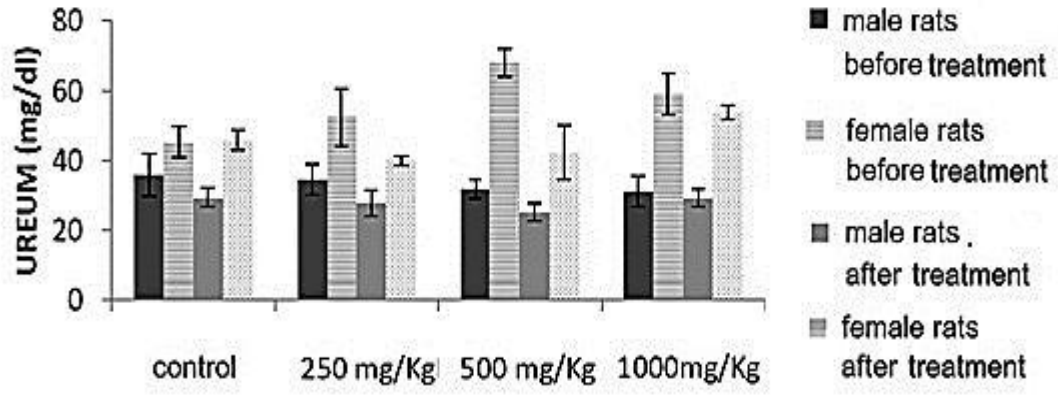

Figure 7 Ureum levels before and after sub-acute administration of BVLE for 28 days. Data: mean ± SEM; n = 5 (5 males, 5 females); p>0.05.

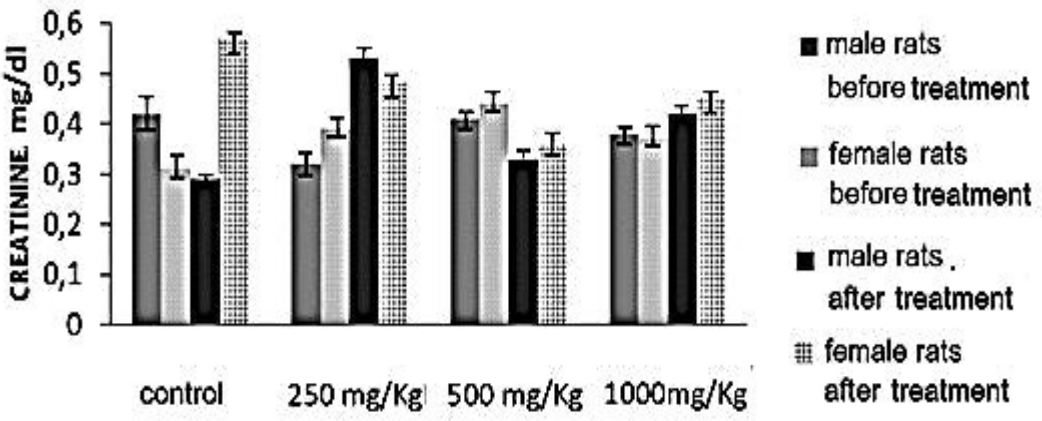

Figure 8 Creatinine levels before and after sub-acute administration of *B. virgata* leaves extract for 28 days. Data: mean ± SEM; n = 5 (5 males, 5 females), p>0.05

Table 7 Hematology analysis data of rat erythrocytes following treatment with BVLE

Hematology	Sex	Treatments			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Erythrocyte ($x10^6$/µl)	Male	8.56 ± 0.33	8.17 ± 0.29	7.13 ± 0.15	6.90 ± 0.99
	Female	6.70 ± 0.69	6.80 ± 0.47	7.60 ± 0.24	7.40 ± 0.24
MCV (fl)	Male	49.40 ± 0.77	48.43 ± 1.07	52.33 ± 1.02	53.26 ± 2.86
	Female	56.50 ± 2.91	56.80 ± 3.83	51.46 ± 0.66	54.16 ± 0.26
MCH (pg)	Male	17.60 ± 0.15	17.16 ± 0.26	18.00 ± 0.30	17.73 ± 0.17
	Female	18.80 ± 0.17	19.50 ± 0.90	18.30 ± 0.20	18.96 ± 0.08
MCHC (g/dl)	Male	35.63 ± 0.50	35.43 ± 0.26	34.46 ± 0.18	33.50 ± 1.70
	Female	33.40 ± 1.36	34.40 ± 0.79	34.73 ± 0.28	35.00 ± 0.11

Data indicated are mean of five replicates ± SEM; There was no statistically significant deviation in these data compared with those in the control group, p>0.05

Table 8 Hematology analysis data of rat leucocytes following treatment with BVLE

Hematology Profile	Sex	Treatments			
		Control	250 mg/kg	500 mg/kg	1000 mg/kg
Leucocyte (x 10^3/µl)	Male	22.88 ± 3.52	22.90 ± 1.26	23.90 ± 0.49	24.56 ± 4.14
	Female	22.76 ± 2.97	14.46 ± 2.30	16.79 ± 1.90	14.58 ± 1.79
Neutrophil (%)	Male	15.76 ± 1.07	29.33 ± 1.20	23.7 ± 2.07	22.1 ± 11.75
	Female	11.26 ± 0.98	18.66 ± 2.19	13.46 ± 1.47	17.33 ± 2.45
Lymphocyte (%)	Male	43.6 ± 2.99	55.73 ± 0.88	45.06 ± 2.74	37.03 ± 19.7
	Female	49.93 ± 2.00	61.93 ± 1.35	64.7 ± 2.00	51.53 ± 2.77
Monocyte (%)	Male	6.86 ± 0.55	5.76 ± 1.44	5.9 ± 0.95	7.83 ± 1.21
	Female	3.76 ± 1.93	5.96 ± 0.88	8.56 ± 0.811	6.7 ± 0.43
Eosinophil (%)	Male	4.2 ± 1.68	2.53 ± 0.96	5.3 ± 1.21	2.8 ± 0.81
	Female	3.36 ± 0.85	3.43 ± 0.58	6.26 ± 1.13	4.43 ± 0.17

Data indicated are mean of five replicates ± SEM; There was no statistically significant deviation in these data compared with those in the control group, p>0.05

Table 9 Hematology analysis data of rat thrombocytes following treatment with BVLE

Hematology Profile	Sex	Treatments			
		Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Thrombocyte (X 10^3 /µL)	Male	1122.6 ± 53.07	1099 ± 58.62	1129.6 ± 63.18	1229 ±71.27
	Female	805.33 ± 2.16	873.33 ± 2.81	1112.3 ± 58.32	1196 ± 1.08
MPV (fl)	Male	7.66 ± 0.12	7.23 ± 0.08	7.76 ± 0.03	7.7 ± 0.05
	Female	8.03 ± 0.47	7.76 ± 0.29	7.56 ± 0.17	7.5 ± 0.15
PDW (fl)	Male	7.93 ± 0.17	7.63 ± 0.03	8.31 ± 0.33	8.33 ± 0.27
	Female	8.9 ± 0.77	8.33 ± 0.32	8.06 ± 0.14	8 ± 0.25
P-LCR (%)	Male	7.66 ± 0.90	6.2 ± 0.80	9.03 ± 0.51	8.9 ± 0.24
	Female	11.3 ± 3.19	9.13 ± 1.70	8.23 ±1.47	7.36 ± 0.97

Data indicated are mean of five replicates ± SEM; There was no statistically significant deviation in these data compared with those in the control group, $p>0.05$

Table 10 Hematology analysis data of rat hemoglobin, hematocrit, RDW-SD, and RDW-CV following treatment with BVLE

Hematology Profile	Sex	Treatments			
		Control	250 mg/kg	500 mg/kg	1,000 mg/kg
Hemoglobin (g/dl)	Male	15.06 ± 0.57	14.01 ± 0.28	12.83 ± 0.14	12.26 ± 1.81
	Female	12.56 ± 1.18	13.23 ± 0.76	13.9 ± 0.32	13.53 ± 0.28
Hematocrit (%)	Male	42.26 ± 1.02	39.50 ± 0.62	37.23 ± 0.33	36.26 ± 3.70
	Female	37.46 ± 2.17	38.46 ± 1.94	40.03 ± 0.71	38.66 ± 0.71
RDW-SD (fl)	Male	29.56 ± 1.18	29.90 ± 0.66	36.5 ± 3.72	36.56 ± 4.76
	Female	36.40 ± 8.06	32.23 ± 2.77	30.33 ± 1.16	29.06 ± 0.38
RDW-CV (%)	Male	20.06 ± 0.80	20.20 ± 0.69	21.43 ± 1.31	21.33 ± 0.92
	Female	19.93 ± 3.14	17.80 ± 0.98	18.76 ± 0.29	17.13 ± 0.32

Data indicated are mean of five replicates ± SEM; There was no statistically significant deviation in these data compared with those in the control group, $p>0.05$

Discussion and Conclusion

The threat of cervical cancer is still a serious problem for women's health. Anti-cervical cancer drugs can cure cancer by killing cells. Unfortunately, these drugs also destroy normal cells, which might cause some uncomfortable side effects (Meegan & O'Boyle, 2019), and these side effects depend on the type of the drugs. The development of an effective chemotherapeutics agent with no toxic effects is urgently needed. Active ingredients obtained from plant sources can be an alternative to these anticancer drugs. Previous studies showed that the anti-cancer effect of the isolated active compound (BV103) in human cervical cancer, HeLa cells of BVLE was mediated by the activation of caspase 3 and p53 protein (Manggau et al., 2012). Recently the putative effect of BVLE alkaloid compound 10-(6,6dihydroxyhexyl)-2,3,6-trimetho-xyphenanthrene-9-carboxamide has been established as a potent anticancer agent (Manggau et al., 2018).

There are still no studies that establish the safe doses and possible toxicological reactions to BVLE which has been utilized as traditional medicine by the people of Makassar to cure inflammation and cancer. Therefore, the recent research was conducted using an *in vivo* model to study the toxicity of BVLE acutely and sub-acutely.

Intense administration of BVLE in rats @ 5,000 mg/kg doses led to neither mortality nor signs of toxicity. This suggested that an LD_{50} of BVLE per oral administration in rats is higher than 5,000 mg/kg. Since substances with LD_{50} larger than 5,000 mg/kg by oral administration are non-toxic (Kennedy et al., 1986), so the acute administration of BVLE is non-toxic. The treatment group of 2,000mg/kg showed some degree of hydropic degeneration, and it was reported 39.97% for liver cells and 34.02% for renal cells, that was categorized as "mild damage" (<50%) but such hydropic degeneration in hepatocyte cells was also reported in the control

group (Figure 1a). Further, the group treated with 5,000 mg/kg also showed the presence of hydropic degeneration and it was reported 52.39% for liver cells and 47.13% for renal cells that descriptively categorized as "moderate damage" (Figure 5c).

The process of hepatocyte damage starts from hydropic degeneration. Hydropic degeneration is mild and reversible damage. It can be a response to infection or exposure to toxicity. This toxicity causes a disruption in the mitochondrial organelle that produces ATP required for the gating of the cellular sodium via cell membrane (Na^+) pumps. If mitochondrial organelle does not produce ATP, there will be no osmotic potential increase in cells and water will be attracted into the cells. Sodium ions (Na^+) attract water, so water enters the cell. Vacuoles containing water are clear and small in the cytoplasm. These unite to form larger vacuoles that occupy the cytoplasm and cover cell nuclei and cause cell swelling.

Furthermore, in sub-acute treatment i.e. 250, 500 and 1,000 mg/kg doses of BVLE also didn't show any deaths or toxic symptoms even after 28 days of daily administration. Further, physical weight, as well as food intake, was unaltered during this period. The doses of BVLE are ascertained to be approximately 50 (acute) and 15 (sub-acute) times stronger than other species of *B. virgata*, such as *B.nivea* var. *Tenacissima* and *B. nivea* var. *nivea*, used as a hepatoprotective against liver injury triggered by carbon tetrachloride(CCl_4) (Chun-Ching et al., 1998). Other research has been illustrated that daily extract administration of *B. nivea* of 32g/kg does not lead to embryo or maternal toxicity in mice, even though it is likely to cause cytotoxicity in cultured ESCs when being administered in large doses. *B. nivea* is another species of *B. virgate* that is commonly given to cure organ failure medically (Tian et al., 2011).

Treatment with BVLE didn't show any observable changes in the biochemical parameters. The AST levels also did not differ from normally measured levels (Hall Robert, 1992; Qili et al., 2017). All standard hematological parameters used were within the normal reference range. Normal values of erythrocytes and leucocytes also did not differ from normally measured levels (Harkness et al., 2010). Compared to other studies platelet profile and platelet index levels for all sample groups are still in the normal range (Qili et al, 2017).

The study of effective chemotherapeutics with no toxic effects is urgently needed. This study showed no toxic effect of BVLE acutely and sub-acutely in the tested animals. Consistent with our previous study, the anti-cancer effect of isolated active compound (BVI03) in human cervical cancer HeLa cells of BVLE was mediated by activation of caspase 3 and p53 protein (Manggau et al, 2009; Gufron, 2011; Mohamed et al., 2011).

Further, no significant toxicity was reported in the biochemical, behavioral, histological, and hematological parameters of Wistar rats after the acute and sub-acute administration of BVLE. However, moderate hydropic degeneration can be seen in rat livers following acute administration of 5,000 mg/kg BLVE. Further studies are needed, namely the mechanism of hydropic degeneration, measurement of microsomal enzyme induction parameters, and studies of chronic treatment effects. Therefore, BVLE fulfills a preclinical criterion that is necessary for being a clinically useful extract for cervical cancer drugs.

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Conflict of Interest- Nil

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STABILITY OF THIOAMIDE TYPE OF PIPERINE UNDER ACIDIC AND BASIC CONDITIONS

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KEYWORDS

Thiopiperine

Acidic and basic conditions

Degradation

Chromatography

Cancer

ABSTRACT

Thioamide type of piperine, "thiopiperine" is a derivate of piperine that having high potency against breast cancer cells. This research was intended to evaluate the stability of thiopiperine under highly acidic and basic conditions for 72 h at 60ºC. This study was conducted by the SHIMADZU® UFLC system integrated with a PDA detector, while the analysis was performed in an isocratic separation mode using column C-18 (COSMOSIL®), 150 mm x 4.6 mm, column particle size: 5 µm. Chromatography condition was set using a mobile phase consisting of 50% aqueous acetonitrile with a flow rate of 1 mL/min, while the detection wavelength was 340 nm. The result showed that thiopiperine undergoes extensive degradation under acidic and basic environments.

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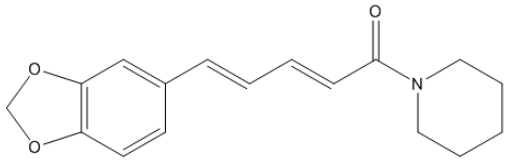
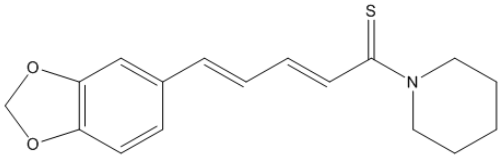
1 Introduction

Piperine, an alkaloid compound derived from pepper (*Piper nigrum* L.), has various pharmacological effects such as anti-inflammatory, anticancer, antiviral, anti-allergic, anti-epileptic, anti-depressant, and anti-Alzheimer's (Shinwari et al., 2019; Stojanović-Radić et al., 2019). Further, it also magnifies the bioavailability of several drugs such as midazolam, diclofenac, resveratrol, isoniazid, domperidone, simvastatin, verapamil, and beta-lactam antibiotic (Tiwari et al., 2020). Structurally piperine has an amide skeleton that can be modified to optimize its pharmacological activities or minimize its adverse effects. Derivatization of piperine has been reported with various analogs and it has various pharmacological effects such as anti-fungal (Souza et al., 2021), PPARγ agonists (Wang et al., 2020), MAO-B inhibitors (Chavarria et al., 2020), anti-neuroinflammatory (Shahbazi et al., 2020), larvicidal (Tantawy et al., 2020), and antitumor (Rifai et al., 2016; Ferreira et al., 2020).

Due to the high medicinal potential of piperine, recently, a derivative compound thioamide-type of piperine (thiopiperine) had been synthesized by modifying the structure of piperine from black pepper. The compound was modified by converting the carbonyl group to a sulfur analog (thiocarbonyl) using *lawesson's* reagent (Figure 1). Aswad (2019) suggested that this compound shows activity against 4T1 breast cancer cells, therefore, this compound can be potentially used in the development of cancer treatment drugs.



a



b

Figure 1 Structure of piperin (a) and Thioamide-type of piperine (b)

In the manufacturing of any drug, data about its stability as candidate active pharmaceutical ingredients must be considered, because it will affect various qualities such as safety and efficacy before it becomes a drug product. According to ICH guidelines, the quality of drug substances and drug products changes with storage time and is influenced by several stress conditions such as acidic, basic, peroxide, temperature, and lights. Furthermore, the determination of stability has only focused on drug products, while data on the stability of the active pharmaceutical ingredients (API) is still less (Blessy et al., 2014). Therefore, this research aimed to determine the stability of thiopiperine as a candidate of API under highly acidic and basic environments.

2 Materials and Methods

2.1 Materials

The materials used in this study are black pepper, piperine (Wako, Japan), acetonitrile (Merck), water pro-HPLC (Merck), Lawesson's Reagent (Wako, Japan), Tetrahydrofuran (THF) (Merck), Dichloromethane (DCM) (Merck), NaOH (Sigma-Aldrich), HCl (Merck). All of the chemicals were used without further purification.

2.2 Synthesis of thiopiperine

Thiopiperine was prepared by the semi-synthesis method from black pepper (*Piper nigrum*) as suggested by Aswad (2019). Ground black pepper (10 g) was refluxed in DCM for 30 minutes followed by evaporation of the solvent *in vacuo*. Cold ether was applied to obtain crude piperine as pale brown solid (126 mg). Subsequently, crude piperine, Lawesson's reagent (162 mg), and 2 mL THF were added into the round bottom flask. The mixture was stirred at room temperature for 24 h. After evaporation of the solvent, the product was purified by flash column chromatography with solvent DCM: EtOAc (2:1) to generate thiopiperine (29 mg) as a bright orange solid.

2.3 Preparation of stock solution

1 mg of thiopiperine was dissolved in 10 mL of acetonitrile to make a solution of thiopiperine (100 µg/mL). In addition, 2.5 mL of stock solution (100µg/mL) was placed into a 5 mL volumetric flask, and then acetonitrile was added up to a 5 mL line (50 µg/mL).

2.4 Instrumentation and chromatographic conditions

HPLC analysis was performed on UFLC Shimadzu® with a PDA detector (λ = 340 nm). The separation was conducted on a C-18 column (Cosmosil®, 5 µm, 4.6x150 mm) with aqueous acetonitrile (50% acetonitrile) as a mobile phase. The flow rate was set 1 mL/minute, injection volume was 20 µL, while analytical run time was 20 min.

2.5 Stability stress testing

2.5.1 Acidic degradation

100 µL of stock solution was placed into a vial, then 900 µL of 0.1 N HCl was added (5 µg/mL). The solution was mixed thoroughly, after that it was stored at 60°C for 72 h. Sampling was conducted at 24 h and 72 h by transfer 100 µL of solution into vial then the volume was adjusted with 900 µL of acetonitrile. The prepared mixture was analyzed by HPLC.

2.5.2 Alkaline degradation

Alkaline degradation was prepared as similar to acidic degradation's procedure, in this 0.1 N NaOH was utilized instead of 0.1 N HCl as a degradation agent.

3 Results and Discussion

Forced degradation study is the process of determining stability that involves degradation of drug substances or drug products at conditions more severe than accelerated conditions, in this case, forced degradation studies help in generating degradants in a shorter period, mostly 2 weeks (Teasdale et al., 2018). To quantify the number of degradants within the forced degradation study, an integrated HPLC system was applied as a stability-indicating method. HPLC method can separate, detect and quantify chemical compounds and various drug-related degradants that are possibly derived during the manufacturing or storage, it can also detect any drug-related impurities that may be introduced during the synthesis (Abdelwahab et al., 2019).

Before stress testing, initially, the HPLC system was calibrated for degradation analysis study. The system suitability testing is applied to verify an analytical method that is appropriate for its intended purpose. Figure 2 exhibited HPLC's chromatogram of piperine and thiopiperine that revealed very high-resolution peaks. Piperine appeared at 3.9 minutes while thiopiperine emerged at 16.6 minutes. The analysis was arranged in an isocratic method mode in column C-18 (150 mm x 4.6 mm) with mobile phase of 50% of aqueous acetonitrile with detector PDA at 340 nm.

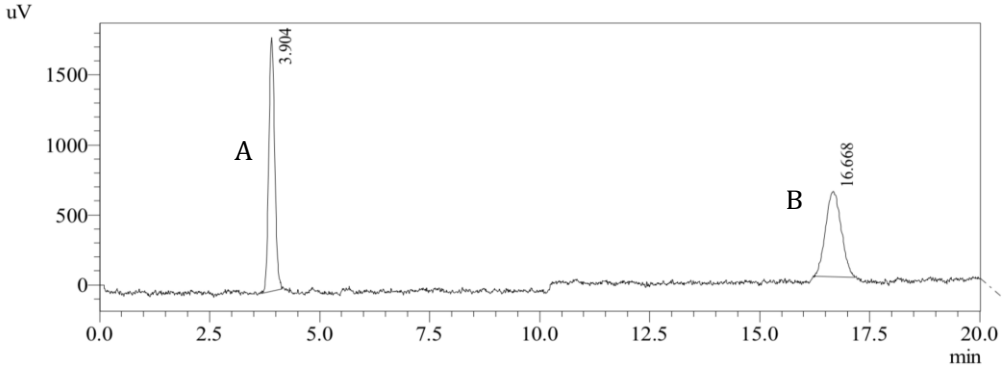


Figure 2 Chromatogram of piperine (A) and thiopiperine (B) on UFLC Shimadzu®, C-18 column (Cosmosil®, 5-µm, 4.6 mm x150 mm) with a mobile phase acetonitrile:water (50:50 v/v) at flow rate 1 mL/min.

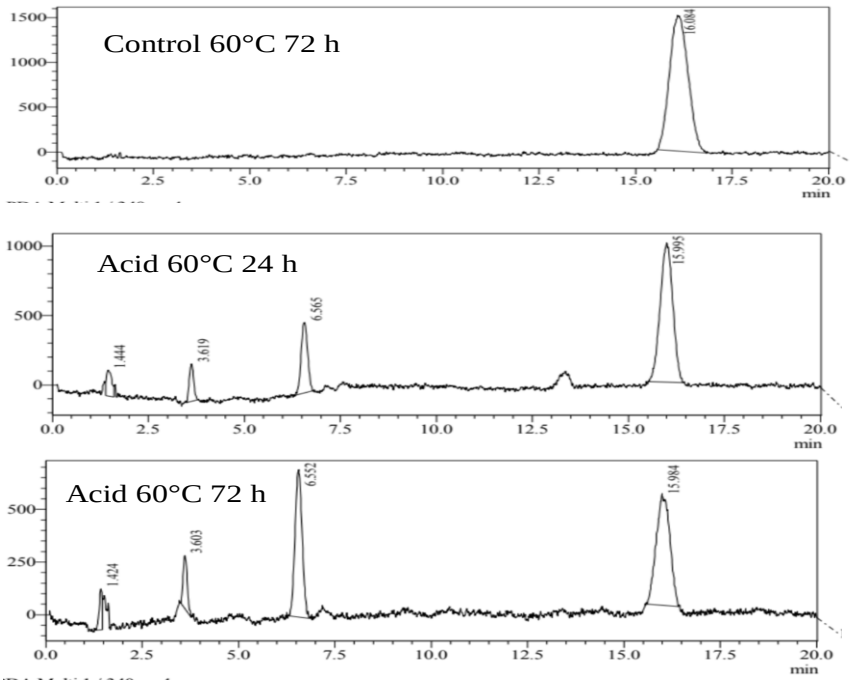


Figure 3 Chromatogram of thiopiperine in 0.1 HCl at 60°C for 24 and 72 h

The data indicated that thiopiperine is more lipophilic than piperine, consider the greater energy of the thiocarbonyl's π-orbital than carbonyl resulting in the electronegativity of thiocarbonyl is less than carbonyl. On the other hand, the polarity of carbonyl is slightly superior to thiocarbonyl (Abboud et al., 1993).

Stress testing of thiopiperine was accomplished in the existence of strong acid (0.1 N HCl), and strong base (0.1 N NaOH) at 60°C for 72h. For acid treatment, thiopiperine solution in 0.1 N HCl environment exhibited degradation process occurred within 24 h after contact with strong acid.

Figure 3 showed that degradants were generated due to the acid environment indicated by new peaks appeared at 1.4 min, 3.6 min, and 6.5 min within 24 h of treatment. In addition, the peak area increased with time. Subsequently, the main peak around 16.0 min was gradually degraded by the time. About 55% of thiopiperine was decayed within 24 h then extensive degradation was occurred up to 75% within 72 h.

Similar to acidic hydrolysis, thiopiperine exhibited a strong degradation under an alkaline environment also (Figure 4). New peaks appeared at 2.3 min, 2.6 min, 3.6 min, and 6.5 min when the thiopiperine was exposed with aqueous NaOH (0.1 N) for 24 h at 60°C. The main peak (16.0 min) was steadily diminished by about 49% within 24 h and decreased 70% within 72 h. In both acidic and basic conditions, thiopiperine underwent a degradation process with time. However, the compound might decay rapidly under acidic conditions than the basic environment.

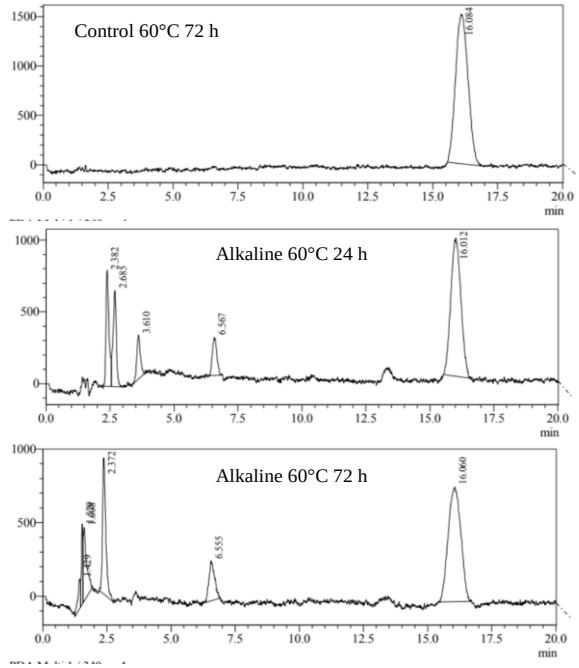


Figure 4 Chromatogram of thiopiperine in 0.1 NaOH at 60°C for 72 h

Conclusion

The stability of thiopiperine investigated through the stability-indicated HPLC method exhibited that the compound was unstable after stress testing for 3 days at 60°C in acidic and basic conditions. The compound was likely to degrade faster under acidic than the basic environment.

Acknowledgment

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Conflict of Interest

The authors declare that there is no conflict of interest

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ANTIBACTERIAL ACTIVITY OF ROBUSTA COFFEE (*Coffea robusta* L.) PEEL EXTRACT AGAINST HUMAN PATHOGENIC BACTERIA

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KEYWORDS

Antibacterial activity

Coffee peel

TLC-Bioautography

Agar diffusion

Pathogenic bacterial

ABSTRACT

Now in these days infectious diseases seriously affect human health and sometimes these infections might become the cause of human mortality. Most of these infectious diseases are caused by bacteria, viruses, and fungi. Although large numbers of antibiotics are available increasing drug resistance in these microorganisms became a serious matter of concern in the scientific community. There is an urgent need for research on alternate natural products that can manage these pathogenic microorganisms without inducing any resistance. The purpose of this study was to determine the antibacterial activity of Robusta coffee (*Coffea robusta* L.) fruit peel extract against 5 human pathogenic bacteria i.e. *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella thypi* NCTC 786. The sample was extracted using the maceration method with methanol as the solvent. The antibacterial activity of fruit peel extract was determined by using the agar diffusion method while the presence of active ingredients was determined by the using TLC-Bioautography assay performed using the mobile phase of n-hexane: ethyl acetate (1 : 3). The results of the study revealed significant antibacterial activity of coffee peel extract against *E. coli* and *B. subtilis* with an inhibition zone of 10.15 mm and 10.96 mm, respectively. Furthermore, results of the TLC-Bioautography revealed that the compounds at Rf 0.76 inhibit the growth of *E. coli* and the compounds at Rf 0.27 inhibit the growth of *B. subtilis* bacteria. These active spots were suspected to be flavonoid and phenolic compounds, respectively but further confirmation detail study is required in the future.

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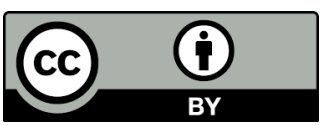

1 Introduction

Infectious diseases caused by bacteria are the most common diseases affecting people in developing countries, including Indonesia. Diseases caused by bacteria are usually treated by administering antibiotics while frequent uses of antibiotics in the treatment of these infections bacteria usually caused resistance development against the most commonly used antibiotic. (Frieri et al., 2017). Recently, the global antimicrobial resistance and use surveillance system suggested that bacteria developed resistance against frequently used antibiotics for the treatment of several infections including diarrhea, sepsis, and urinary tract infection, and it revealed the depletion of effective antibiotics. As per the WHO report, more than 90% of *E. coli* strains are resistant to the most commonly used antibiotic drug ciprofloxacin that is associated with the treatment of urinary tract infection (WHO, 2020). The problem of antibiotic resistance increasing every year, therefore, it is necessary to research the search of alternative natural substances which have antibacterial properties and can serve as a source of new drugs.

The coffee plant (*Coffea* sp.) belongs to the family Rubiaceae, which is a tropical plant and widely consumed as a nonalcoholic beverage throughout the world. As per the report of Indonesia Coffee and Cocoa Research Institute (ICRI), Indonesia is the third-largest coffee-producing country in the world after Brazil and Vietnam (Radydjencole, 2011). However, the large amount of coffee production is not balanced with appropriate waste processing especially in the case of peel and the leaves of the coffee plant. So far, the waste from the peel of the coffee has been used by farmers as animal feed but as per the chemical composition of the coffee cherries, many alternative uses can be generated from the peel of the coffee plant (Pujiyanto, 2007).

The ability to inhibit the growth of pathogenic bacteria can be influenced by the structure of secondary metabolites contained in plants. Widyotomo & Sri (2007) reported the presence of several secondary metabolites such as 1,3,7-Trimethylpurine-2,6-dione (caffeine) and polyphenol compounds from the coffee peel which have antimicrobial properties. Further, research conducted by Ridwan (2018) also reports the presence of flavonoids and polyphenols from the coffee peel and that shows antibacterial activity against *S. aureus* and *E. coli*.

Among the secondary metabolite, phenolic acids have strong antibacterial activity against Gram-positive bacteria. These phenolic compounds having different alkyl chains of hydroxyl groups that can form complexes with bacterial cell membrane proteins. Further, phenolic compounds like gallotannins bind to the membrane protein and inactivate these membrane bounds proteins of bacteria. While flavonoids have multiple cellular targets which inhibit the formation of nucleic acid, attachment, and biofilm formation of bacteria and sometimes disturbed the functioning of the cytoplasmic membrane such as permeability of the membrane, and energy metabolism. (Yixie et al., 2015; Rempe et al., 2017). A study on 2',4'-dihydroxylation of B ring and 5,7-dihydroxylation of A ring in the flavone was affecting the activity of the compound against methicillin resistance bacteria (Cushnie & Lamb, 2005; Patra, 2012). The purpose of this study was to evaluate the antibacterial activity of the coffee peel extract against the selected pathogenic bacteria using the TLC bioautography and agar diffusion method.

2 Materials and Methods

The materials used in this research included nutrient agar (Merck) as the medium, DMSO (Merck), methanol (Merck), n-hexane (Merck), ethyl acetate (Merck), paper disk, TLC plate (Merck), $AlCl_3$, $FeCl_3$, Lieberman Bouchard, and dragendorff reagents. All materials are pro-analytical grade.

2.1 Sample preparation

Coffee peel sample was collected from the Robusta Coffee Factory, Majannang Village Gowa Regency. The sample was air dried, mashed and sieved using a no.18 mesh.

2.2 Extraction

The sample was extracted with the maceration method using methanol as the solvent. The obtained extract was filtered and concentrated using a rotary evaporator until a thick extract was obtained (Vijay et al., 2012).

2.3 Antibacterial Activity Assessment

The antibacterial assay was carried out by using the disk diffusion method on nutrient agar (NA). The methanol extract with a concentration of 2mg/20mL was added to paper discs and these paper discs were left to dry. Dried paper discs were inoculated on the nutrient agar plates pre-inoculated with *B. subtilis* ATCC 6633, *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *S. typhi* NCTC 786 bacterial culture, and these cultural plates were incubated at 37°C for 24 hrs. DMSO was used as a negative control. The diameter of the inhibition zone was measured after 24 hrs of incubation (Somayeh et al., 2014; Denis et al., 2019).

2.4 Contact Bioautography Assay

The methanol extract of Robusta coffee was spotted on a TLC plate (silica gel plate Merck 60 F254) then eluted with a mixture of n-hexane: ethyl acetate (1: 3 v / v) as the mobile phase. The antibacterial activity of active spots was detected using TLC bioautography method (Pandey et al., 2004) on MHA media using the same test microorganism on the previews method and then the plate was left in the refrigerator for 30 minutes for pre-incubation.

After 30 minutes, the TLC was removed from the plate and incubated again for 24 hours at 37°C, and then the inhibition zone was observed. The inhibition zone was observed and matched with the Rf value of the TLC plate (Demetrio et al., 2016).

3 Results and Discussion

This research was used to evaluate the antibacterial activity of Robusta coffee fruit peel extract against pathogenic microorganisms (Figure 1). Robusta coffee fruit extract was prepared by the maceration method using methanol as a solvent, and the obtained amount of extract was 4.27%. The obtained methanol extract was tested against *E. coli, S. typhi, P. aeruginosa, S.aureus,* and *B.subtilis* by agar diffusion method (Brooks et al., 2005). The antibacterial compound will diffuse into the solid media and inhibits bacterial growth, which is indicated by the formation of a clear area which is known as a zone of inhibition around the disc paper. Methanol extract exhibit antibacterial activity against the tested four bacteria i.e. *E.coli, S.aureus, B.subtilis, S.typhi* at a concentration of 2 mg/ disc while it did not show any inhibitory activity against *P. aeruginosa* (Table 1).

Figure 1 Coffee peel from *Coffearobusta*L

The active compound of the extract was detected by contact bioautography method using n-hexane: ethyl acetate (1:3) as the eluent and silica GF_{254} as the stationary phase and then observed under UV 366 nm and UV 254 nm wavelength (Figure 2). The results of the TLC-bioautography revealed antibacterial activity of Robusta coffee peel extract against *E. coli* with a Rf value of 0.76 and *B. subtilis* with a Rf value of 0.27. However, for *S.aureus* and *S. typhi* the inhibition zone was formed at the initial spotting point of the medium containing (figure 3). The polarity properties of silica gel F254 plate bind tightly the polar compounds making them stay on the initial spotting point and not being eluted and this might be a reason that the active compounds at the initial spotting point showed a clear zone as polar compounds (Arcana & Anubha, 2011).

Identification of chemical compounds was carried out to determine the classes of the compounds contained in the coffee peel extract using TLC plates with sprayed reagents**.** The result showed that the methanol extract of coffee peel extract had a visible mark of the dark blue spot after being sprayed with FeCl3 reagents which characterized the presence of the phenolic compound, while the marked with light blue spot after being sprayed with citroboric reagents and observed under UV 366 nm suggested the presence of the flavonoid compound. Along with this, after spraying the Lieberman Bouchard reagents, the spots on the TLC plate turn to

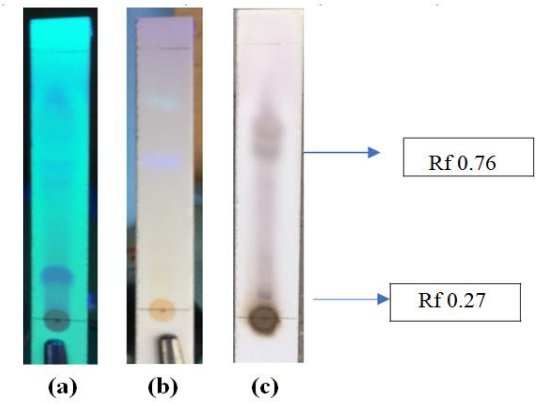

Figure 2 Chromatogram of methanol extract (a) UV Appearance 254 nm, (b) UV Appearance 366 nm and (c) After spraying H_2SO_4

brown which indicated the existence of terpene compounds in the extract. The active spot on the TLC-bioautography test that inhibits *E. coli* was located at Rf 0.76 while the *B. subtilis* spot was located on Rf 0.27 and the active ingredients associated with these spots could be flavonoid and phenolic compounds respectively (Choma IM, Edyta, 2011; Tiwari, 2011; Elzbieta et al., 2016).

The possible mechanism of antibacterial activities of flavonoid compounds was the formation of complex compounds with extracellular proteins which inhibiting cell membrane function and energy metabolism by inhibiting the use of oxygen by bacteria (Cushnie & Lamb, 2005). While phenolic compounds denaturing the bacterial cell proteins and inhibit the multiplication of bacterial cells. The membrane bonds formed between phenol and protein damaged the protein structure. Further, the hydrogen bonds will affect the permeability of the cell wall and cytoplasmic membrane because both are composed of proteins. The disturbed permeability of the cell wall and cytoplasmic membrane can cause an imbalance of macromolecules and ions in the cell so that the cell becomes lysis (Cowan, 1999).

Table 1 Antibacterial activity of methanol extract against five different test bacteria.

	Zone of Inhibition diameters (mm)					
	E.coli	*S.aureus*	*S.thypi*	*B.subtilis*	*P.aeruginosa*	*DMSO*
Methanol extract	10.15±1.02	9.70±0.85	8.96±0.29	10.96±1.00	6.00±0.92	5.95±0.90

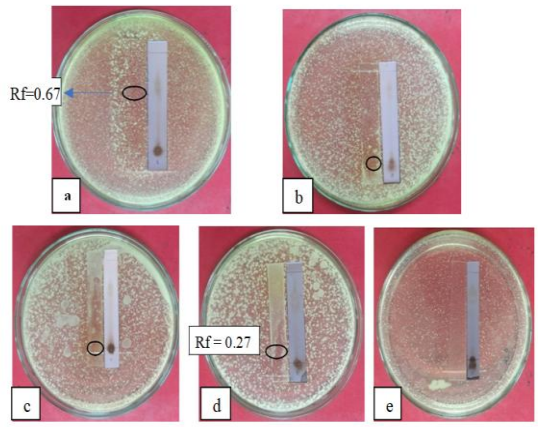

Figure 3 TLC-Bioautography of the extract against (a) *E. coli*, (b) *S. aureus*, (c) *S. thypi*, (d) *B. subtilis*, and (e) *P. aeruginosa*

Conclusion

The methanol extract of Robusta coffee peel exhibited antibacterial activity against of *E.coli*, *S.aureus*, *S. typhi*, and *B.subtilis* bacteria. Further, the TLC-bioautography test revealed that the methanol extract has antibacterial activity against *E.coli* with Rf 0.76 and *B. subtilis* with Rf 0.27 and it was suspected the presence of flavonoids and phenolic compounds, further detailed studies required for establish the presence of these active ingredients (Figure 4).

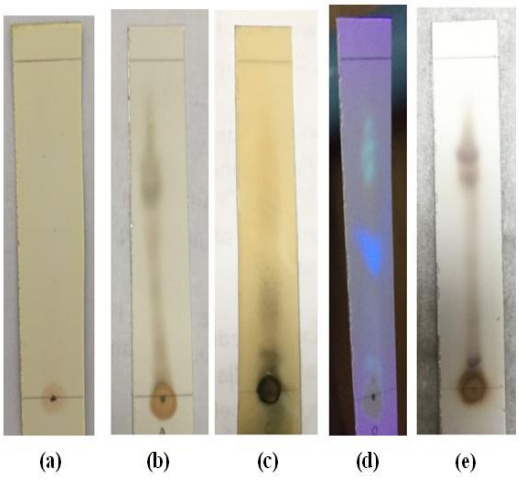

Figure 4 Identification of chemical compound (a) Dragendorff reagent, (b) LB reagent, (c) $FeCl_3$ reagent, (d) cytoborate reagent and (e) Vanillin H_2SO_4 reagent

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Conflict of Interest

The authors declare that there is no conflict of interest in this manuscript.

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COMPARISON OF α –GLUCOSIDASE INHIBITORY ACTIVITY OF *Moringa oleifera* ETHANOLIC EXTRACT

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KEYWORDS

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Moringa oleifera

Ethanol extract

IC50

Acarbose

ABSTRACT

The purpose of this research was to determine the α-glucosidase enzyme inhibitory activity of *Moringa oleifera* plant samples collected from the three geographical areas viz., Saragi, Bacuhau, and Batumatongka of Southeast Sulawesi Indonesia. Ethanol extract of *Moringa* leaves was prepared by the maceration method using 95% ethanol. The estimation of α –glucosidase inhibitory activity of this extract was performed *in vitro*. The results of the study showed that ethanolic extract of three Moringa samples i.e. Sarangi, Bacuhau, and Batumatongka had the IC_{50}value of 18.62, 10.18, 10.58 ppm, respectively while IC_{50}value for the acarbose positive control was reported 11.54ppm. From the results of this study, it can be concluded that ethanolic extract of *Moringa* could inhibit α –glucosidase and this potential was similar to the commercial α –glucosidase inhibitor acarbose.

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1 Introduction

Now in these days prevalence of diabetes mellitus is rapidly increasing throughout the world. The prevalence of diabetes mellitus in adults (20–79 years) was 6.4% or 285 million in 2010 and is expected to reach 7.7% or 439 million up to 2030 (Shaw et al., 2010). While in the case of Indonesia, the incidence of diabetes mellitus is projected to reach up to 21.3 million by 2030 (Kementerian Kesehatan, 2020). The increasing prevalence of diabetes mellitus needs serious attention for the treatment of this disorder. For a healthy routine diabetes mellitus infected people take insulin or oral antidiabetic drugs piles daily. The oral hypoglycemic drug is one of the most important pharmacological therapies for the treatment of type 2 diabetes mellitus. One of the alternate popular choices is the alpha-glucosidase enzyme inhibitor drugs because these are associated with lesser side effects. The *α*-glucosidase is an enzyme found in the intestine and catalyzes the breakdown of polysaccharide groups to monosaccharides and helps in the glucose absorption by the intestine (Palanuvej et al., 2009). The mechanisms of hypoglycemic action of α-glucosidase inhibitors are the reduction in the digestive process of complex carbohydrates and their reduced intestinal absorption; therefore, it decreases the glucose levels in people with diabetes mellitus (WHO, 1999; Saha et al., 2011).

Treatment with synthetic drugs often fails because these drugs have lots of side effects including the development of insulin resistance, or the huge costs for long-term therapy. Many efforts have been made to search for alternative antidiabetic agents with better efficacy, minimal side effects, controlled blood sugar levels, and relatively cheaper cost. (Fahey, 2005; Rante et al., 2019).

Indonesia has abundant natural resources and among these, medicinal plants as a source of traditional medicine are the most common ones. *M. oleifera* belongs to the family Moringaceae and is widely used by traditional healers for the treatment of diabetes mellitus due to its anti-diabetic properties (Jaiswal et al., 2009; Giridhari et al., 2011). Moringa is well known for its more than 90 types of nutrients in the form of essential vitamins, minerals, amino acids. Further, it contains almost 539 active compounds which have been made this plant a popular choice for the African and Indian traditional healers. Further antidiabetic properties of this plant is also well reported (Toripah et al., 2014). The leaves extract of *M. oleifera* contains various active ingredients including flavonoids, tannins, anthraquinones, cardiac glycosides alkaloids, triterpenoids, and saponins which help in reducing sugars. Tende et al. (2011) reported that the hypoglycemic effect of flavonoids is associated with the stimulation of pancreatic β cells which enhance insulin secretion.

2 Material and Methods

2.1 Sample preparation

Leaves samples of *M.oleifera* were collected from the three different regions namely Saragi, Bacuhau, and Batumatongka of the Southeast Sulawesi, Indonesia. The collected leaves were cleaned, shaded dried and a fine powder was made with the help of an electronic mixture & grinders.

2.2 Extraction

Ethanolic extract of *Moringa* leaf powder was extracted by using the maceration method with 95% ethanol. The obtained extracts were filtered by using a Buchner vacuum filter and evaporated using a rotary evaporator to thicken the solvent, and this drying procedure was continued until the crude extract was obtained.

2.3 Thin Layer Chromatography (TLC) profile

The ethanol extract was analyzed by using thin-layer chromatography (TLC), and for this, toluene: ethyl acetate (7:3) mixture was used as a mobile phase to qualitatively identify the presence of bioactive substances. The TLC profiles were observed under visible light and UV light at a wavelength of 366 nm. The identification of chemical compounds was performed using spray reagents Dragendorff and sitroborat for alkaloid and flavonoid respectively.

2.4 α-Glucosidase Inhibitory Activity Test

The activity of the α-glucosidase enzyme was analyzed using the Sancheti et al. (2009) method with some necessary modifications. The enzyme stock solution was prepared in a phosphate buffer solution (pH 7). The enzymatic reaction was performed in 96 well plate by mixing 15 µL of 25 mM p-NPG as substrate, 60 µL phosphate buffer solutions, and 10 µL of *M. oleifera* ethanol extract, this was followed by the 5 minutes incubation of plate at 37^{o}C and mixing of 15 µL of α-glucosidase. This was followed by the incubation of the reaction mixture at 37°C for 30 minutes, the reaction was stopped by adding 100 µL of 0.2 M Na_2CO_3 solution. The resulting P-nitrophenol was measured at λ = 405 nm using an Elisa reader. The experiment was conducted with 3 replications and % inhibition was calculated using the following formula:

$$\% \text{ Inhibition } = \frac{\text{Absorbance without sample} - \text{Absorbace with sample}}{\text{Absorbance with sample}} \text{ X } 100\ \%$$

3 Results and Discussion

The extraction process was carried out using a maceration method with 95% ethanol solvent. From all three sampling sites, a similar yield of extract was obtained. Ethanol solvent was chosen because

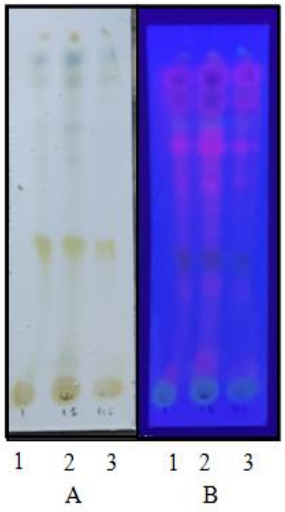

Figure 1 Chromatogram of ethanol extract of *Moringa* leaves using mobile phase toluene : ethyl acetate (7:3), under visible light (A) and 366 nm UV light (B), 1: Saragi, 2: Bacuhau, 3: Batumatongka study sites

Table 1 Screening phytochemicals of ethanolic extract from *M. oleifera* leaves from different origins

Compound class	*Moringa* Extract		
	Saragi	Bacuhua	Batumatongka
Flavonoid	+	+	+
Alkaloid	+	+	+

it can dissolve and extract a wide range of nonpolar to polar active ingredients (Saifuddin et al., 2011). According to Suryanto & Wehantouw (2009), the separation of the compounds depends on the solubility of the components to be separated in the solvent. Apart from the type of solvent, the size of the sample subjected to any extraction method also affects the yield of extraction. A smaller sample surface area will promote the contact surface of the sample substance which increases its interaction with the solvent (Sineke et al., 2016). The *Moringa* ethanolic extract from three different areas was analyzed using a TLC method to determine the presence of alkaloid and flavonoid compound class using Dragendorff and sitroborat spray reagents respectively (Figure 1; Table1).

The in vitro antidiabetic properties of *Moringa* extract was estimated by measuring the α-glucosidase enzyme inhibition capacity. Acarbose is a competitive, reversible inhibitor of pancreatic alpha-amylase and membrane-bound intestinal *α* -glucoside hydrolase, and because of these characteristics, it had been used as a standard antidiabetic positive control in this study. Results of the α-glucosidase inhibitory assay revealed that the ethanolic extract of *Moringa* leaves collected from the three different areas of Southeast Sulawesi had different levels of α-glucosidase inhibition (Table 2). *Moringa* extracts prepared from the leaves collected from the Saragi area had an IC_{50} value of 18.62, while this was reported 10.19 and 10.58 for the leaves samples collected from Bacuhau and Batumatongka localities of the southeast Sulawesi (Table 2). In the case of standard control acarbose, the IC_{50} value for the α-glucosidase inhibitory activity was reported 11.54. Results of the study revealed that *M.oleifera*

Table 2 Comparison of α-glucosidase inhibitory activity of *Moringa* leaf extracts prepared from the leaves collected from three different localities of Southeast Sulawesi

Moringa leaves Sample localities	Concentration (ppm)	Percentage inhibition	Linear regression	IC_{50}
Saragi	10	6.13	y = 2,4924x + 3,5924 R² = 0,9746	18.62
	20	8.98		
	30	10.84		
	40	12.62		
	50	16.77		
Bacuhau	10	3.22	y = 4,7124x - 2,0035 R² = 0,9817	10.19
	20	7.09		
	30	10.88		
	40	18.28		
	50	21.19		
Batumatongka	10	8.01	y = 4,5503x + 1,8573 R² = 0,9622	10.58
	20	9.96		
	30	13.60		
	40	20.48		
	50	25.49		
Acarbose	10	7.51	y = 4,0136x + 3,6695 R² = 0,9782	11.54
	20	12.09		
	30	16.25		
	40	18.15		
	50	24.55		

leaves extract to have significant α-glucosidase inhibitory activity and this was at par the standard positive control.

Acarbose inhibits the action of α -glucosidase hydrolase enzyme in the small intestine which significantly inhibit the breaks down of oligosaccharides, trisaccharides, and disaccharides (sucrose, maltose) to monosaccharides (glucose, fructose), which leading to a depletion of carbohydrate absorption by the brush border cell of the small intestine (Schnell et al., 2016). By making a delay in the digestion of carbohydrates, acarbose slows down the glucose absorption, which resulted in a reduction of postprandial blood glucose concentration (Schnell et al., 2016).

According to Adewole et al. (2006) α -glucosidase hydrolase inhibitory activity of the *Moringa* leaves extract might be due to the presence of flavonoids (quercetin and kaempherol) and triterpenoids. This has been already established in a rat model of diabetes mellitus using a streptozotocin (STZ) as the diabetes-inducing agent (Adewole et al., 2006). Flavonoid compounds can regenerate pancreatic β cells in STZ-induced diabetes mellitus rats. Further, the presence of the quercetin was also reported from the *Moringa* leaves, and it was found beneficial to stimulate progenitor cells in the pancreatic duct to promote cellular differentiation. This, subsequently, facilitates the formation of the new islet of Langerhans cells or endocrine cells in diabetic mice that has been subjected to induced pancreatic damage (Rifaai et al., 2012). In addition, *Moringa* leaves are also rich in vitamins, minerals, and essential amino acids that will be useful in cell regeneration (Farooq et al., 2012). Therefore, the results of the present study supported the use of *Moringa* leaves extract as an antidiabetic agent, mainly for its inhibition of α-glucosidase activity.

Conclusion

Ethanolic extract of *M. oleifera* had α- glucosidase inhibitory activities that may differ based on the origin of the sample. The lowest IC_{50} value was found for the *Moringa* extract originated from Bacuhau (IC_{50}= 10.18 ppm), but this is not significantly different from the IC_{50} value of *Moringa* extract of Batumatongka areas leaves (IC_{50}= 10.58 ppm). The IC_{50} value was even lower compared to the positive control acarbose (IC50= 11.54 ppm), that suggesting a greater α- glucosidase inhibitory activity of the *Moringa* leaves extract.

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Conflict of Interest

The authors declare that they have no conflict of interest

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EFFECT OF HONEY CONSUMPTION AFTER PHYSICAL EXERCISES ON ELECTROLYTES AND BLOOD GLUCOSE LEVELS

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KEYWORDS

Honey

Blood glucose

Electrolytes

Physical exercise

ABSTRACT

Honey has been used as food and medicine for thousands of years. The purpose of this study was to determine the effect of honey in restoring the levels of electrolytes and blood glucose after performing physical exercises. The effect of honey consumption on electrolyte and blood glucose levels was examined on12 healthy male subjects, which were divided into 4 groups with three members in each group. Group I was treated as control and provided only 250 ml of mineral water after exercise, while group II was provided 250 ml commercial isotonic beverage, group III provided honey solution 1 (15 ml honey/250 ml water), and group IV provided honey solution 2 (45 ml honey/250 ml water). The physical exercise performed by the subjects is running on a treadmill at a speed of 5.6 km/h for 40 minutes. Measurements of electrolyte and blood glucose levels were performed 24 hrs before as initial baseline, and these were also measured after the physical exercise as well as after the treatment. Results of the study revealed that honey played a significant role in the restore electrolyte and blood glucose levels in people who have performed physical activities such as exercising and the effect of honey is similar to the commercial isotonic beverage. However, no significant difference (p-value > 0.05) was reported between the honey solution 1 and 2 and other treatment groups in elevating sodium and chloride level.

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1 Introduction

Honey is a natural substance of a sweet thick brown, a light yellow or brown-red liquid that is produced by honey bees from the flower nectar, or secretions of plants, or excretions from plant-sucking insects (Talebi et al., 2020; Ilia et al., 2021). Honey has been used by humans for thousands of years as food and medicine, particularly it is well known for its nutritional, antimicrobial, and medicinal properties (Yusof et al., 2018; Hadju et al., 2020). Honey has a relatively complete nutrient diet and it has carbohydrates, proteins, vitamins, minerals, enzymes, polyphenols, and many other nutritive substances in sufficient concentrations. The main component of honey (ranged from 85% - 95%) consists of carbohydrates, which materialize in the form of blood glucose and fructose and are easily absorbed by the gastrointestinal tract (Eteraf-Oskouei & Najafi, 2013). Other components, such as proteins (amino acids and enzymes), vitamins, and minerals, are also important for the human body (Miguel et al., 2017). Honey, with its various essential components, has been widely used for teeth and gum care, insomnia, anemia, fatigue, vertigo, energy supplement, and hepatoprotective against drug toxicity (Pasupuleti et al., 2017; Aliyah et al., 2019).

About 60% of the adult human body consists of body fluids. Regulation of body fluids is particularly important and can be affected the body's salt and pH balance. Excessive discharge of body fluid, as happened when one is performing physical activities, sweating, vomiting, and suffering from diarrhea, can cause the decline of body fluids and electrolytes that leads to dehydration (Edwards & Noakes, 2009). Dehydration might cause delirium, lack of concentration, and convulsions in moderate cases while it can cause coma or even death under severe conditions (Pross, 2017). When performing physical activities such as exercising, it is not uncommon for a person to lose 6-10% of his body weight due to sweating. Liquid loss due to excessive sweating can lead to dehydration if it is not replenished with the appropriate liquid (rehydration) (Popkin et al., 2010). At present, the consumption of isotonic beverages (sports drinks) to maintain body fluids and electrolytes during or after exercising has been adopted by many people in society (Larson et al., 2015). However, a previous study showed that low pH 2.4 – 4.5 of isotonic drinks can cause demineralization of tooth enamel. In addition, the acidity of less than pH 5.5 around the tooth's surface dematerializes the tooth enamel and it depends on the carbohydrate content, type, and acid concentration of the isotonic drink (Panigoro et al., 2015).

Honey could be an alternative to replace body fluids and electrolytes, apart from consuming an isotonic beverage. According to Abeshu & Geleta (2015), honey is a reach source of minerals and nutrients and can be used as a nutritional intake for people engaging in physical activities, such as exercise. Further, honey has a higher concentration of carbohydrates (85-98%) which are an effective replacement for blood glucose lost during exercising. Moreover, it also contains minerals, mainly sodium, potassium, and chlorine, which can be used as a substitute for lost body electrolytes (Eteraf-Oskouei & Najafi, 2013; Abeshu & Geleta, 2015).

Based on the aforementioned reasons, the current study aimed to determine the effect of honey consumption on electrolytes and blood glucose levels specifically following physical exercises. This is mainly based on the fact that honey can be used to replace body fluids and electrolytes, without having the disadvantages of commercially available isotonic drinks. The study was carried out on non-athlete to view whether honey can be utilized for people requiring additional nutrients after regular physical exercise.

2 Materials and Methods

2.1 Materials and Devices

The devices used in this study included treadmill (BH Fitness®), stopwatch, digital scales (iscale®), holder vacutainer, a tourniquet (ONEMED®), sphygmomanometer (OMRON® Hem-7130), glucometer (Nesco® Multicheck), electrolyte analyzer (Roche® 9180), Cobas®C111 analyzer, and laboratory glassware. All the measurement devices used were calibrated to ensure the accuracy of the experimental equipment.

The materials used in this study include aqua®, honey "X" (produced by the Faculty of Forestry of Hasanuddin University), isotonic beverage "Y" obtained from supermarkets, reagent for Cobas® GLUC2, alcohol swab (ONEMED®), round plaster (PLESTERIN® Round), vacutainer needle (BD® Vacutainer), plain vacutainer tube of 3 cc (ONEMED® Vaculab).

2.2 Study Sample

A total of 12 healthy adult male subjects, aged between 18-30 years with body mass index between 18.5 - 27.0 were randomly selected. Subjects included in this study are physically fit and met the following criteria: Willing to be involved in the current study (and therefore should be willing to fill out the informed consent letter), do not have any allergies to honey or isotonic beverages, willing to consume honey or isotonic beverages, not a professional athlete, having normotension or blood pressure at/or approximately 110/70 mm Hg. Subjects were not included in this study if they were on an extreme dietary restriction, consuming honey daily, taking drugs that can change fluid and electrolyte balance, having an acute or chronic disease that can affect the results of the study (e.g., renal dysfunction), having abnormal levels of serum electrolyte and blood glucose.

2.3 Honey Solution

The type of honey used in this study was Trigona honey produced by *Trigona species*. The honey solution was made into two concentrations using 1 and 3 tablespoons of honey, respectively (6% v/v and 18% v/v). The honey solution 1(HS1) was prepared by dissolving 15 ml of honey with aqua® in an Erlenmeyer flask of 250 ml volume and stirred homogeneously while the honey solution 2 (HS2) was prepared in the same way using 45 ml of honey.

2.4 Examining the Effect of Honey Consumption on Electrolyte and Blood Glucose Levels

For estimation of blood glucose level and electrolytes after honey consumption was determined by the Armstrong et al.(2012) method with some modification. This method was approved by the Commission of Ethics for Health Research, Faculty of Medicine, Hasanuddin University (883/H4.8.4.5.31/ P36-KOMETIK/2017). Briefly, before conducting the study, subjects were directed to not eat for at least 8 hrs and not drink for at least an hour before the study began. The respondents were divided into 4 groups, and each group consisted of 3 respondents, details of divided groups was as Group I was considered as control and given 250 ml of aqua® (G1), while Group II was given 250 ml of commercial isotonic drink (G2), Group III was given 250 ml of HS1 (G3), Group IV was given 250 ml of HS2 (G4).

Before exercising, the blood pressure of each respondent was measured by professional healthcare while the body mass indexes (BMI) of the respondents were calculated by using the following formula (Table 1).

$$\frac{\text{wieght (kg)}}{\text{height (m)}^2}$$

Initial blood glucose and electrolyte (Na^+, K^+, Cl^-) levels of the selected respondents were measured one day before the day of exercise (H-24) by taking a blood sample from the venous by using BD® vacutainer system of blood collection. All the selected respondents were asked to run for 40 minutes on a treadmill (5.6 km/hour). After the exercise ends, they were allowed to rest for 5 minutes, and this was followed by the weighed and measured their blood glucose and electrolytes (Na^+, K^+, Cl^-) levels as the baseline (H0). Later on, each group was provided an appropriate drink according to the predicted group treatment. After 60 minutes without any heavy physical activity, the respondents from each group were reweighed, and 3 ml of venous blood sample was taken for the estimation of blood glucose and electrolyte (Na^+, K^+, Cl^-) level (H+1). The same procedure was also carried out after 2 hours of exercise (H+2). The collected blood samples were centrifuged for 15 minutes at 3000 rpm to obtain serum. The electrolyte levels were measured by Roche® 9180 electrolyte analyzer, and the blood glucose level was measured by using the Cobas® C 111 analyzer.

2.5 Data Collection and Analysis

The variables analyzed in this study were electrolytes and blood glucose levels with different treatment groups at different periods, before and after the physical exercise. The obtained data were analyzed using the statistical program SPSS Ver. 16. To assess the effect of the treatment, one-way ANOVA (Analysis of Variance) and Post-Hoctest were performed to see its differences.

3 Results and Discussion

The effects of honey consumption on electrolyte and blood glucose levels of the respondents who perform physical exercise was studied before and after running and 1 and 2 hours after taking the assigned drink.

All the selected respondents have met the age criteria of 18-30 years with an average of 22±0.1 years old. Data given in table 1 suggested that all respondents had blood pressure that met the inclusion criteria of being normotension or an average 110/70±12.64/7.61 mmHg. The body mass index (BMI) data also shows that all respondents involved in this study, could not be categorized as obese (BMI >30) or underweight (BMI <17).

Table 1 Body Mass Index (BMI), and blood pressure of volunteers prior to exercise testing

Subject code	Age (years)	Blood pressure (mmHg)	Body Mass Index
1	21	105/68	27.0
2	22	107/69	18.5
3	20	128/69	26.9
4	21	105/75	20.9
5	22	106/73	18.0
6	22	126/68	24.4
7	23	110/70	21.2
8	24	112/78	19.7
9	22	121/86	21.7
10	21	106/69	18.8
11	22	107/68	22.1
12	22	127/81	20.7

Initial vein blood withdrawal from the respondents was used as the basal value to minimize the effects of the diet on electrolyte and blood glucose levels in the respondents who would perform the exercise. The results of the initial electrolyte and blood glucose level measurement of the respondents are presented in table 2. The average initial electrolyte and blood glucose levels of the respondents are within the range of reference values (Na^+ 136-145 mmol/L; K^+ 3.5-4.5 mmol/L; Cl^- 98-107 mmol/L; Blood glucose 80-120 mg/dL).

Tabel 2 Initial serum level of electrolytes and glucose in volunteers before exercise

No	Group of treatment	Serum levels			
		Sodium (mmol/L)	Potassium (mmol/L)	Chloride (mmol/L)	Glucose (mg/dL)
1.	G1	136.3 ± 3.8	4.1 ± 0.7	102.7 ± 2.1	107.0 ± 2.6
2.	G2	141.0 ± 1.7	4.3 ± 0.6	105.0 ± 3.5	93.0 ± 28.4
3.	G3	137.3 ± 2.1	4.1 ± 0.2	103.3 ± 2.9	100.7 ± 4.0
4.	G4	136.0 ± 2.0	4.1 ± 0.2	103.3 ± 2.3	103.3 ± 14.6

Values are mean±SD, G1 was given 250 ml of aqua®, G2 was given 250 ml of commercial isotonic drink, G3 was given 250 ml of HS1, G4 was given 250 ml of HS2. Reference values in blood healthy adult man of Na^+136 – 145 mmol/L; K^+ 3.5 – 4.5 mmol/L; Cl^- 98 – 107 mmol/L; Glucose 80–120 mg/dL.

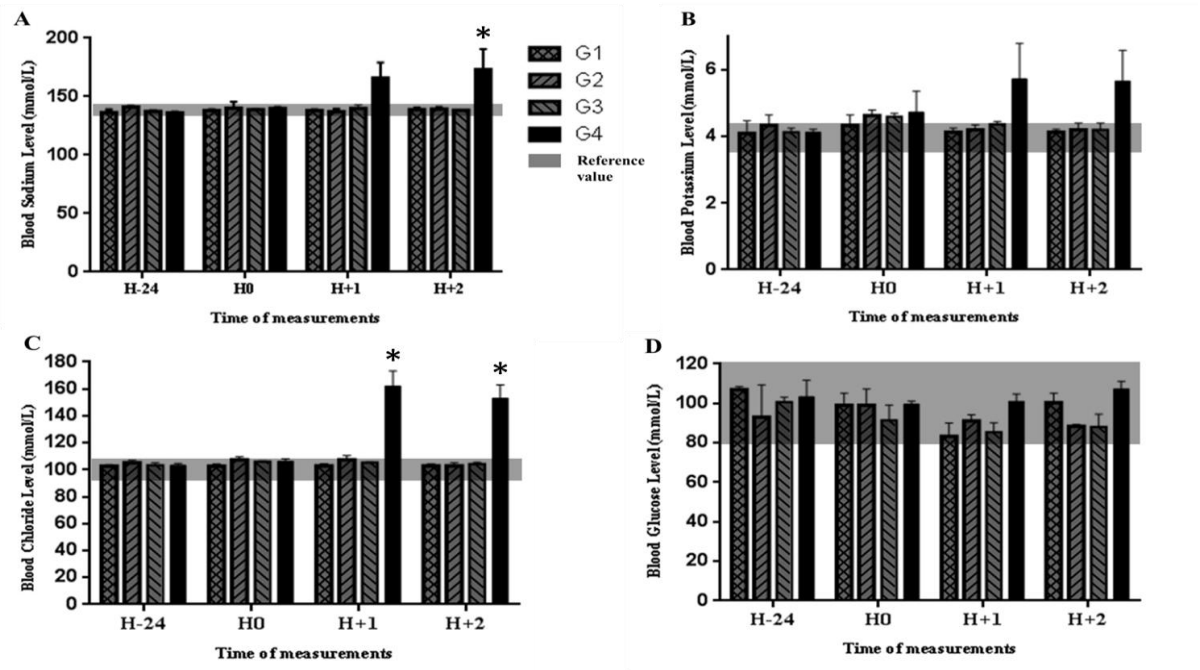

Figure 1 Blood electrolyte and glucose levels in the selected respondents, measured at 24hrs before physical exercise (H-24), before treatment (H0), an hour after treatment (H+1), and 2 hrs after treatment (H+2); Values are mean±SD, all the used treatment abbreviations are similar to table 2 (*p <0.05: significant difference compared to other treatments by one-way ANOVA)

Results presented in figure 1 revealed that one hour after the treatment of water (G1), isotonic beverage (G2), or the honey solution 1 (G3), the levels of electrolyte and blood glucose were relatively stable and are in the range of reference value. Based on the results of statistical data analysis of electrolyte levels, there was no significant difference was reported between the treatment groups ($p > 0.05$). While in the case of G4, a rapid increase was reported in the level of the electrolyte after one hour of the treatment by honey solution 2 and this elevation remained high even after 2 hours of treatment. This increased in electrolyte levels might be due to the intake of isotonic beverage or honey because both solutions having electrolyte ions which can be used to replace lost electrolytes during exercising. Further, 250 ml of the commercial isotonic beverage has 5.25 mmol of sodium, 1.25 mmol of potassium, and 4 mmol of chloride while in the case of 100 ml of honey contains 0.03 - 1.74 mmol of sodium, 0.26 - 12.05 mmol of potassium, and 0.06 - 0.6 mmol of chloride (Ajibola et al., 2012). However, after the electrolyte level increases, the body's homeostatic system will balance the intracellular and extracellular level of electrolytes to the normal reference values (Na^+ 136 – 145 mmol/L; K^+3.5 – 4.5 mmol/L; Cl^- 98 – 107 mmol/L).

This effect showed relatively slower on the G4 group which still has a high level of electrolytes even after 2 hours of consuming the solution containing 3 tablespoons of honey. Similarly, Saat et al. (2002) reported that the electrolyte levels (sodium, potassium, and chloride) increased after physical activity including exercises. After drinking, the electrolyte levels in the respondent's blood decreased over time to resume the initial electrolyte levels in all treatment groups (Saat et al., 2002).

During running, the body experiences hypertonic and hypovolemic states due to sweating. Previous studies revealed that the released sweat contains 20 - 80 mmol/L of sodium, 4 - 8 mmol/L of potassium, and 20 - 60 mmol/L of chloride. To maintain the osmolarity of the body, intracellular fluid shifts to the extracellular space. Plasma volume will decrease, and plasma osmotic pressure rises to maintain the lack of fluid in the body (Sawka et al., 2015). Osmotic pressure increased along with the increased levels of sodium and chloride in the plasma and declining water concentrations (Kenefick et al., 2012; Singh, 2003). Intracellular to the extracellular space transfer of potassium also occurs because the extracellular potassium is released along with sweat as the result of increased body heat. Therefore, to maintain extracellular potassium levels, intracellular potassium shifts to extracellular space (Gröber, 2009).

The average blood glucose level of the respondents seemingly decreased after running as shown in figure 1D. A decrease in blood glucose levels after physical exercise occurs since the body needs energy in the form of ATP. The largest source of energy is obtained from carbohydrates which are broken down into glucose and converted these glucose molecules in ATP through glycolysis, oxidative decarboxylation, Krebs cycle, and electron transfer (Rodwell et al., 2018). Although the blood glucose level of all respondents of G1, G2, and G3 groups remained lower than the initial level two hrs after isotonic beverage or honey solutions intake, the levels were still within reference value of 80-120 mg/dL. In contrast with this, in the G4 group, the subjects experienced a rapid increase in glucose levels one hr after the honey solution 2 consumption. However, based on the results of statistical analysis of glucose levels ($p<0.05$), the Post-Hoc test analysis showed that there was no significant difference between the treatment groups of honey solution 1 and honey solution 2 with other treatment groups.

Electrolytes and glucose play an important role in the body such as the formation of energy and action potentials processes. Overactive people can lose fluids and electrolytes excessively. Lack of fluids and excessive electrolytes can cause dehydration which can lead to delirium, seizures, and even death in certain cases (Sherwood, 2012). It is important to replace lost fluids and electrolytes in the body so that the cells can carry out their functions properly. Rehydration to replenish lost body fluids and electrolytes is necessary to keep our body cells nourished. Still, this study has some limitations, including small sample size and a short study duration. This emphasizes the requirement for further study with a larger sample size and longer duration.

Conclusion

From the results of this study, it can be concluded that mixing 250 ml honey in water can restore electrolyte and blood glucose levels in people who have performed physical activities including exercise. However, there is no significant difference between other treatments, except for sodium and chloride level elevation within the administration of 18% v/v honey solution. Nevertheless, it is important to note that these findings show that honey possesses the ability to restore electrolyte and glucose levels without necessarily having the side effect of commercial isotonic drinks.

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Conflict of interest

The authors declare no conflict of interest in this study.

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ANTIOXIDANT ACTIVITY OF FOREST MANGGOSTEEN (*Garcinia hombroniana* Pierre) FRACTION USING DPPH AND ABTS METHOD

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KEYWORDS

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DPPH

ABTS

Fraction Active Antioxidant

ABSTRACT

This study was carried out to determine the active antioxidant fraction of *Garcinia hombroniana* bark extract by the DPPH and ABTS method. Along with this, the study also attempts to identify the class of compounds present in the various extract of *G. hombroniana* by the active fraction. The bark extract of *G. hombroniana* was prepared by the multilevel maceration method by using three solvents including n-hexane, ethyl acetate, and 96% ethanol. Results of study suggested that n-hexane (HSE), ethyl acetate (EASE) and ethanol 96% extract (ESE) have antioxidant activity with IC_{50} value of 423 ± 16.72 µg/mL, 284.89 ± 2.7µg/mL, and 10.49 ± 0.19 µg/mL in DPPH assay, while these extracts showed IC_{50} value of 560.92 ± 48.86 µg/mL, 430.18 ± 16.65 µg/mL, and 13.92 ± 0.57 µg/mL respectively in ABTS assay. Further, fractionated using vacuum column chromatography revealed the presence of five fractions viz., A, B, C, D, and E. Among these, fractions D showed the highest antioxidant activity with an IC_{50} value of 4.83 ± 0.18 µg/mL and 6.82 ± 0.31 µg/mL in DPPH and ABTS assays. Results of the fractioned analysis and qualitative determination showed that the active fraction of *G. hombroniana* contained flavonoid, triterpenoid, alkaloid, and saponin compounds, and antioxidant activities of these extracts might be due to the presence of these active ingredients.

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1 Introduction

Nowadays, people's lifestyles have been changed to practical and instant life, especially on their daily consumption. Recently, consumption of fast foods has been increased tremendously and excess consumption of instant or fast food is the way of high quantity free radical (FR) production (Poumorad et al., 2006; Lobo et al., 2010). Accumulation of these FR in the human body may cause cancer or cardiovascular disease like serious diseases which might be a leading cause of death in the current scenario. Further, oxidative stress may also contribute to the dysfunction and death of neurons which might be caused by neurodegenerative disease at higher oxidative stress (Bernd & Christian, 2002). Recent studies showed that phytochemicals being considerable for the prevention of atherosclerosis which also contributes to the pathophysiology of cardiovascular disease (Sen et al., 2010; Silvana & Christina, 2020). Therefore, recently, antioxidants that can neutralize the effect of various FR are strongly needed. Antioxidants are substances that react and neutralize FR by donating their electrons and can prevent oxidative damage of molecules in the human body. Natural antioxidants are in high demand, among these natural antioxidants, phenolic compounds are most common and are abundantly exist in plants, these antioxidants can donate their hydrogens to FR, and inhibit oxidation of low-density lipoproteins by chelating metal ions (Lobo et al., 2010).

Garcinia sp. has been well known for the higher concentrations of xanthone, benzophenone, and flavonoid compounds (Klaiklay et al., 2013; Nargis et al., 2014). Listyani et al. (2017) has been reported that the ethyl acetate extract of *G.hombroniana* stem bark has antioxidant activity and it can inhibit the lipoxygenase enzyme with EC_{50} and IC_{50} values of 15.34 µg/mL and 0.26 µg/mL, respectively. Further, Nargis et al. (2015) succeeded in isolating cycloartane, triterpene, and five other triterpenoid compounds from dichloromethane *G. hombroniana* extract. Antioxidant activities of the natural antioxidant can be determined by various methods but the two most common methods are 1,1-diphenyl-2 picrylhydrazyl (DPPH) and the 2,2‘azinobis (3-ethylbenzthiazoline-6-sufonic acid) method (ABTS). Therefore, in this study, both methods have been used to test the potency of forest mangosteen extract as a natural antioxidant. Further, this study was also conducted to obtain an active antioxidant fraction of *G.hombroniana* stem bark.

2 Materials and Methods

The materials required in this study are Chambers (CAMAG), exicators, UV lamps, micropipettes (Nesco®), simplicial ovens, and analytical scales (Sartorius®). All reagents used are pro analytic reagents. The bark of the forest mangosteen (*G. hombroniana*) was collected from the Rompegading Village. The collected bark samples were sorted wet, cleaned, and washed with running water, then dried in an oven at 40°C. The dried sample was used for the extraction. Identification of the collected sample was carried out at the Botany Laboratory of the Faculty of Mathematics and Natural Sciences, Hasanuddin University.

2.1 Extraction process

Dried forest mangosteen stem bark was macerated using the multilevel maceration method with 3 kinds of solvents, namely n-hexane, ethyl acetate, and 96% ethanol. The obtained 3 extracts viz., hexane soluble extract (HSE), ethyl acetate soluble extract (EASE), and 96% ethanol soluble extract (ESE) were evaporated using a rotary evaporator (Buchii®).

2.2 Determination of Chromatogram Profiles Using Thin Layer Chromatography (TLC)

About 100 mg of HSE, EASE and ESE extract were dissolved with suitable solvents, then spotted on the GF254 silica plate (Merck®), and eluted using 3 eluent mixtures which were hexane-ethyl acetate (1: 1), chloroform-ethyl acetate-formic acid (5: 2: 0.5), chloroform-ethyl acetate-formic acid (5: 4: 0.25), respectively in the different chambers. The spot was visualized under a UV 254 nm, 366 nm and observed directly after being sprayed with 10% H_2SO_4 reagent (Merck®) accompanied by heating and also sprayed with DPPH solution.

2.3 Fractionation of Ethanol Extract

Fractionation of ethanol extract was done using vacuum liquid chromatography (VLC). Silica gel 60 PF_{254} were used as stationary phase and 8 different mixtures of ethyl acetate and methanol viz., ethyl acetate 100%, different ratio of ethyl acetate-methanol (20:1; 10:1; 5:1; 3:1; 1:1; and 1:3), and methanol 100% were used as a mobile phase. After undergoing VLC of ESE, the fractions that resulted were qualitatively analyzed for their TLC profile. The same TLC profile was used to obtain 5 fractions which were fractions A, B, C, D, and E. The fractions were then tested for their antioxidant activity.

2.4 Determination of antioxidant activity

2.4.1 DPPH Method

Five different concentrations i.e. 2.5 ppm; 5 ppm; 7.5 ppm; 10 ppm and 12.5 ppm of HSE, EASE, and ESE were prepared and followed by the addition of 80 µL DPP @ 4 mM. While Trolox® was used as a positive control. Subsequently, this mixture was incubated at room temperature in the dark for 30 minutes, and absorption was measured using a microplate reader (Biorad®) at a wavelength of 515 nm (Nargis et al., 2014; Rajkumar et al., 2015).

2.4.2 ABTS Method

Like DPPH, here also five different concentrations i.e. 2.5 ppm, 5 ppm, 7.5 ppm, 10 ppm, and 12.5 ppm of HSE, EASE, and ESE were prepared and 120 µL of mixtures of ABTS and Potassium Persulfate solution was added with 200 µL MeOH. While Trolox® was used as a positive control. Subsequently, the mixtures were left for incubation at room temperature in the dark for 30 minutes. This was followed by the measurement of the absorption by using a microplate reader at a wavelength of 650 nm. The free radical scavenging activity is expressed as percent inhibition which can be calculated by the following formula:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100$$

The sample concentration and the probit value of percent inhibition are plotted on the x and y axes, respectively, to obtain a linear regression equation. This equation is used to determine the IC_{50} value of each sample (Nargis et al., 2014).

3 Results and Discussion

The current study was carried out to obtained active antioxidant fractions from the natural source of *G.hombroniana*. In this research, the multilevel extraction process yielded a different amount of extract. Results presented in table 1 revealed that ESE is the most yield-giving extract. Further, the thin layer chromatography (TLC) profile of HSE, EASE and ESE can be seen in figure 1. It revealed that all extracts undergo a good separation process using the appropriate solvent. The ethanol extract was the most abundant and active extract which fractionated the compounds based on their polarity. The fractions with the same TLC profiles were combined and resulted in 5 fractions (Fraction A, B, C, D, and E), these can be seen in the chromatogram given in figure 3. The chromatogram showed a variety of compounds contained in each fraction which showed the different colors of spots under UV observation at 254 and 366 nm wavelength.

Table 1 Percent Rendement and Organoleptic properties of Forest Mangosteen Bark Extract

Extracts	Percent Rendement (%) w / w	Organoleptic
Hexane Soluble Extract (HSE)	0.12	Odorless, tasteless, light brown
Ethyl Acetate Soluble Extract (EASE)	0.34	Odorless, tasteless, light brown
Ethanol96% (ESE)	7.03	Odorless, tasteless, dark brown

3.1 Antioxidant Activity

Qualitative and quantitative analyses of the antioxidant activity of *G.hombroniana* extracts and fractions were carried out by ABTS and DPPH assay. Quantitative antioxidant activity analysis of the extracts showed that the ESE has the highest antioxidant activity to reduce the ABTS and DPPH radicals with IC_{50} of 13.92 and 10.49 µg/mL respectively. As shown in figure 2, the ESE has a potential activity as a natural antioxidant as it has a relatively similar activity to Trolox in scavenging the ABTS and DPPH radicals (IC_{50} 10.9 µg/mL against ABTS and 5.58 µg/mL against DPPH). While the lowest antioxidant activity was reported in the HSE, and EASE extracts, these extracts have an IC_{50} value of 423 µg/mL (HSE) and 284.89 µg/mL (EASE) in DPPH and 560.92 µg/mL (HSE) and 430.18 µg/mL (EASE), in ABST radical scavenging assay (Figure 1). In a previous study, Rajkumar et al. (2015) evaluated the antioxidant potential of *Garcinia imberti* stem bark methanol extract against DPPH and reported the IC_{50} value of 274.24 µg/mL, and this value was reported lower than the current study *G. hombroniana* bark extract.

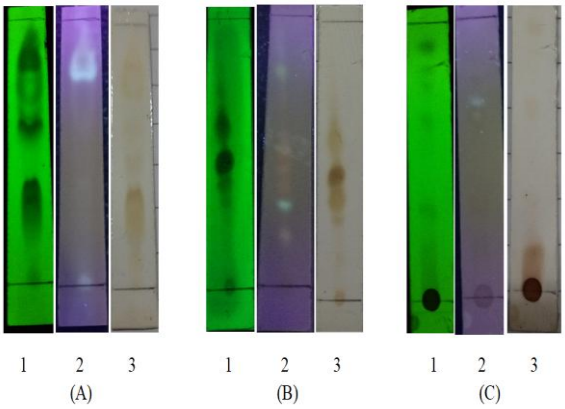

Figure 1 (A) Chromatogram profile of n-hexane extract with an eluent mixture of hexane: ethyl acetate (1: 1), (B) Chromatogram profile of ethyl acetate extract with eluent mixture of chloroform: ethyl acetate: formic acid (5: 2: 0.5), (C) Chromatogram profile of 96% ethanol extract with eluent mixture Chloroform: ethyl acetate: formic acid (5: 4: 0.25); here 1 is visualized under 254 nm UV Lamp; 2 under 366 nm UV Lamp; and 3 Visualized after spraying with 10% H2SO4 + heating

Furthermore, qualitative analysis of antioxidant activity was also done to obtain the fraction from different extracts. The chromatogram was sprayed with 0.2% DPPH solution (Figure 3). The chromatogram has shown five fractions A, B, C, D, and E as yellow spots on purple background in the TLC stationary phase. These results are in line with the qualitative antioxidant activity shown by the hexane fraction of red dragon fruit that faded the purple color of DPPH on the TLC plate (Budilaksono et al., 2014). To prove the qualitative results, a quantitative analysis of fractions A, B, C, D, and E was also conducted (Figure 4). ABTS assay to the fifth fraction showed that fraction D has the highest activity with IC_{50} of 6.82 µg/mL. While, fraction B, C, E, and A showed antioxidant activity with IC_{50} value of 8.04 µg/mL, 8.39 µg/mL,

16.91 µg/mL, and 102.37 µg/mL, respectively (Figure 4). According to Phongpaichit et al. (2007) compounds that have IC50 values <10 µg/mL is in the very active category and based on these recommendations, the antioxidant activity of fraction B, C, and D are in the very active category while fraction A has weak antioxidant activity.

Antioxidant analysis using the DPPH method showed that fraction D has the highest antioxidant activity with IC_{50} of 4.83 µg/mL. While, fractions C, B, and E showed antioxidant activity with IC50of 6.87 µg/mL, 8.15 µg/mL, and 9.93 µg/mL, respectively. While among the tested fraction, a weak antioxidant activity was shown by fraction E (IC_{50} Value of 186.64 µg/mL).

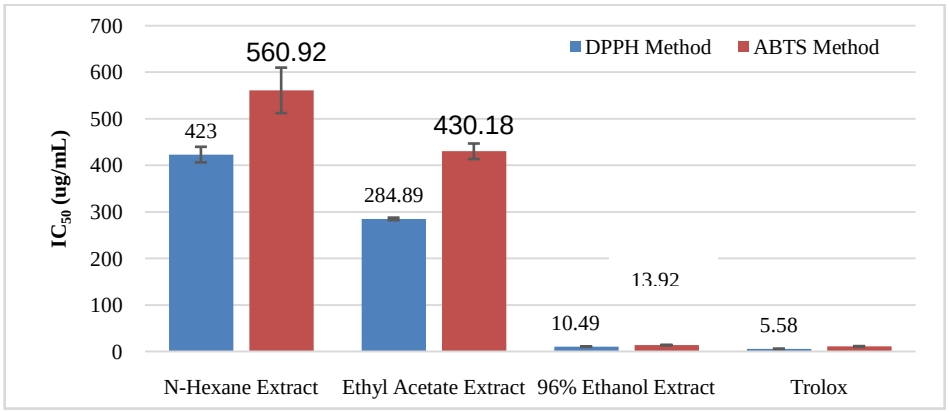

Figure 2 IC_{50} values of different extracts on free radical inhibitory activity using the ABTS and DPPH methods

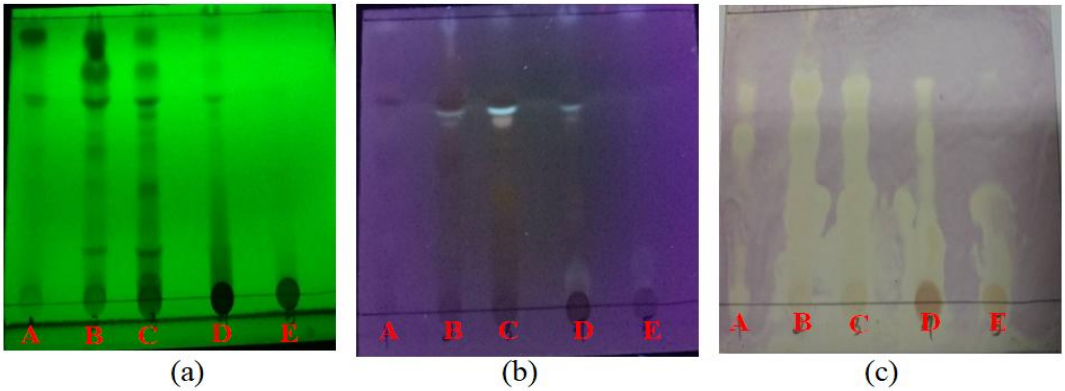

Figure 3 TLC chromatogram profile Fraction A, B, C, D, and E using Chloroform: Ethyl acetate: formic acid (2: 5: 0.5) as eluent (a) visualized at UV 254 nm, (b) visualized at UV 366 nm, (c) DPPH spraying

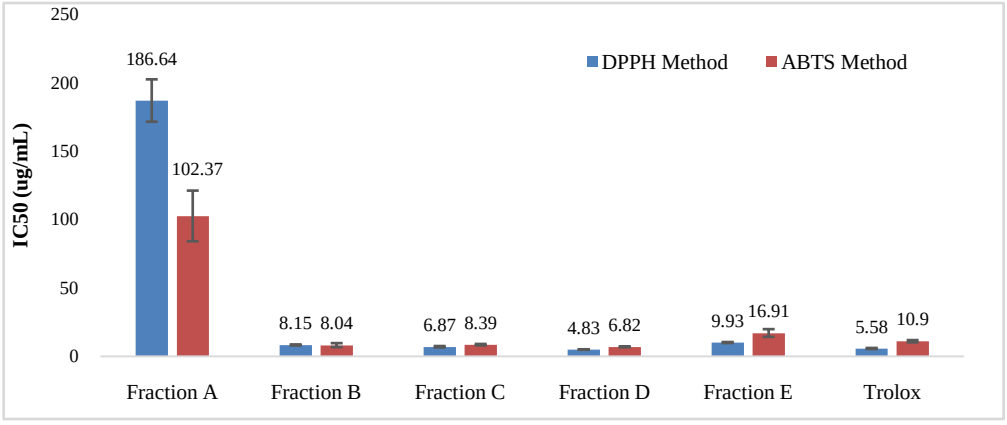

Figure 4 IC_{50} values of free radical inhibition activity for 96% ethanol fraction using the ABTS and DPPH methods

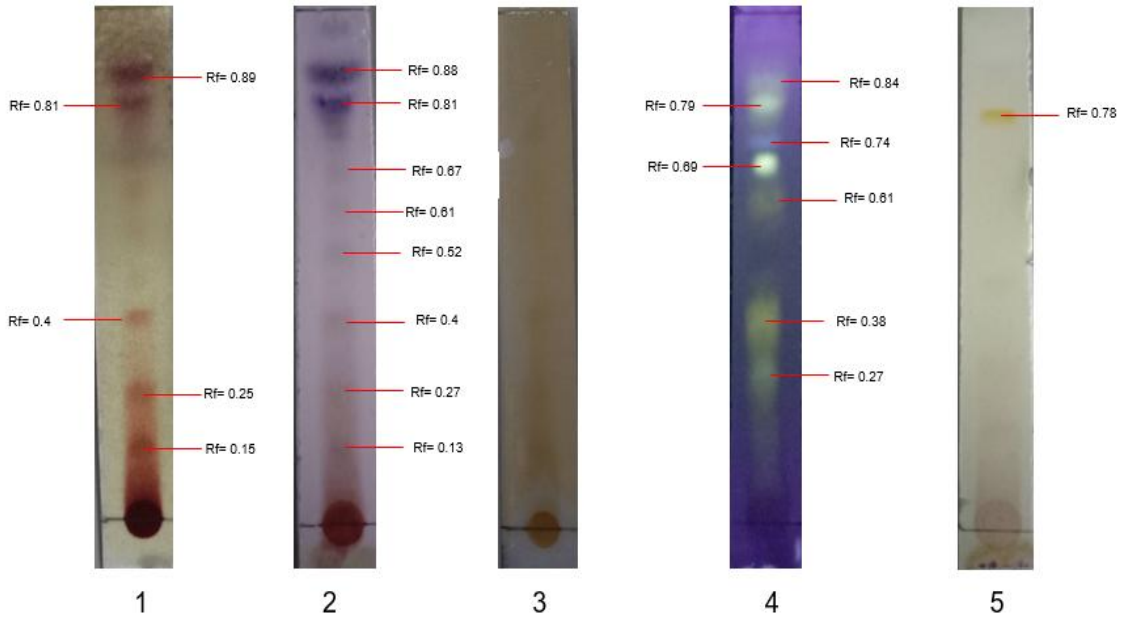

Figure 5 Chromatogram profile of active fraction using sprayed reagents after elution of fraction using mobile phase ethyl acetate-chloroform-formic acid (5: 1: 0.5). (1) Vanillin sulfate, (2) Anisaldehyde, (3) $FeCl_3$ (4) $AlCl_3$ at UV 366 nm, (5) Dragendroff

The identification of chemical compounds contained in the active fraction of forest mangosteen stem bark was done by spraying chemical reagents on the TLC plates spot. The results showed that the active fraction contains flavonoids, triterpenoids, alkaloids, and saponins (Figure 5). Identification of flavonoids using AlCl3 reagent, positive results are indicated by yellow, green, and blue stains that fluoresce at a wavelength of 366 nm after spraying the reagent (Wagner & Bladt, 1996; Galuh et al., 2018). The identification of alkaloid compounds using dragendroff reagent is marked with a brown or brownish-orange stain after spraying the reagent (Archana & Anubha, 2011). Identification of triterpenoid compounds was carried out using anisaldehyde solution marked with a blue-purplish stain, while the identification of saponin compounds was carried out using the vanillin-sulfuric acid reagent marked with a blue or red color change on the TLC stain (Sarker et al., 2006; Elzbieta et al., 2016). These results are in the line of previous research which found some terpenoid and saponin compounds in thymi oil on the TLC profile showed purple and blue spots (Elzbieta et al., 2016). However, qualitative identification of chemical compounds in the active fraction showed that compounds accumulation still occurred in this fraction. Further study needs to be done to isolate and characterized active antioxidants in this fraction.

Conclusion

Among the tested *G. hombroniana* bark extracts, 96% ethanol-soluble extract (ESE) has the highest antioxidant activities in both DPPH and ABTS assay. Further, among the tested five fractions, fraction D from ethanol extract is the active antioxidant fraction that has the highest free radical inhibition activity using both the DPPH and ABTS methods with IC_{50} of 4.83 ± 0.18 µg / mL and 6.82 ± 0.31 µg/mL respectively. Identification of positive chemical components indicates the presence of alkaloids, flavonoids, saponins, and terpenoids in fraction D.

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