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EDITORIAL WORDS

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Editor in Chief
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Assalamu'alaikum Wr. Wb.

Alhamdulillahirabbil 'alamin. The next edition of **Borneo Journal of Pharmacy** (*Borneo J Pharm*), has been published at May 2023. This edition contains ten articles: Pharmacology-Toxicology, Pharmacognosy-Phytochemistry, Pharmaceutical, Analytical Pharmacy-Medicinal Chemistry, Natural Product Development, and Management Pharmacy. This edition includes writings from five countries: Indonesia, Nigeria, Philippines, Taiwan, and United States. The authors come from several institutions, including Universitas Ahmad Dahlan, Universitas Muhammadiyah Mataram, University of California, Los Angeles, National Research and Innovation Agency Republic of Indonesia, Universitas Muhammadiyah Prof. Dr. HAMKA, Universitas Muhammadiyah Kalimantan Timur, Universitas Gadjah Mada, Universitas Sembilanbelas November Kolaka, Universitas Halu Oleo, Universitas Lambung Mangkurat, Universitas Jenderal Achmad Yani, Ahmadu Bello University, Institut Kesehatan Helvetia, Taipei Medical University, Sekolah Tinggi Ilmu Farmasi Riau, University of the Philippines Manila, and Universitas Diponegoro.

Editorial boards are fully aware that there are still room for improvement in this edition, hence with all humility willing to accept constructive suggestions and feedback for improvements to the publication for the next editions. The editorial board would like to thank all editors and reviewers, and contributors of the scientific articles who have provided the repetoire in this issue. We hope that all parties, especially the contributors, could re-participate for the publication in the next edition on August 2023.

Wassalamu'alaikum Wr. Wb.

Palangka Raya, May 2023

Editor-in-Chief

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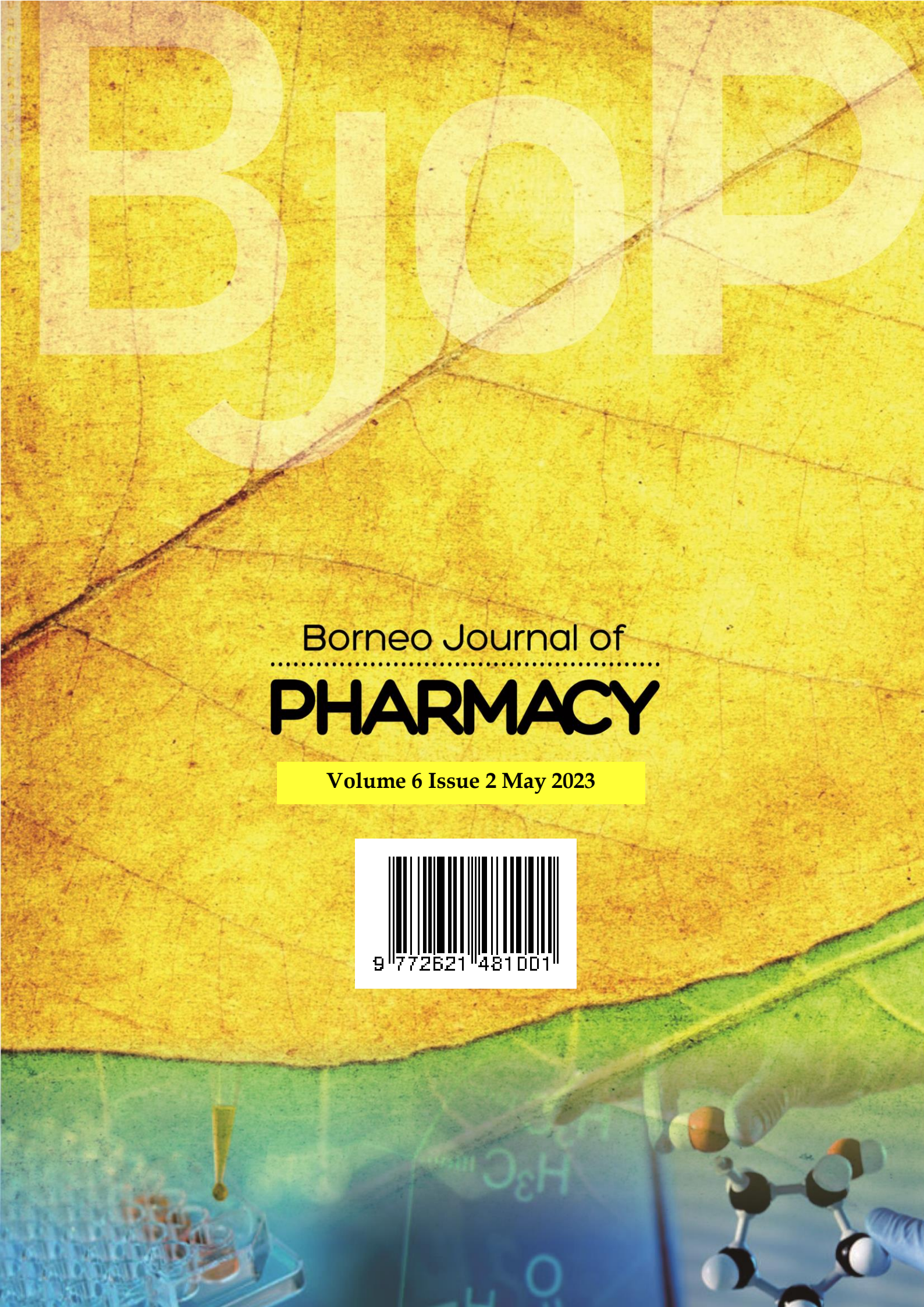
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Research Article

Identification of Biological Risk Genes and Candidate Drugs for Psoriasis Vulgaris by Utilizing the Genomic Information

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Autoimmune disease
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Genomic variant
Psoriasis vulgaris

Abstract

Psoriasis is an autoimmune disease that causes inflammation on the skin's surface, characterized by the appearance of pink plaques covered with white scales. Currently, the availability of psoriasis vulgaris therapy is still limited. Therefore, considering the discovery of new drug candidates by utilizing genetic variations, such as single nucleotide polymorphisms (SNP) through drug repurposing, is a profitable method. The SNP associated with psoriasis was obtained from Genome-Wide Association Studies (GWAS) and Phenome-Wide Association Studies (PheWAS) databases. We identified 245 SNPs associated with psoriasis vulgaris with criteria of r^2 >0.8. To prioritize the candidate of a gene associated with psoriasis, we used five criteria of functional annotation (missense/nonsense, cis-eQTL, PPI, KEGG, and KO mice) where if there were more than two criteria of assessment, they were defined as the risk gene of psoriasis vulgaris. Fifty-two genes were identified as the risk gene of psoriasis vulgaris, then expanded using the STRING database to obtain more gene candidates of drug targets. The result is 104 genes candidates for drug targets, of which 24 overlapped with 96 drugs, according to DrugBank. Of the 96 drugs that have been approved for other indications, we found that five drugs (ustekinumab, tildrakizumab, risankizumab, guselkumab, and etanercept) are currently in clinical trials for the treatment of psoriasis that target two genes (*IL23A* and *TNF*). We argue that these two genes are the most promising targets based on their high target scores on functional annotations. This research explains the potential that utilizing genomic variation can contribute to drug discovery.

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INTRODUCTION

Psoriasis is an autoimmune disease that can cause inflammation on the skin surface[1]. Psoriasis vulgaris disease is characterized by erythematous plaques such as scaly localized or scattered all over the skin surface[2]. Areas of skin that often occur lesions are the elbows, knees, and the skin surface of the head. Psoriasis is classified into eight, including Plaque Psoriasis (Psoriasis Vulgaris), Guttate Psoriasis, Pustular Psoriasis, Generalized Pustular Psoriasis, Palmoplantar Pustulosis, Acrodermatitis Continua of Hallopeau, Erythrodermic Psoriasis, Inverse Psoriasis[3]. Of the many types of psoriasis vulgaris described above, psoriasis is the most widely reported species. Psoriasis vulgaris is characterized by red or pink plaques covered with white or gray scales, large or small plaques, thick or thin, with clear boundaries. Psoriasis vulgaris affects about 2% of the population in North America and Europe. Women are more likely to experience psoriasis vulgaris than men, with a ratio of 9 : 1[4]. Psoriasis vulgaris is experienced by sufferers starting from the age of 33 years, with 75% of cases occurring before 46 years of age[5].

The pathogenesis of psoriasis vulgaris involves inflammatory mechanisms, especially the pathway of T-helper cell pathway[6]. In the pathogenesis of psoriasis vulgaris, the genetic factor becomes an essential part of developing psoriasis itself. Therefore, the genetic factor plays a vital role in developing psoriasis vulgaris. Several studies have described successfulness in identifying locus susceptibility of psoriasis vulgaris[7-9]. In addition to the genetic factor, other studies explain involvement in other things, such as smokers with an incidence rate of 95% more affected by psoriasis vulgaris than non-smokers[1].

Treatment options for psoriasis include topical corticosteroids, vitamin D analogs, calcineurin inhibitors, keratolytic, and phototherapy[10]. There are several barriers to the treatment of psoriasis, and resistance can occur due to several factors, such as depression and psychological anxiety, that can worsen the severity level of psoriasis and duration of clinical symptoms[11]. In addition, ineffective dosing and poor adherence, such as severe psoriasis requiring multiple drugs, be a factor in resistance[12,13]. Therefore, it is necessary to find an alternative drug that has the potential for the treatment of psoriasis vulgaris. The development of new drugs is generally long-term, which not only costs much money but also requires research both clinically and preclinically[14]. Recently, many methods have been applied to accelerate the discovery and development of new drugs. One of these methods is the drug repurposing approach. The concept of drug repurposing utilizes old drugs (orphan drugs) for new indications[15]. The development of current health technology based on bioinformatics and big data in the health sector has become an advantage to integrate into the drug repurposing for psoriasis vulgaris disease. In several previous studies, the concept of drug repurposing has been used for the development of new drugs in tuberculosis[16], chronic hepatitis B, asthma, depression, colorectal cancer[17], and atopic dermatitis[18], with a system of score assessment and to find the candidate of drug repurposing. Our research aims to utilize a genomic database with a drug-repurposing approach for psoriasis vulgaris. Our methodology is based on utilizing a genomic database and genomic variant drive drug repurposing.

MATERIALS AND METHODS

Materials

The database used includes Genome-Wide Association Studies (GWAS; https://www.ebi.ac.uk/gwas/), Phenome-Wide Association Studies (PheWAS; https://phewascatalog.org/), HaploReg v4.1 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg.php), Drugbank (https://go.drugbank.com/), and ClinicalTrial.gov (https://clinicaltrials.gov/).

Methods

Psoriasis vulgaris risk genes

Analysis of psoriasis vulgaris disease used GWAS[19] (accessed on February 3rd, 2022) with total 49 single nucleotide polymorphisms (SNP) and PheWAS[20] (accessed on February 3rd, 2022) with total 196 SNP. We obtained SNP associated with psoriasis vulgaris using these two databases. Furthermore, SNP obtained from GWAS and PheWAS Catalog were developed with HaploReg v4.1 (accessed on February 3rd, 2022). Single nucleotide polymorphisms associated with psoriasis vulgaris were identified with significance criteria of $<10^{-8}$ for the GWAS Catalog and a significance of <0.05 for the PheWAS Catalog (**Figure 1**).

Biological psoriasis vulgaris risk genes

The genes obtained were prioritized using five functional annotations: Missense, Cis-eQTL, KEGG, Komice, and PPI. Genes that have been prioritized and according to significance were given one point. Each gene was assigned a point based on matching criteria, and the score ranged from 0-5[21,22] (**Figure 1**).

Candidate drugs for psoriasis vulgaris

The genes were mapped based on the Drugbank (accessed on March 19th, 2022) to find a candidate drug for psoriasis vulgaris. The Drugbank database provides information about drugs and gene targets for discovering new drugs. Drugbank is a data source that provides detailed information on drug action with comprehensive drug targets. Drugbank is often used in education to find drug targets, drug design, drug screening, drug metabolism prediction, and drug interactions prediction[23]. Drugbank has detailed information about 1467 drugs approved by Food Drug Association (FDA) that have been matched with 28.447 brand names and synonyms. Furthermore, all drug data was confirmed to ClinicalTrial.gov (accessed on March 19th, 2022). ClinicalTrial.gov is a web-based resource that provides easy access for a person, both the general public, patients, family members of patients, researchers to obtain information regarding clinical studies of various diseases and conditions[24] (**Figure 1**).

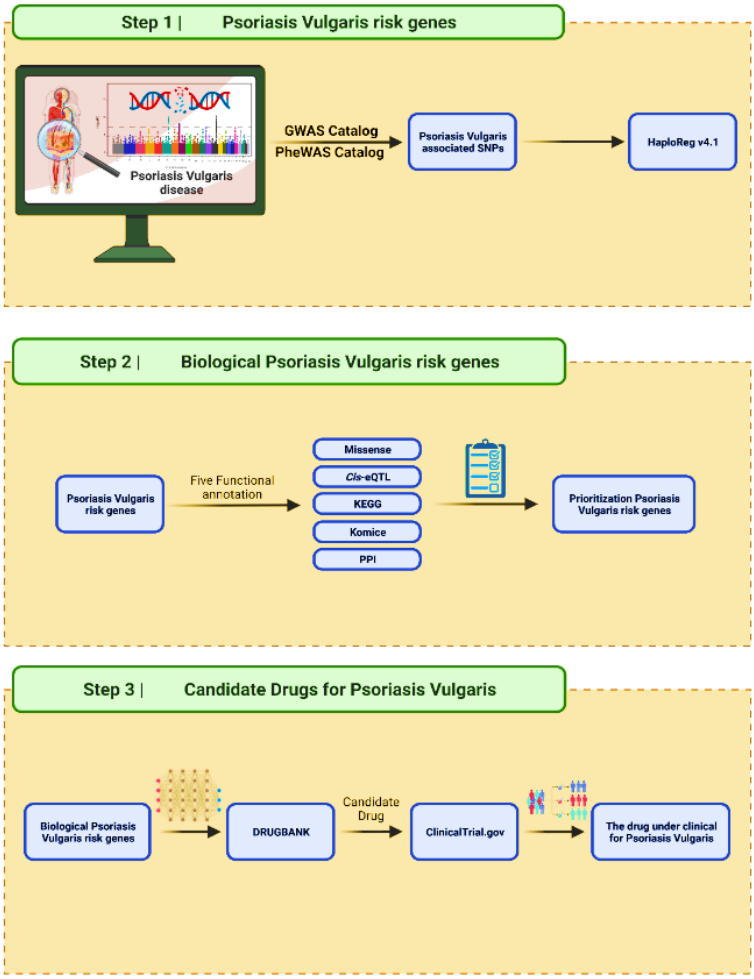

Figure 1. Schematic of drug repurposing by utilizing the database of the genome for psoriasis vulgaris

RESULTS AND DISCUSSION

We systematically identified 245 SNP associated with psoriasis vulgaris, retrieved from GWAS and PheWAS. We set the criteria from the GWAS catalog database with inclusion criteria $<10^{-8}$ and the PheWAS catalog with inclusion criteria <0.05, developed with HaploReg V4.1. We further expanded the SNP based on the criterion of LD >0.8, in this step, we identified 245 SNP.

Functional annotation of the risk gene of psoriasis vulgaris

Five functional biological annotations were prioritized for the risk gene of psoriasis vulgaris. One point is assigned for each functional annotation. Assessment of each of the 359 genes used the following five criteria: genes with risk variant of psoriasis vulgaris missense (n=8); genes of cis-eQTL (n=20); genes involved in the KEGG pathway (n=36); genes in the knockout/KO mice phenotype (n=14); and genes involved in GO terms used for PPI application (n=40) (**Table I**). After the data is collected, the next step is to determine the biological score. Genes with a score of 0 have 0 genes, genes with a score of 1 have 0 genes, genes with a score of 2 has 39 genes, genes with a score of 3 has 12 genes, and genes with a score of 4 has one gene. The calculated genes with a score >2 are 52 genes (**Figure 2**). One risk gene of the top biological psoriasis vulgaris is *Cluster of Differentiation 247* (*CD247*), with a gene score 4 with muromonab drug candidate data.

Table I. Assessment of genes for the risk gene of psoriasis vulgaris

Genes code name	Missense	Cis-eQTL	KEGG	KO mice	PPI	Total Score
CD247	0	1	1	1	1	4
CDK6	0	0	1	1	1	3
CXCR2	0	1	1	0	1	3
DDX58	0	0	1	1	1	3
HLA-DQB1	0	1	1	1	0	3
NFKBIA	0	0	1	1	1	3
NFKBIZ	0	1	1	0	1	3
PLCL2	0	1	1	0	1	3
RFTN1	0	1	1	0	1	3
RUNX3	0	0	1	1	1	3
SH2B3	1	1	0	0	1	3
TNF	0	0	1	1	1	3
TNFAIP3	0	0	1	1	1	3
ADIPOQ	0	0	1	0	1	2
BAK1	0	1	0	1	0	2
C4B	1	0	0	0	1	2
C6orf57	1	1	0	0	0	2
CCDC88B	0	0	1	0	1	2
CCND3	0	0	0	1	1	2
DENND1B	0	0	1	0	1	2
EDC4	0	1	0	0	1	2
ELMO1	0	0	1	0	1	2
EOMES	0	1	0	0	1	2
ERAP1	0	1	1	0	0	2
ESR1	0	0	1	0	1	2
ETS1	0	0	1	0	1	2
GP1BA	1	0	1	0	0	2
HCLS1	0	0	1	0	1	2
HLA-DRA	0	0	1	1	0	2
HRG	0	0	1	0	1	2
HSPA1L	1	1	0	0	0	2
IFIH1	0	0	1	0	1	2
IL13	0	0	1	0	1	2
IL23A	0	0	1	0	1	2
IL23R	0	0	1	0	1	2
IRF4	0	0	1	0	1	2
IRF8	0	1	0	0	1	2
LDLR	0	0	1	0	1	2
MAP3K14	0	0	0	1	1	2
MICB	0	1	1	0	0	2
PIK3R2	0	0	1	1	0	2
PPT2	1	0	0	0	1	2
PSMB10	1	0	0	0	1	2
PSMB8	1	1	0	0	0	2
PSMB9	0	1	0	0	1	2
REL	0	1	0	0	1	2
STAT2	0	0	0	1	1	2
TNIP1	0	0	1	0	1	2
TRAF3IP2	0	0	1	0	1	2
TRIM38	0	1	1	0	0	2
TSLP	0	0	1	0	1	2
UBASH3B	0	1	1	0	0	2
Total	**8**	**20**	**36**	**14**	**40**	

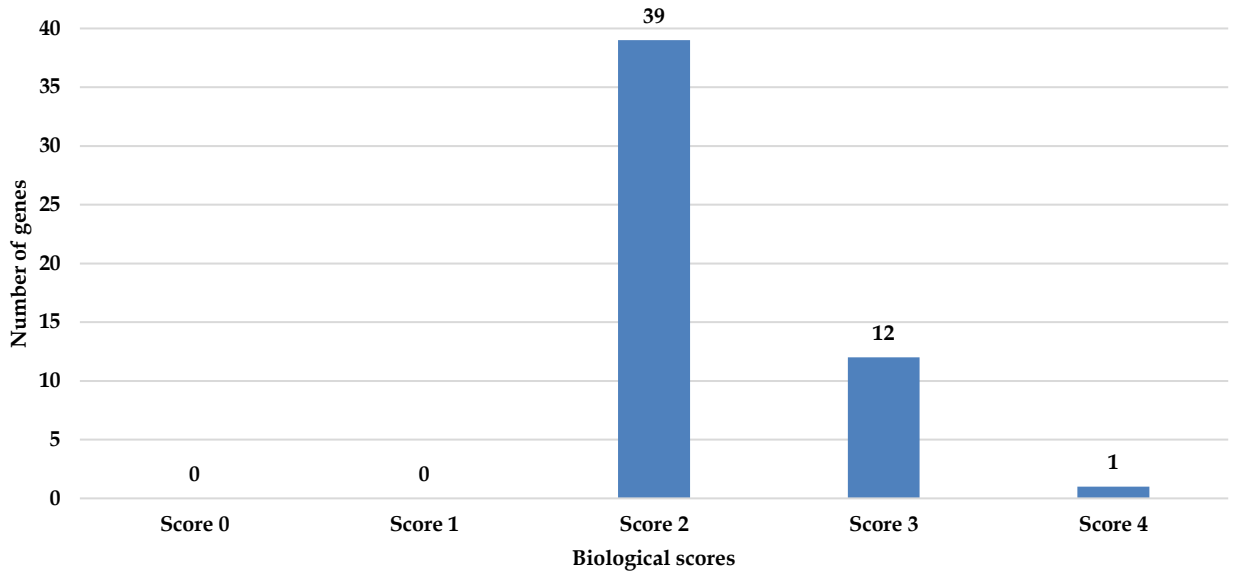

Figure 2. The number of genes (*y*-axis) that fulfill each of the five biological criteria (*x*-axis) for drug priority. The genes with a score of 0 and 1 are 0, while genes with a score of 2 and more (39 + 12 + 1 = 52 genes) are described as the risk gene of biological psoriasis vulgaris.

Expanding the risk list of biological psoriasis vulgaris

This study uses the STRING database. There are 104 genes listed in this study. These genes are a list of genes for new drug candidates that are used for further analysis in psoriasis vulgaris.

Discovery of psoriasis vulgaris drug target

New drug candidates for psoriasis vulgaris were mapped to the Drugbank to find candidates for psoriasis vulgaris drugs. From the search results in the Drugbank, by entering 24 genes and 96 drug targets, it was found that two genes were used for psoriasis. Those that have been approved for psoriasis vulgaris disease have been clinically approved are the *Interleukin-23A* (*IL23A*) gene, and the drug targets consist of ustekinumab, tridakilzumab, risankizumab, and guselkumab (**Figure 3**).

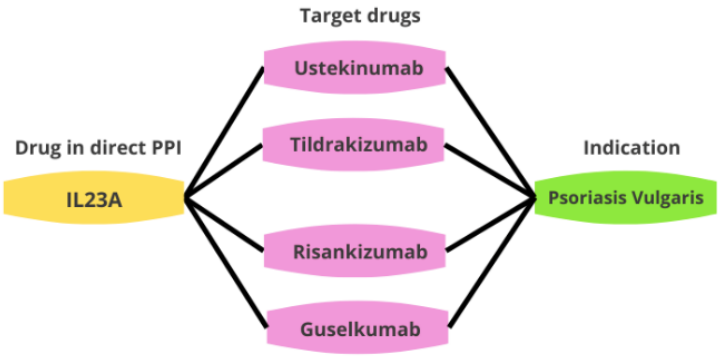

Figure 3. Relationship between psoriasis vulgaris risk genes and available drugs for psoriasis vulgaris.

In this study, two genes of the drug target were found that bind for each gene with two drugs that are approved for other diseases and currently under clinical investigation for psoriasis vulgaris, including guselkumab, tildrakizumab, etanercept, and infliximab. These drugs are likely used to treat psoriasis vulgaris (**Table II**). This study focuses on finding new drugs for psoriasis vulgaris based on candidates of genes identified from the catalog of GWAS and PheWAS. This study also prioritizes genes at risk for psoriasis vulgaris with an assessment that uses five functional annotations to predict new drug candidates. This research found 24 genes target that bind to 96 drug targets, of which two genes bind to drug targets related to psoriasis vulgaris, including the *IL23A* and the *Tumor Necrosis Factor* (*TNF*) genes. The *IL23A* is the drug target of guselkumab, ustekinumab, tildrakizumab, and risankizumab. At the same time, the *TNF* is a drug target of etanercept.

Table II. The drug in clinical investigation for psoriasis vulgaris

Drugs	Target	Mecanism in psoriasis vulgaris	Original indication	Development status	Identifier (ClinicalTrial.gov)	Recruitment status
Guselkumab	*IL23A*	Blocking the signal of cascade inflammation, which elevates abnormally, drives epidermal abnormalities, including keratinocyte hyperproliferation and psoriatic plaque formation	Plaque psoriasis	Phase 4	NCT04080648	Recruiting
Ustekinumab	*IL23A*	Interferes with the actions of proteins, interleukin 12 (IL12) and interleukin 23 (IL23), which reduces inflammation (swelling) in the skin	Plaque psoriasis	Phase 2	NCT01999868	Completed
Tildrakizumab	*IL23A*	IL23 is a natural cytokine involved in inflammatory responses and immune. Tildrakizumab inhibits the release of cytokines and proinflammatory chemokines	Plaque psoriasis	Phase 4	NCT05683015	Recruiting
Risankizumab	*IL23A*	Binds to the P19 IL-23 subunit to prevent the cytokine from binding to its receptor. IL23 is involved in peripheral inflammation, particularly in T-cell responses, so this inhibition aims to reduce psoriatic skin lesions	Plaque psoriasis	Phase 4	NCT05685940	Recruiting
Etanercept	*TNF*	TNF is a natural cytokine involved in inflammatory responses and normal immunity. The elevated level of TNF is found in the network and fluid of those who experience rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis	Rheumatoid arthritis	Phase 4	NCT00640393	Completed

Interleukin-23 is a member of the *Interleukin-12* (*IL12*) family of cytokines with pro-inflammatory properties[25]. Both *IL12* and *IL23* have different immunological pathways and have separate functions but complete each other. *Interleukin-12* is required for antimicrobial response against intracellular pathogens, whereas *IL23* is essential for the recruitment and activation of various inflammatory cells required to induce chronic inflammation and granuloma formation. These two cytokines regulate the cellular immune response necessary for host defense and tumor suppression[26]. *Interleukin-23* is secreted by activated macrophages and dendritic cells (DCs) in peripheral tissues (skin, intestinal mucosa, and lungs). The *IL23* receptor has therapeutic potential for autoimmune diseases, including psoriasis vulgaris, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis[27]. Based on our study, it was found that *IL23* is the drug target of tildrakizumab and guselkumab for psoriasis vulgaris. Tildrakizumab is a humanized IgG1 monoclonal antibody that targets *IL23* p19, and it is approved for use in moderate to severe psoriasis vulgaris[28]. Meanwhile, guselkumab is a specific inhibitor of *IL23* through binding to the *IL23* p19 subunit, significantly improving psoriasis vulgaris signs and symptoms[29].

Tumor Necrosis Factor is a multifunctional cytokine that plays a vital role in cellular events such as cell survival, proliferation, differentiation, and death. *Tumor Necrosis Factor* is also a pro-inflammatory cytokine, so *TNF* is secreted by inflammatory cells that may be involved in carcinogenesis related to inflamation[30]. Currently, *TNF* plays a role in the pathogenesis of several inflammatory conditions, which has led to increased use of target therapies with *TNF* to treat inflammatory bowel disease (IBD), rheumatoid arthritis, psoriasis vulgaris, and psoriatic arthritis[31]. Currently, five *TNF* inhibitors are approved by the Food and Drug Administration (FDA), including etanercept, infliximab, adalimumab, certolizumab pegol, and golimumab[32]. This is in line with the research that we have done, in which of the 115 drug targets found, a *TNF* gene binds to the target associated with psoriasis vulgaris, i.e., etanercept and infliximab.

Etanercept is a fully human *TNF* receptor that reduces the inflammatory response by inhibiting the interaction between *TNF* and receptors of *TNF* on the cell surface[33]. The working mechanism of etanercept is inhibiting *TNF* activation and binding it competitively, resulting in antagonistic interactions with receptors on the cell surface and preventing inflammatory activation[34]. The average half-life of etanercept is about 4.3 days (70-100 hours) and peaks at 48-60 hours with a bioavailability of 58%[35]. Infliximab is a murine-human IgG1 monoclonal antibody that binds to the transmembrane precursor *TNF*[36]. The

mechanism of infliximab is by binding specificity, affinity, and avidity to *TNF*, and through its inhibition, neutralizing and cytotoxic activity, it interferes with the pathology of psoriasis vulgaris and other inflammatory diseases characterized by overproduction of *TNF*[37]. Infliximab is effective in the induction and treatment phase of psoriasis vulgaris. Infliximab not only clears skin lesions but also significantly improves the quality of life that is related to health. The work concept of infliximab is shorter at 3.5 weeks than other biologic drugs such as adalimumab, etanercept, and alefacept[38]. Efficacy rates of infliximab were significantly higher up to week 24 than etanercept[39].

The advantage of drug repurposing by utilizing a gene variation from the GWAS and PheWas catalog database is finding potential ways to use new drug candidates for psoriasis vulgaris. However, this approach has limitations, in which not all identified gene targets have pharmacological activity, and these genes can potentially miss the drug target. Therefore, further investigation is needed to determine the effect of drugs candidate in clinical applications.

CONCLUSION

In this research, 52 genes were identified as biological risk genes of psoriasis vulgaris and obtained one gene with the highest score of 4: *CD247*, with candidate data of muromonab drug. In this case, the involvement of genes in drug discovery against psoriasis vulgaris is likely to be very significant, so further research is needed.

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AUTHORS' CONTRIBUTION

LN and **LMI** conceived and designed the study. **LN** and **LMI** performed the computational analysis. **LN** wrote the manuscript. **LN**, **ANP**, **ARA**, **DAP**, **WA**, **RC**, and **LMI** revised the manuscript. **LMI** and **WA** supervised and coordinated this study. All authors have read and approved the manuscript and made significant contributions to this study.

DATA AVAILABILITY

The data presented in this study are available in supplementary material by contacting the corresponding author.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Short Communication

The Effect of Long Exposure Reed Diffuser Essential Oil *Plumeria alba* on Cortisol Levels of Male Wistar Rats

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Abstract

Stress can occur due to a person's inability to respond to a stressor, resulting in bodily or mental disorders. Anxiety can be characterized by increased levels of cortisol, which is regulated by the Hypothalamus-Pituitary-Adrenaline (HPA-axis). Aromatherapy is a therapy using essential oils that give a distinctive aroma to plant parts such as flowers, roots, leaves, and stems. Aromatherapy can be done through a reed diffuser. The frangipani (*Plumeria alba*) is one of the plants in Indonesia that has a particular scent in its flowers. This study aims to determine the effect of prolonged exposure to *P. alba* essential oil reed diffuser on cortisol levels in rats. Twenty-four rats were divided into four groups: the control group without exposure to a reed diffuser for 5 and 10 days and the other group with exposure for 5 and 10 days. Each blood was drawn through the retro-orbital, centrifuged to obtain serum, and tested using LC-MS to determine cortisol levels. The group with ten days of essential oil exposure showed lower cortisol levels. It can be concluded that the duration of aromatherapy exposure is connected to cortisol levels and that aromatherapy can be utilized as a stress-reduction therapy.

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INTRODUCTION

Stress levels affect memory function. Stress is a condition where the demands that must be met exceed their capabilities, the cause of stress is called a stressor[1]. Stress can occur due to a person's inability to respond to a stressor. It can result in bodily or mental disorders. Stress can be characterized by the increased hormone cortisol, regulated by the Hypothalamus-Pituitary-Adrenaline (HPA-axis), causing cortisol levels to rise[2,3]. Some therapies to prevent dementia and decreased memory function include using drugs, physical exercise, and aromatherapy[4].

Aromatherapy is a therapy using essential oils that give a distinctive aroma to plant parts such as flowers, roots, leaves, and stems. Aromatherapy can be done through a diffuser either electrically or using a reed[4]. Several studies have shown that aromatherapy using rosemary and lemon essential oils spread through a diffuser in the morning and lavender and orange at night can improve cognitive function in dementia patients[5]. Aromatherapy alleviated stress and improved sleep quality in intensive care unit patients after two days of the experimental treatment by exposure to lavender aroma oil[6]. Essential oil (or volatile or flying oil) is a compound of natural ingredients with a distinctive aroma. It can be found in the roots, stems, twigs, flowers, fruit, seeds, or rhizomes of plants with an aroma[7]. Essential oils are obtained through several extraction methods, such as distillation, certain solvents, or pressing the scented plant parts[8,9]. Essential oils are used as additives in

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medicine, cosmetics, perfumes, aroma enhancers in food and beverages, cleaning agents, and others[10]. Essential oils have aromatherapy benefits and pharmacological effects such as antibacterial, antioxidant, anti-inflammatory, antifungal, and antiviral[11].

One of Indonesia's plants with a distinctive aroma in its flowers is the *Plumeria alba*. *Plumeria alba*, also known as frangipani, is widely planted as decoration in the house's yard because the flowers are attractive and have an aroma. *Plumeria alba* also grows wild on roadsides and in burial areas. Several studies have shown that aromatherapy can improve memory function and reduce stress. Therefore, this study aims to determine the effect of *P. alba* essential oil as aromatherapy on male white rats' memory function and stress levels.

MATERIALS AND METHODS

Materials

Plumeria alba was harvested from a Botanical Garden Balai Penelitian Tanaman Rempah dan Obat (BALITTRO), Bogor, West Java, Indonesia, and identified at Herbarium Bogoriense of Indonesia Institute of Sciences with specimen number B-830/V.

Methods

Essential oil extraction

As much as 1 kg of *P. alba* flowers was distilled in a steam container at a temperature of ± 100°C for two hours. The collected steam was then separated using a separatory funnel to obtain the essential oil. *Plumeria alba* essential oil compound was detected using GC-MS and dissolved in an alcohol solvent, and placed in a reed diffuser bottle.

Animal treatment

Twenty-four male rats weighing 150-200 g were provided by the Center of Laboratory Animal Breeding, Kemuning Karanganyar, Indonesia. During the experiment, the animals were housed under standard environmental (25±3^{o}C temperature and 12:12 hours light and dark) and nutritional (standard pellet diet and water ad libitum) conditions. The Medical and Health Research Ethics Committee Universitas Muhammadiyah Prof. Dr. HAMKA (KEPKK-UHAMKA) approval was obtained, and all procedures performed in the study complied with the desired ethical rules, with ethical approval number 02/22.07/01740.

Experimental design

The rats were divided into four groups (n=6): the group without exposure to the reed diffuser for 5 and 10 days and exposure to the reed diffuser for 5 and 10 days. Rats were placed in standard cages containing three rats. The cages were placed in a 2 x 1 m box and given a reed diffuser. The box still had air vents but was not too big so that the intensity of the aroma inhaled from the reed diffuser was more and limited the use of the scent to the outside air. The group was exposed to the reed diffuser for five days, then on the 6th day, their blood was drawn and their cortisol levels checked. In the group that was exposed to the reed diffuser for ten days, on the 11th day, their blood will be drawn and their cortisol levels checked. Data were analyzed using a t-test to determine the significance.

Blood sampling

The blood sample was drawn retro-orbitally for each rat and centrifuged at 4000 rpm for 10 minutes to obtain serum on the fifth and tenth days. The serum was then tested for cortisol levels using the LC-MS method. The results of cortisol levels were tested using the ANOVA test to see the significance.

Data analysis

The experimental data were expressed as the mean±SEM. Data were analyzed by t-test. The significance of the difference between means was determined, where a p-value <0.05 was considered significant.

RESULTS AND DISCUSSION

Cortisol test levels (**Figure 1**) between the control (no exposure) and treatment (with exposure) groups significantly differed and decreased. Exposure to essential oils for ten days reduced cortisol levels more than exposure to essential oils for five days. The group exposure within ten days has a significant difference from others.

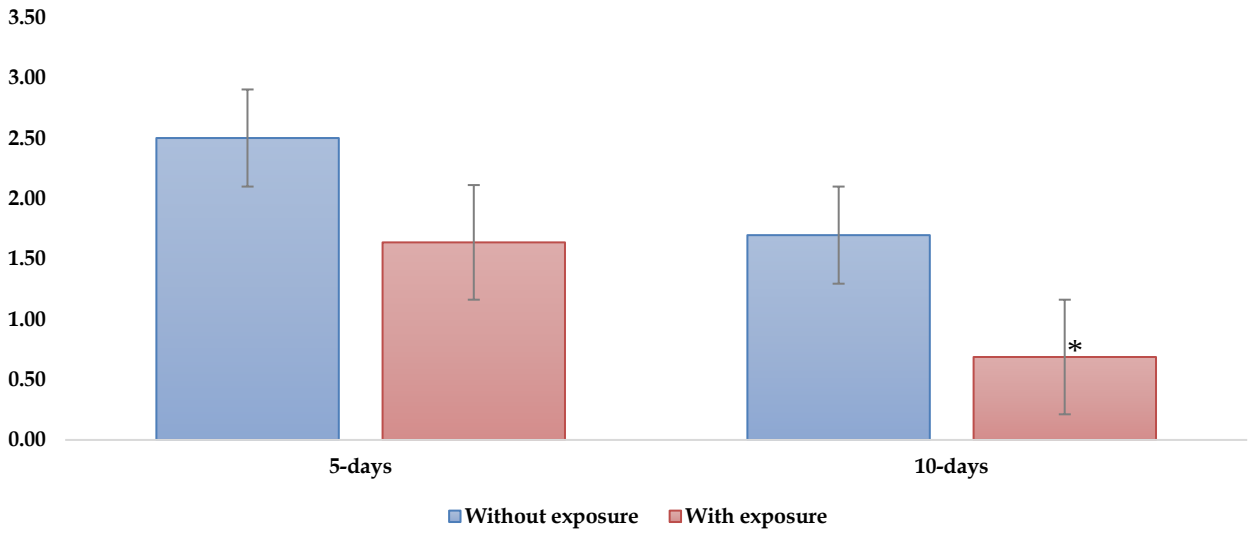

Figure 1. Cortisol levels (ug/mL) on the blood serum of male white rats (*p <0.05).

The body reacts to stress by releasing two chemical messengers: hormones in the blood and neurotransmitters in the nervous system[12]. Stress can be defined as a condition where the demands that must be met exceed the existing capabilities of the object. Stress can also be interpreted as a condition that shows changes due to responding to a stressor[2].

Each type of body response to physical and psychological stress can increase ACTH secretion, increasing cortisol levels. The release of stress hormones begins with corticotrophin-releasing factor (CRF) secretion[13]. The CRF is first released from the hypothalamus in the brain into the bloodstream, reaching the pituitary gland, which is located just below the hypothalamus[14]. In this place, CRF stimulates the release of adrenocorticotrophin hormone (ACTH) by the pituitary, which stimulates the adrenal glands to release various hormones. One of them is cortisol. Cortisol circulates in the body and plays a role in coping mechanisms. When the stressor received by the hypothalamus is robust, the CRF secreted will increase so that the stimulation obtained by the pituitary also increases, and the secretion of cortisol by the adrenal gland also increases. If the emotional state has stabilized, the coping mechanism becomes positive, then signals in the brain will inhibit the release of CRF, and the stress-hormone cycle will repeat[14,15]. In conditions of restlessness, anxiety, and depression, cortisol secretion can increase up to 20 times due to stress[16].

Essential oils are compounds of natural ingredients with a distinctive aroma due to the nature of their blends that contain fragrance and are volatile, so essential oils are also known as volatile oils[7]. In clinical applications, trans nasal inhalation of essential oils can be used nasal inhaler, vapor diffuser, spraying into the air, vapor balms, or direct inhalation by evaporation using tissue or cotton round[17]. Notably, inhalation of essential oils or aromatic plant volatile oils can send signals directly to the olfactory system and trigger the brain to produce neurotransmitters, e.g., serotonin (5-hydroxytryptamine, 5-HT) and dopamine, influence the neuroendocrinological system, neurophysiological brain activity, sympathetic and parasympathetic nervous system, biomarkers changes, psychological and behavioral effects, and modulate mental disorders further. This indicates that the effects of aromatherapy on mental diseases are attributable to the pharmacological action brought on by systemic absorption or act on the neurological system rather than just the psychological sense of the scent[5].

The research data showed increased cortisol levels in the normal group with the group exposed to aromatherapy (**Figure 1**). This is in line with previous studies that stated conditions of restlessness, anxiety, and depression, and cortisol secretion can increase up to 20 times due to stress[16]. The rats exposed to aromatherapy for ten days had lower blood serum cortisol

levels than rats treated for only five days. It means the stress level also decreases. These results align with previous research on the impact of aromatherapy on stress and sleep quality in ICU patients. The experimental group received aromatherapy treatment, and the control group significantly changed subjective and objective stress indexes[6]. According to previous research, *P. alba* oil contains linalool and linalyl acetate[18,19]. Linalool contains sedative and narcotic properties, whereas linalyl acetate possesses narcotic properties. In addition, it can also activate the parasympathetic nervous system [20]. These results showed a relationship between the duration of exposure to essential oils in cortisol levels.

CONCLUSION

This research shows that the length of exposure to *P. alba* aromatherapy is related to cortisol levels. The group with exposure for ten days has the lowest cortisol levels and can be used as a therapy to reduce stress.

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AUTHORS' CONTRIBUTION

SS, **TB**, and **SP** revised the initial manuscript before submission to the journal website. **SS** and **TB** managed the experiment in the laboratory during the research. All authors read, reviewed, and approved the manuscript and English language.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Research Article

Phytochemical Analysis and Anti-Inflammatory Activity of The Combination of *Trigona apicalis* propolis Extract and Honey

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Inflammatory
Propolis

Abstract

Chronic inflammation is common in infectious diseases, rheumatoid arthritis, gout, and autoimmune diseases. However, using non-steroidal anti-inflammatory drugs (NSAIDs) is accompanied by dangerous side effects. Therefore, searching for safer alternative therapies without side effects is very important. A natural blend of ingredients produced by stingless bees from plants was potential as a remedy. Meanwhile, the potential of kelulut bee products from East Kalimantan as an anti-inflammatory is still unknown. This study aimed to compare the chemical composition of kelulut bee (*Trigona apicalis*) products and evaluate the anti-inflammatory effect of honey, propolis, and their combination. Propolis extract and honey were determined as secondary metabolites. An anti-inflammatory *in vivo* assay triggered the edema using carrageenan on male mice and measured its anti-inflammatory power value. Propolis extract and honey from *T. apicalis* have a promising anti-inflammatory effect and are significantly higher than the positive control. Meanwhile, combining propolis extract and honey did not enhance the anti-inflammatory effect. In addition, combining honey and propolis preparations with a ratio of 75 : 25 has a better effect on reducing edema volume than the other two combinations. Still, it is not better than the treatment with propolis extract or honey alone. The content of polyphenol compounds found in honey and propolis preparations is thought to have an important role in reducing edema volume.

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INTRODUCTION

Inflammation is an essential process for our body to defend against infection. However, excessive inflammation can lead to pain and chronic diseases such as rheumatoid arthritis and damage body tissues. Limitation of oxidative stress is one way to control inflammation. Natural ingredients are a source of antioxidants and can reduce aspects of the inflammatory response[1].

Indonesia is rich in natural resources that are potentially used as medicine[2]. One of the natural resources found in Indonesia, especially in East Kalimantan, is stingless bee or kelulut bee (*Trigona apicalis*) products[3]. Among 500 known stingless bee species, 40 of them have the potential to produce honey[4]. The bee products from *T. apicalis*, such as propolis, honey, and bee pollen, have been known to have health benefits[5]. Propolis is a resin-like product produced by bees and exhibits biological activity, for instance, antioxidant, antibacterial, and empirically used as an immunomodulator. Propolis is an essential component of bee hives. It is a hive's defense against bacteria, fungi, and viruses[6]. Propolis is produced by bees using its

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surrounding natural resources. Therefore, various chemical compositions are contained in propolis, such as flavonoids, tannins, phenolic compounds, terpenes, and several antioxidant compounds[7]. Interestingly, the surrounding environment also gives the diversity of propolis composition, and this difference in chemical composition affects its biological activity. In addition, Mulyati *et al*.[8] stated that the content of flavonoids produced by *T. apicalis* bee propolis is higher than *Apis* spp.
In East Kalimantan, the *T. apicalis* species is one of the stingless bee species commonly cultivated by the community[9]. Its propolis production is higher than stinging honey bees (*Apis* spp.). This species is also cultivated because of its ability to produce a distinctive honey taste (sour) and is liked by consumers. The most prominent chemical composition of *T. apicalis* produced honey is phenolic compounds such as p-coumaric acid, naringenin, caffeic acid, quercetin, and taxifolin[10].
The uniqueness of *T. apicalis* products, which have the most colonies, has encouraged further research on honey and propolis, a type of *T. apicalis* native to East Kalimantan, as potential natural ingredients to be developed as an anti-inflammatory agent. This study aims to evaluate the chemical constituent of *T. apicalis* propolis and honey extract native to East Kalimantan using qualitative phytochemical determination and compound profiles. It was continued to determine the bioactivity of propolis extract, honey, and their combination as anti-inflammatory using carrageenan-induced edema in male mice.

MATERIALS AND METHODS

Materials

The sample used was *T. apicalis* propolis and honey obtained from kelulut bee farmers in Tanah Merah, Samarinda, East Kalimantan, provided by Rendri Arista Avimaro as an apiary. Identification of bee species was carried out at the Forest Protection Laboratory of Universitas Mulawarman in April 2021. Based on the bee specimens and the characterization of the hives, it was inevitable that the sample used in this study was *Tetrigona apicalis*, better known as *Trigona apicalis*. The characterization of the *T. apicalis* nest entrance is shown in **Figure 1**.

Figure 1. *Trigona apicalis* nest. Characterizing the *T. apicalis* nest entrance differs from other stingless bee nest entrances. Nest characterization was used for bee species identification.

Methods

Extraction and fractionation

Methanol was used as a solvent to extract *T. apicalis* bee propolis samples, and used the maceration method to obtain crude propolis methanol extract. After obtaining the concentrated extract of *T. apicalis* bee propolis, liquid partition was carried out using a solvent with a ratio of ethyl acetate : *n*-hexane (1 : 1). Then it was shaken and allowed to stand until it formed two phases. The results of the ethyl acetate filtrate from the partition were collected and evaporated to obtain the ethyl acetate fraction of propolis.

Phytochemical determination

Alkaloids: As much as 5 mL of the ethyl acetate fraction was put into a test tube, 2 mL of concentrated HCl, and 1 mL of Dragendorff's reagent was added. A change in color to red or orange indicates alkaloid content.

Flavonoids: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then a few drops of ferric chloride solution were added. A change in color to yellow, red or brown after exposure to light indicates the presence of flavonoids.

Triterpenoids/steroids: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then 0.5 mL of chloroform and a few drops of hydrogen peroxide were added. A color change to a reddish-brown intersurface indicates the presence of triterpenoids, while the red top layer and yellow H_2SO_4 layer indicate the presence of steroids.

Saponins: As much as 1 mL of the ethyl acetate fraction was put into a test tube, then 2 mL of distilled water was added, then the mixture was heated on a hotplate for 10 minutes, filtered, and the filtrate was put into a test tube which had been added 10 mL of distilled water and shaken for two minutes. The presence of stable foam on the surface of the mixture indicates the presence of saponins.

Tannins: As much as 3 mL of the ethyl acetate fraction was put into a test tube, then two drops of 1% ferric chloride were added. A change in the solution from yellow to a greenish-black color indicates the presence of tannins.

Preparation of animals' test

This research was approved by the Health Research Ethics Committee Health Polytechnic of Ministry of Health East Kalimantan, Indonesia, with ethical clearance statement number DL.02.03/4.3/18854/2022. The test animals were male mice (*Mus musculus*) with a body weight of 20-30 g quarantined to adapt to the laboratory environment. There were 33 mice as test animals divided into 11 groups; each group contained three mice. One group served as positive control, which was treated with sodium diclofenac orally. The negative control group was treated with a blank. Nine groups serve as the treatment groups. Three groups were treated with 30, 60, and 120 mg/kg honey, another three groups were treated with 30, 60, and 120 mg/kg propolis extract, and the last three groups were treated with the combination of honey and propolis with the composition of 25 : 75; 50 : 50; and 75 : 25.

Preparation of 1% carrageenan suspension

As much as 100 mg of carrageenan was weighed then homogenized using 0.9% NaCl solution and then put into a volumetric flask and added 0.9% NaCl solution up to 10 mL[11].

Preparation of diclofenac sodium

A total of 100 mg of diclofenac sodium powder was weighed and then put into a 100 mL beaker glass and added water little by little while stirring with a stir bar until homogeneous, then put into a volumetric flask and made up to 100 mL.

Anti-inflammatory test

The anti-inflammatory test was done using carrageenan-induced paw edema[12]. The edema volume was measured using a mercury plethysmometer. The initial stage of the study was to measure each test animal's body weight and foot volume with a scale and a plethysmometer as initial data (baseline). After that, each mouse was given treatment according to its group. Each mouse was injected with carrageenan solution in the sole of its right foot as much as 0.1 mL. Carrageenan injection was carried out subplantar. After being injected with carrageenan, the mice were given the test solution according to the group orally. Thirty minutes later, the foot volume was measured using a mercury plethysmometer by dipping the sole of the rat's right foot until the mark, and then the edema volume was recorded. Measurements were made every 30

minutes until 150 minute after the treatment. Inflammation volume was the difference in the volume of the paws of the rats after and before being injected with carrageenan. At the time of measurement, the volume of the liquid in the plethysmometer must be kept at the same level each time the measurement, and the mark on the rat's foot must be visible. The measurement was done by submerging the rat's foot until the level of the mark on the foot.

Data analysis

The results of anti-inflammatory measurements were summarized using mean and standard error. The edema data of each group was plotted in a time course graph. The area under the curve (AUC) was calculated using the trapezoid method from the graph. The anti-inflammatory effects were measured using the following **Equation 1**. The statistical comparison between groups was analyzed using the ANOVA method and continued with a post hoc test.

$$Anti - inflammatory\ effect: \frac{AUCn - AUCt}{AUCn} \times 100\% \qquad [1]$$

AUC_n: Area under curve of negative control group
AUC_t: Area under curve of treatment group

RESULTS AND DISCUSSION

Phytochemical determination

The phytochemical determination of the secondary metabolite of *T. apicalis* propolis and honey was examined by reagent, while their secondary metabolite demonstrated color change. **Table I** compares the phytochemical analysis of *T. apicalis* propolis and honey. Fadzilah *et al*.[13] reported the activity of antioxidants in propolis from Malaysia of type *Heterotrigona itama*, *T. apicalis*, *T. thoracica*, and others. Our previous study[14] also showed that *Trigona* bee species originating from East Kalimantan have antioxidant and antibacterial activity. The activity is also influenced by the compounds contained therein. Propolis extract has positive phytochemical test results for alkaloid compounds. Alkaloid compounds have anti-malarial, anti-cancer, antioxidant, antimicrobial, anti-inflammatory, anti-obesity, and anti-HBV properties[15].

Flavonoid compounds are generally found in every part of the plant, such as seeds, fruit, stamens, roots, and stems. *Trigona apicalis* propolis extract and honey also showed positive test results for containing flavonoids. Flavonoid compounds have antioxidant activity[16]. Propolis and honey by *T. apicalis* are obtained from plants. Triterpenoid constituents from plants act as protective agents against insect and bacterial attacks[17]. Terpenoids were detected in both *T. apicalis* propolis and honey. Tannins are polyphenolic compounds that are naturally found in vegetables. Polyphenols comprise a large family of secondary metabolites stored in plant cell vacuoles, such as esters or glycosides. Tannins are considered high molecular-weight polyphenols[18].

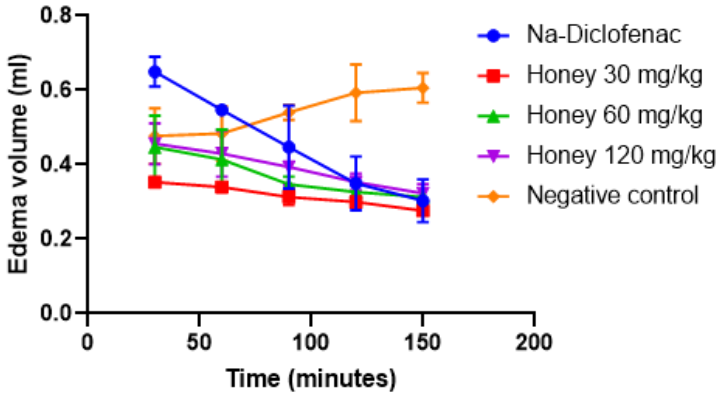
Table I. Phytochemical analysis of *T. apicalis* propolis and honey

Phytochemical content	Propolis	Honey
Alkaloid	+	-
Flavanoid	+	+
Triterpenoids/steroids	+/-	+/+
Saponin	+	+
Tannin	+	+

Anti-inflammation determination

Figure 2 shows the time course change of the edema volume in mice paws induced by carrageenan. Sodium diclofenac was a positive control for the assay, and the negative control group was treated with a vehicle. The treatment of honey and propolis suppresses the edema volume for 30 minutes after treatment. Meanwhile, in the positive control group, the edema volume is higher compared to the other group, including the negative control. The late onset of sodium diclofenac could cause this effect as the edema volume dropped drastically after 60 minutes. The negative control group showed increased edema volume and peaked after 150 minutes. The honey, propolis, and a combination treatment showed lower edema volume 30 minutes after treatment than the positive control. The different compositions of honey and propolis combination do not affect the anti-inflammatory effect, as seen in **Figure 2C**. All the compositions have a similar slope for reducing edema

volume. The treatment of 30 mg/kg honey gives better inflammation inhibition than the other concentrations; meanwhile, all propolis doses provide a similar anti-inflammation effect.



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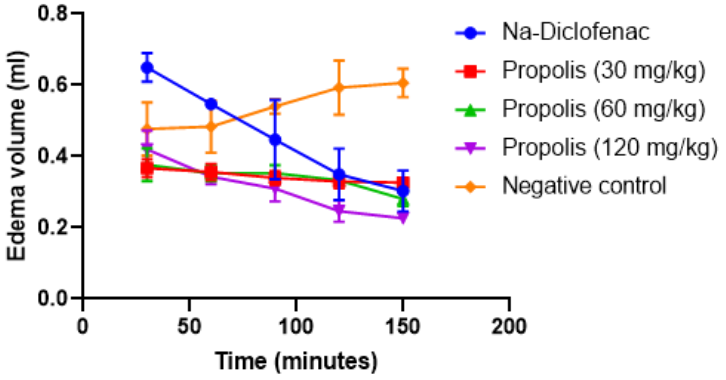

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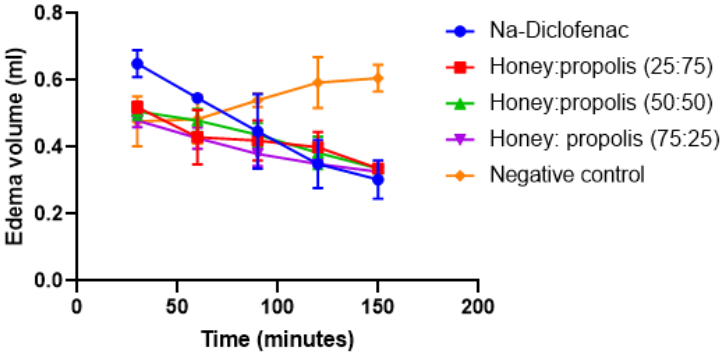

c

Figure 2. The time course change of rat paw edema volume after treatment with (**a**) honey, (**b**) propolis, and (**c**) a combination of honey and propolis. Sodium diclofenac 10 mg/kg served as a positive control, and the negative control group was treated with blank.

The anti-inflammatory effect was obtained by calculating the AUC from each group and compare to the AUC of the negative control group. The percentage of anti-inflammatory effects is shown in **Figure 3**. The result showed that the treatment of ethyl acetate fraction of propolis at doses 30, 60, and 120 mg/kg had a significant difference compared to sodium diclofenac. In contrast, treatment with honey only showed a significant difference at 30 mg/kg. Combining honey and ethyl acetate fraction of propolis did not enhance the anti-inflammatory activity. All honey and ethyl acetate fraction combinations of propolis (25 : 75; 50 : 50; and 75 : 25) have no significant difference compared to the sodium diclofenac group. The highest anti-inflammatory effect was obtained from the ethyl acetate fraction of propolis treatment at 120 mg/kg.

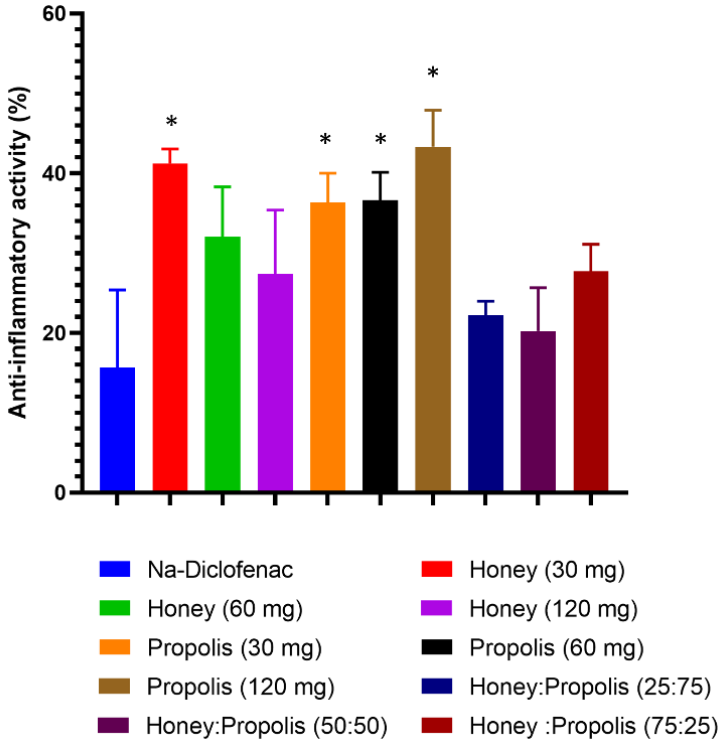

Figure 3. Anti-inflammatory effect of honey, propolis, and the combination of honey and propolis given to the mice induced with carrageenan compared to sodium diclofenac treatment (*p <0.05).

Carrageenan-induced edema is the most used method to generate acute inflammation in vivo and is suitable for evaluating the effect of oral non-steroidal anti-inflammatory agents. The injection of carrageenan elevates inflammatory mediators such as bradykinin, histamine, prostaglandins, and reactive oxygen species[19]. This study showed that treating the ethyl acetate fraction of propolis and honey exhibits anti-inflammatory activity. One of the most important compositions of propolis is caffeic acid phenethyl ester (CAPE). The CAPE has exhibited anti-inflammatory properties by reducing the expression of cyclooxygenase (COX) enzyme and blocking the release of arachidonic acid, decreasing prostaglandins and leukotrienes synthesis. At the molecular level, CAPE is known to reduce the activity of nuclear factor-kappa B (NF-kb) and reduce various inflammatory mediator cytokines such as IL-8[20]. Furthermore, the flavonoid contents of propolis also enhance the anti-inflammatory effect by inhibiting the cyclooxygenase activity[21].

Honey contains various phenolic compounds due to the natural diet of the bees. The phenolic compounds such as quercetin, chrysin, ferulic acid, hesperetin, and ellagic acid are responsible for the anti-inflammatory effect of honey by modulating the activity of cyclooxygenase 2 as well as nitric oxide production by inhibiting inducible nitric oxide synthase (iNOS)[22-24]. In addition, several compounds found in stingless bee honey, for instance, kaempferol and caffeic acid, have been shown to have an anti-inflammatory effect on ear edema-induced mice[25]. Interestingly, the combination of propolis and honey in all compositions showed a lesser effect on anti-inflammatory activity than treatment with propolis and honey alone. This effect can be caused by the chemical interaction that occurs when honey and propolis are mixed. However, the anti-inflammatory effect of this combination is still slightly higher than the positive control group but not significantly different.

CONCLUSION

Administration of honey and propolis had a better effect on reducing edema volume than the positive control. The combination of honey at a dose of 30 mg and ethyl acetate fraction of propolis at a dose of 120 mg gave the best effect as an anti-inflammatory in experimental animals. Combining honey and propolis preparations with a ratio of 75 : 25 has a better effect on reducing edema volume than the other two combinations. However, this combination was not significantly different than positive control.

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AUTHORS' CONTRIBUTION

Conceptualization and Methodology: Paula Mariana Kustiawan, Sinta Ratna Dewi, Chaerul Fadly Mochtar Luthfi M. **Extraction and phytochemical determination**: Paula Mariana Kustiawan. **Anti-inflammatory test**: Jati Pratiwi, Chaerul Fadly Mochtar Luthfi M, Alfin Syahrian Dwi Nugraha, Novia Misnawati Aisyiyah. **Data analysis and statistics**: Irfan Muris Setiawan, Paula Mariana Kustiawan. **Writing, review, and editing of manuscript**: Paula Mariana Kustiawan, Irfan Muris Setiawan.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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Research Article

Tentative Identification of Compounds, Antioxidant, and Antimicrobial Activity of the Edible Part of *Benincasa hispida* L. Fruit (Cucurbitaceae)

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Keywords:
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Abstract

The edible part of *Benicasa hispida* (Thunb.) Cogn. fruit is traditionally used in Southeast Sulawesi to treat high blood pressure, typhoid fever, and body cooling. The present study evaluated the chemical compounds present in the 80% ethanol of the edible part of the plant using phytochemical screening and an LC-MS analysis, antioxidant activity based on assays on total phenolics content (TPC), total flavonoids content (TFC), and DPPH, and antimicrobial activity towards *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. Phytochemical screening revealed the presence of tannins, flavonoids, terpenoids, steroids, and saponins in the extract. As many as eighteen compounds (**1-18**) were tentatively identified in the extract, including sugars, a simple phenolic, a tricarboxylic acid, a peptide, flavonoids, quinic acid derivatives, phytosterols, triterpenoids, and saponins. The extract exhibited remarkable antioxidant activity with an SC_{50} value of 23.4 µg/mL, although its TPC (1.1±0.1 mg GAE/g extract) and TFC (1.0±0.1 mg QE/g extract) values were considered in low amounts. The extract was found inactive to inhibit the microbial growths of all tested microbes. However, raffinose (**3**) present in the extract might be beneficial as a prebiotic to promote a healthy human gut. The study concludes that the 80% ethanol extract of the edible part of *B. hispida* fruit could be used to develop natural antioxidant agents and nutraceuticals.

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INTRODUCTION

Benincasa hispida (Thunb.) Cogn. is a herbaceous climber that belongs to the Cucurbitaceae family. It is one of the Cucurbitaceae crops known as wax gourd due to its white wax-covered fruit. The fruit's edible part (mesocarp) is white, spongy, and succulent. This part is the main part of the plant used by natives in making food[1]. In some countries like Malaysia, India, and China, the plant is traditionally used to treat coronary diseases, gastrointestinal tract problems, urinary tract and kidney stones problems, metabolism ailments, constipation, fever, diabetes, and obesity[2,3]. In Southeast Sulawesi, the plant is known by different vernacular names by locals, such as *konduru* (Bombana, Konawe, Baubau, Muna, Kolaka), *kundur* (Kendari), and *sudeng* (Bugis, Kolaka). Traditionally, the edible part of *B. hispida* fruit is processed as soup or juice and given orally to treat high blood pressure[4,5], typhoid fever[6], and for cooling the body. For daily food consumption, this part is processed as vegetable soups like *sayur bening* (clear soup) and *sayur santan* (coconut soup). Hence, based on medicinal uses and functional nutrition, the fruit of *B. hispida* is easy to find in local markets.

Despite its traditional uses, studies on chemical compounds and biological activities of *B. hispida* from Southeast Sulawesi are still a handful, which only one report on antibacterial activity[7]. Hence, the current status of *B. hispida* emerges for further investigation. The present study evaluated the chemical compounds of the 80% ethanol extract of edible part of *B. hispida*

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fruit using phytochemical screenings and an LC-MS analysis, antioxidant activity based on assays on total phenolics content (TPC), total flavonoids content (TFC), and DPPH, as well as antimicrobial activity towards *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*.

MATERIALS AND METHODS

Materials

Chemicals

Ethanol (cat. no: 1.00983), HPLC grade of acetonitrile (cat. no: 1.00030), Folin-Ciocalteu (FC) reagent (cat. no: 1.09001), ascorbic acid (cat. no: 1.00468), dimethyl sulfoxide (DMSO, cat. no: 1.02952), sodium bicarbonate (cat. no: 1.06239), aluminum sheet thin layer chromatography (TLC) plate (silica gel 60 GF254, 0.25 mm, 20 × 20 mm, cat. no: 1.05554), Mueller-Hinton agar (MHA, cat. no: 1.05437), and potato dextrose agar (PDA, cat. no: 1.10130) were obtained from Merck (Darmstadt, Germany). Gallic acid (cat. no: G7384) dan quercetin (cat. no: G4951) were obtained from Sigma-Aldrich (St. Louis, US). Meanwhile, aluminum chloride hexahydrate (cat. no: 898) was purchased from Loba Chemie (Mumbai, India), and 2,2-diphenyl-1-picrylhydrazyl (DPPH, cat. no: MB263) was purchased from HiMedia (Mumbai, India). Deionized water (Waterone) was purchased from OneMed (Jakarta, Indonesia). Chloramphenicol and ketoconazole were purchased from Kimia Farma (Jakarta, Indonesia).

Microbial cultures

Microbial cultures used were *S. typhi* ATCC 14028, *E. coli* ATCC 35218, *S. aureus* ATCC 25923, and *C. albicans* ATCC 10231, which represent Gram-negative bacteria, Gram-positive bacteria, and fungi, respectively.

Plant sample

Fruits of *B. hispida* were freshly collected from local markets in Kolaka regency, Southeast Sulawesi, Indonesia, in February 2022. The sample was taxonomically authenticated using a literature1, and its voucher specimen (number BH001) was deposited at the Laboratorium Terpadu USN Kolaka. The morphology of the fruit is displayed in **Figure 1**.

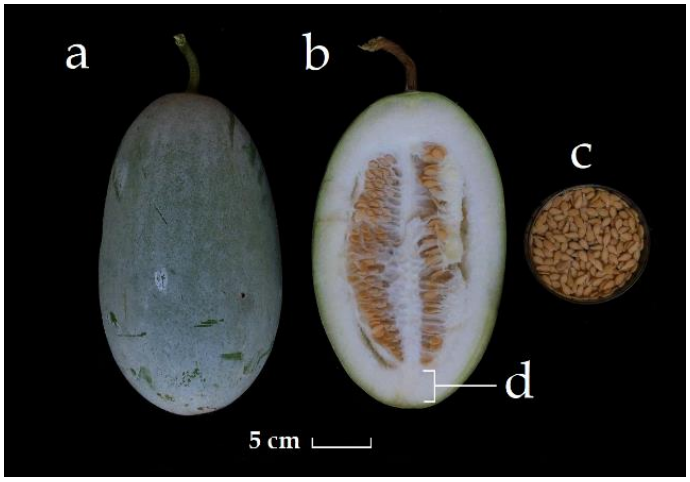


Figure 1. Fruits of *B. hispida* (**a**), cross-sectional of the fruit (**b**), seeds (**c**), and the edible part (**d**).

Methods

Sample preparation

A sample of the fruit was washed using tap water and drained. The edible part (**Figure 1d**) was separated from the skin and seeds by cutting. This part (26.514 kg) was dried at an optimized temperature of 50°C for three days using an oven (Maksindo, Indonesia). The dried edible part was coarsely powdered using a dry blender and yielded coarse powder (0.822 kg) with a percentage yield of 3.1% and a water content of 96.9%. The coarse powder was then kept in a zip lock pouch until use.

Sample extraction

The coarse powder of the edible part of *B. hispida* fruit (0.274 kg) was macerated using 80% ethanol as the solvent. Maceration of the sample was executed for 3 × 48 hours at room temperature. The 80% ethanol extract was obtained after manual filtration, and the solvent was evaporated using a vacuum rotary evaporator (Biobase, China). Then, the extract was weighed and stored in a refrigerator at 4°C until use.

Phytochemical screening

Phytochemical screenings were performed according to a previous work[8] to detect the presence of alkaloids, tannins, flavonoids, terpenoids, steroids, and saponins in the 80% ethanol extract of the edible part of *B. hispida* fruit. The qualitative observation was also made based on results on color intensities for alkaloids (reddish orange), tannins (brownish green/dark blue), flavonoids (red), terpenoids (reddish brown), steroids (fluorescence greenish-yellow), and the formation of foam for saponins with the scale of high (+++), fair (++), and low (+) of their presence in the extract.

LC-MS/MS analysis

Chemical compounds in the 80% ethanol extract were evaluated using a UPLC aligned with an MS analyzer (Waters Xevo Tandem Quadrupole (TQD) Mass Spectrometer, Waters, Ireland) according to our previous method[9] with some adaptations. Separation of the compounds was performed using a reverse phase UPLC BEH C18 column (2.1 × 50 mm, particles 1.7 µm) and gradiently eluted with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The elution was performed for 20 minutes with a flow rate of 0.4 mL/minute (**Table I**). The extract was dissolved in ethanol (90%) and filtered using a Millex membrane filter of 0.22 µm. The injection volume was 5 µL. For mass analysis, a positive mode electrospray ionization (ESI) was performed using the following parameters: mass range of m/z 50 to m/z 1200, cone voltage 30 V, capillary voltage 3.0 kV, and source temperature 500°C. The m/z values of separated peaks and their fragmentations were analyzed using online mass databases (PubChem, ChemSpider, CheBI, KEGG, mzCloud, and Mass Bank). The identification of tentative compounds as natural products was cross-checked using the Dictionary of Natural Products and LOTUS (Natural Products Online).

Table I. Gradient elution

Time	%A (water-0.1% formic acid)	%A (acetonitrile-0.1% formic acid)
0 minute	95	5
15 minutes	0	100
5 minutes	95	5

Total phenolics content (TPC) and total flavonoid content (TFC) assays

Both TPC and TFC values in the 80% ethanol extract were spectrophotometrically evaluated using Folin-Ciocalteu and aluminum chloride methods, respectively, according to previous reports[9,10], with some modifications. The 80% ethanol extract was diluted in ethanol (96%), and the final concentration in the reaction mixture was 1000 µg/mL. For TPC, 20 µL of diluted extract in a 96-well microplate was added with 100 µL of Folin-Ciocalteu (10%, v/v, in water) and incubated for 5 minutes at room temperature. Then, the mixture was added with 80 µL of sodium bicarbonate (7.5%, w/v, in water) and re-incubated for 30 minutes at room temperature in the dark. The absorbance of the reaction mixture was read at 765 nm using a microplate reader (Spectrostar Nano, BMG Labtech, Germany). Meanwhile, for TFC, the reaction mixture was 100 µL of diluted extract and µL of aluminum chloride (2%, w/v, in water). The mixture was incubated for 15 minutes, and its absorbance was read at 435 nm using a microplate reader. Both TPC and TFC experiments were repeated three times. Concentrations of phenolics and flavonoids in the diluted extract were calculated using equations from their respective linear standard curves, that is, a gallic acid standard curve (absorbance = (0.083 × concentration of gallic acid in µg/mL) + 0.2006, $r^2 = 0.990$) for TPC and a quercetin standard curve (absorbance = (0.0459 × concentration of quercetin in µg/mL) + 0.0189, $r^2 = 0.999$) for TFC. Both curves were established by plotting the absorbance versus concentrations ranging from 1 to 31.2 µg/mL. Then, TPC (mg GAE/g of extract) and TFC (mg QE/g of extract) values were calculated using the **Equation 1**, where C was the concentration of gallic acid or quercetin plotted using the linear standard curves, FV was the final volume used for dilution, d was the dilution factor, and W was the weight of 80% ethanol extract.

$$mgGAE\ or\ mg\ QE: \frac{(C \times FV \times d)}{W} \qquad \textbf{[1]}$$

DPPH assay

The radical scavenging activity of the 80% ethanol extract was evaluated using qualitative and quantitative DPPH assays according to the previous methods[9,10] with slight modifications. For qualitative analysis, five serial solutions of the extract in 96% ethanol were spotted at 20 µL on the TLC plate, then dipping the plate into a DPPH solution (0.4 mM in 96% ethanol) for 10 seconds. After drying with an air dryer, the plate was incubated for 30 minutes in the dark at room temperature. The white zone formed around the spot against the purple DPPH on the plate, indicating radical scavenging activity. The final sample concentrations in the plate ranged from 12.5 to 200 µg/spot. Ascorbic acid was used as the positive control of the assay. For quantitative analysis, the reaction mixture consisted of 100 µL of extract solution (0.8 to 100 µg/mL, in 96% ethanol) and 100 µL of DPPH solution (40 µg/mL, in 96% ethanol) was incubated for 15 minutes in the dark at room temperature. Then, the reaction mixture absorbance was read at 515 nm. The percentage of radical scavenging activity (%RSA) was calculated using the **Equation 2**. The absorbance of the sample was corrected using the sample blank containing sample and ethanol. The 50% radical scavenging concentration (SC_{50}) values of the extract and positive controls (ascorbic acid, gallic acid, and quercetin) were obtained from a nonlinear regression (curve fit) using GraphPad Prism 5.

$$\%RSA: \frac{(Absorbance\ of\ DPPH - Absorbance\ of\ sample)}{Absorbance\ of\ DPPH} \times 100\% \qquad \textbf{[2]}$$

Antimicrobial assay

The antimicrobial activity of the 80% ethanol extract was evaluated using an agar well diffusion method according to the previous studies[11,12]. The extract solution was made in DMSO-normal saline (1-2%). The activity was first screened at a high concentration of 10,000 µg/well. Chloramphenicol (20 µg/well) and ketoconazole (0.5 µg/well) were used as the positive controls of the assay for bacteria and fungi, respectively. The surface of solidified media MHA or PDA in a Petri dish was inoculated with 1 mL of microbial culture (0.5 McFarland). Wells for extract, positive control, and solvent control were prepared by aseptically punching the inoculated media using yellow tips and discarding the tips. After that, samples were added to the wells accordingly (40 µL/well), and the Petri dish was incubated at 37 °C for 24 hours for bacteria and 48 hours for fungi. The observation of a clear zone around the well was done two times at 18 and 24 hours for bacteria and 24 and 48 hours for fungi. The diameter of the clear zone as the inhibition zone indicated the antimicrobial activity.

Data analysis

Data from TPC, TFC, DPPH, and antimicrobial assays were obtained from three repeated experiments (n = 3) and presented as mean ± standard deviation (SD). SC_{50} values were determined using GraphPad Prism 5 (GraphPad Inc., California, US).

RESULTS AND DISCUSSION

Yield of extraction and phytochemical screening

The maceration of the edible part of *B. hispida* fruit using 80% ethanol as the solvent yielded a reddish-brown extract with a percentage yield of 17.3% (47.49 g). Phytochemical screenings of this extract showed the occurrence of tannins, flavonoids, terpenoids, steroids, and saponins (**Table II**). Meanwhile, alkaloids were not detected, possibly due to either their absence or low amounts in the extract since previous studies have reported the presence of pyrazine derivatives and volatile nitrogen-containing compounds in *B. hispida* fruit[13]. Additionally, considering the polarity of the solvent used, these compounds might not be extracted during extraction. On the contrary, terpenoids were qualitatively present in high amounts by observing an intense reddish-brown layer during the experiment, suggesting the presence of polar terpenoids in the extract.

Table II. Phytochemical groups detected in the 80% ethanol extract of edible part of *B. hispida* fruit

Phytochemical group	Detection	Qualitative observation
Alkaloid	Absent	-
Tannin	Present	+
Flavonoid	Present	+
Terpenoid	Present	+++
Steroid	Present	+
Saponin	Present	+

Tentative identification of compounds

The LC-MS/MS analysis of the 80% ethanol extract of the edible part of *B. hispida* fruit yielded variable peaks of compounds in the total ion mass chromatogram starting from the retention time (T_R) of 0.52 to 16.20 minutes (**Figure 2**). Some major peaks are also observed based on their peak height, such as at the retention time of 0.52, 9.52, 9.71, and 14.19 minutes. The analysis of molecular ion and fragmentation patterns of the separated peaks resulted in the tentative identification of 18 compounds, including sugars (**1, 3**), a simple phenolic (**2**), a tricarboxylic acid (**4**), a peptide (**5**), flavonoids (**6, 7, 8**), quinic acid derivatives (**9, 10**), phytosterols (**11, 12, 13**), triterpenoids (**14, 18**), and saponins (**15, 16, 17**). These compounds are listed in **Table III**, and their structures are displayed in **Figure 3**.

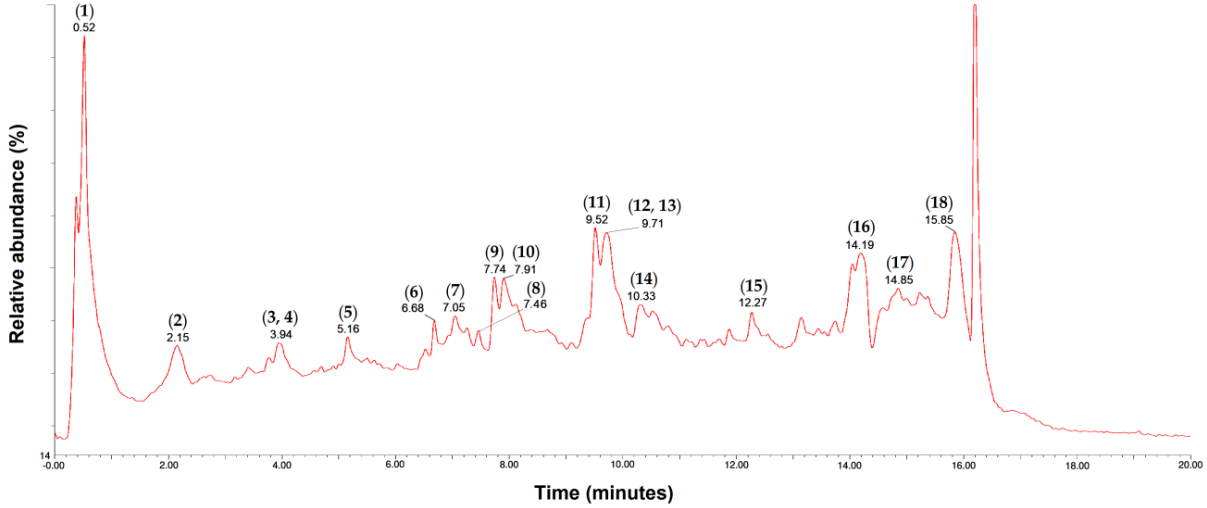


Figure 2. Total ion chromatogram (TIC) of compounds in the 80% ethanol extract of edible part of *B. hispida* fruit.

Table III. Tentative compounds identified in the 80% ethanol extract of edible part of *B. hispida* fruit

T_R (min)	ES(+)	Ion type	MS/MS; m/z (% base peak)	Tentative compound name	Structure number	Formula	MW
0.52	381.69	$[M+K]^+$	291, 148, 118, 104 (100)	Sucrose	**1**	$C_{12}H_{22}O_{11}$	342.29
2.15	171.44	$[M+H]^+$	171 (100)	Gallic acid	**2**	$C_7H_6O_5$	170.11
3.94	622.10	$[M+4H_2O+2Na]^+$	522 (20), 504	Raffinose	**3**	$C_{18}H_{32}O_{16}$	504.43
3.94	229.54	$[M+2H_2O]^+$	193 (60)	Citric acid	**4**	$C_6H_8O_7$	192.12
5.16	532.05	$[M+H]^+$	309 (40), 225	3-*S*-glutathione-dihydrosinapic acid	**5**	$C_{21}H_{29}N_3O_{11}S$	531.53
6.68	291.71	$[M+H]^+$	261 (100), 167	Catechin	**6**	$C_{15}H_{14}O_6$	290.26
7.05	478.99	$[M+2H]^+$	381, 327, 311 (100), 301, 229, 208, 167	Chrysoeriol-7-*O*-glucuronide	**7**	$C_{22}H_{20}O_{12}$	476.38
7.46	480.99	$[M+H]^+$	301 (100), 229, 208, 167	Myricetin-3-O-glucoside	**8**	$C_{21}H_{20}O_{13}$	480.37
7.74	339.67	$[M+H]^+$	323 (100) , 241, 218, 167	3-*p*-coumaroylquinic acid	**9**	$C_{16}H_{18}O_8$	338.30
7.91	427.79	$[M+H]^+$	411, 339, 323 (100), 241, 218, 167	4-hydroxybutyl-chlorogenate	**10**	$C_{20}H_{26}O_{10}$	426.41
9.52	409.91	$[M+H]^+$	343, 327 (100), 313, 273, 241, 213	Stigmasta-4,22*E*,25-trien-3-one	**11**	$C_{29}H_{44}O$	408.65
9.71	410.95	$[M]^+$	343, 327 (100), 313, 273, 241, 213	5-dehydroavenasterol	**12**	$C_{29}H_{46}O$	410.67
				Stigmasta-7,22*E*,25-trien-3β-ol	**13**	$C_{29}H_{46}O$	410.67
10.33	443.79	$[M+H]^+$	427 (40), 327, 233, 208	Cucurbita-5,23-diene-3β,25-diol	**14**	$C_{30}H_{50}O_2$	442.72
12.27	576.76	$[M]^+$	469, 377, 337, 279 (10), 208, 152	Daucosterol	**15**	$C_{35}H_{60}O_6$	576.84
14.19	1023.76	$[M+H+Na]^+$	815, 798, 697, 664, 614, 566, 464, 436 (20), 338, 327, 284, 217, 152	Oleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-*a*-L-rhamnopyranosyl-(1→2)-*a*-L-arabinopyranoside	**16**	$C_{51}H_{82}O_{19}$	999.18
14.85	1059.78	$[M+H_2O]^+$	815, 798, 686, 664, 588, 566, 464, 436, 381, 181, 152	3β-O-acetyloleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-*a*-L-rhamnopyranosyl-(1→2)-*a*-L-arabinopyranoside	**17**	$C_{53}H_{84}O_{20}$	1041.22
15.85	571.10	$[M+H]^+$	554 (100), 532, 441, 338, 152	25-O-acetyl-16,20-dihydroxy-3-methoxy-cucurbita-5(10),6,23-triene-3,11,22-trione	**18**	$C_{33}H_{46}O_8$	570.71

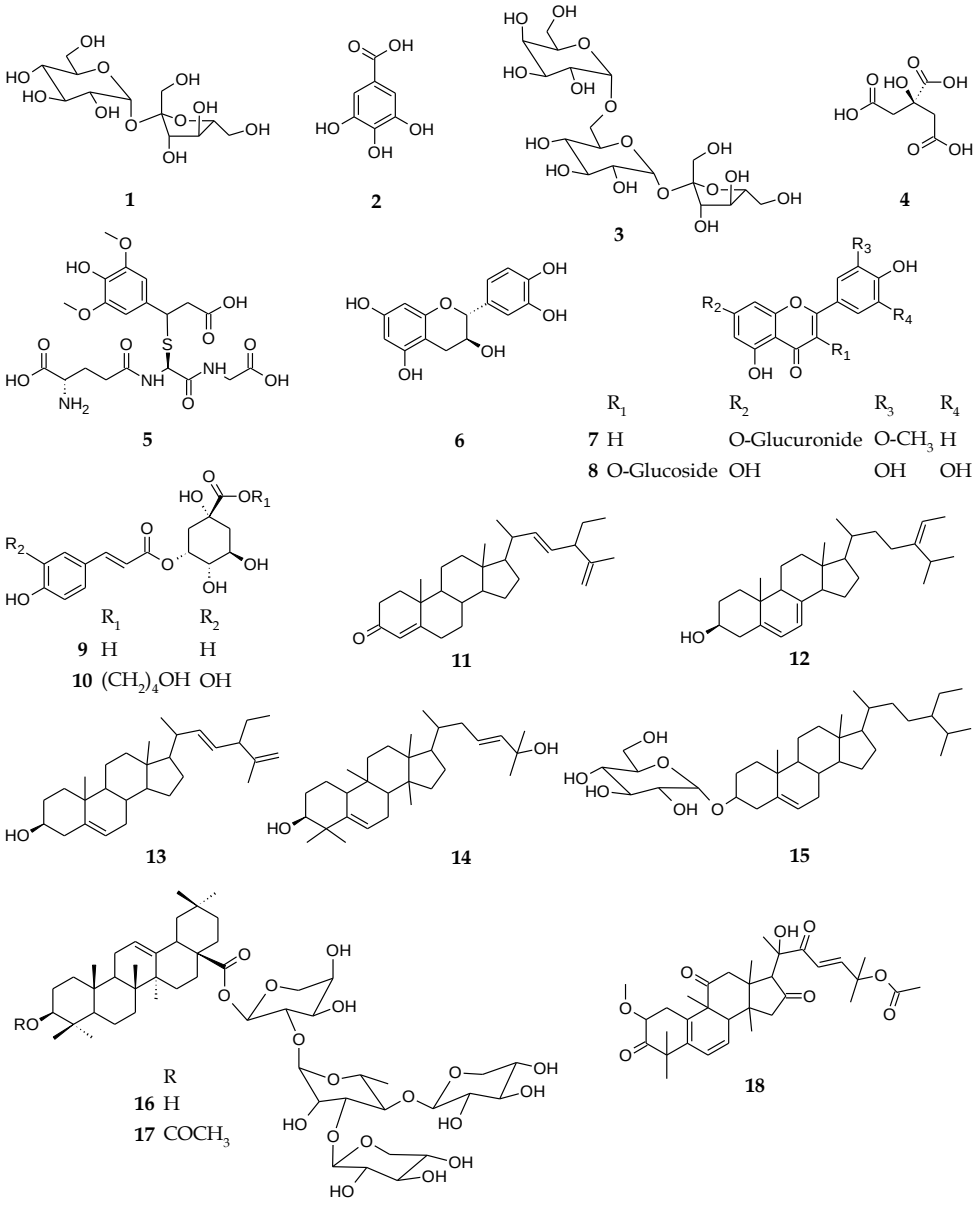


Figure 3. Structure of tentative compounds (**1**-**18**) in the 80% ethanol extract of edible part of *B. hispida* fruit.

The presence of sugars (**1, 3**) in the 80% ethanol extract of the edible part of *B. hispida* fruit validated its nutritional components. The LC-MS/MS spectrum (**Figure 2**) showed a major peak at a retention time of 0.52 minutes, having a sodium adduct ion at m/z 381.69 $[M+Na]^+$. Fragment ions of this peak at m/z 118 and 104 indicated the pattern of disaccharides[14], for which sucrose (**1**) is tentatively assigned for the peak. A recent study has reported sucrose (**1**) as a component for the taste and nutrition of *B. hispida* fruit, along with glucose and fructose. This study shows that sucrose (**1**) composition decreased with fruit development while glucose and fructose increased[15]. In line with our present study, the fruit sample used was unripened at the age of approximately four months. Hence sucrose (**1**) is detected as a major compound in the extract, while glucose and fructose are undetectable. Another sugar was found at the retention time of 3.94 minutes and had an adduct ion of $[M+4H_2O+2Na]^+$ at m/z 622.10, followed by fragment ions at m/z 522 $[M+H_2O]^+$, m/z 504 $[M]^+$ and m/z 143 as the base peak. This pattern is typical of trisaccharides[14], which based on previous studies[16,17], raffinose (**3**) is tentatively identified. The family of Cucurbitaceae has been reported to produce oligosaccharides, mainly raffinose (**3**) and stachyose. Both of them are galactosyl-sucrose which plays a role as transport sugars in plants of this family[17]. Related to biological activity, polysaccharides from *B. hispida* fruit showed an antiglycation effect and antioxidant activity, which benefit for reducing glucose levels in diabetic patients[18]. Hence, more studies on the antidiabetic activity of *B. hispida* fruit are a noble challenge.

In addition to sugars, organic acids contribute to the taste and nutrition of the *B. hispida* fruit such as citric acid (**4**) and malic acid[15]. The peak of citric acid (**4**) was observed at the retention time of 3.94 minutes, overlapping with raffinose (**3**) and having a base peak of m/z 193 for a protonated molecular ion $[M+H]^+$. The citric acid (**4**) and malic acid are organic acids accumulated in the Cucurbitaceae family during fruit development. Their contents are higher in the young fruits stage and gradually decrease during maturity. The antimicrobial activity of this compound (**4**) has been reported[19].

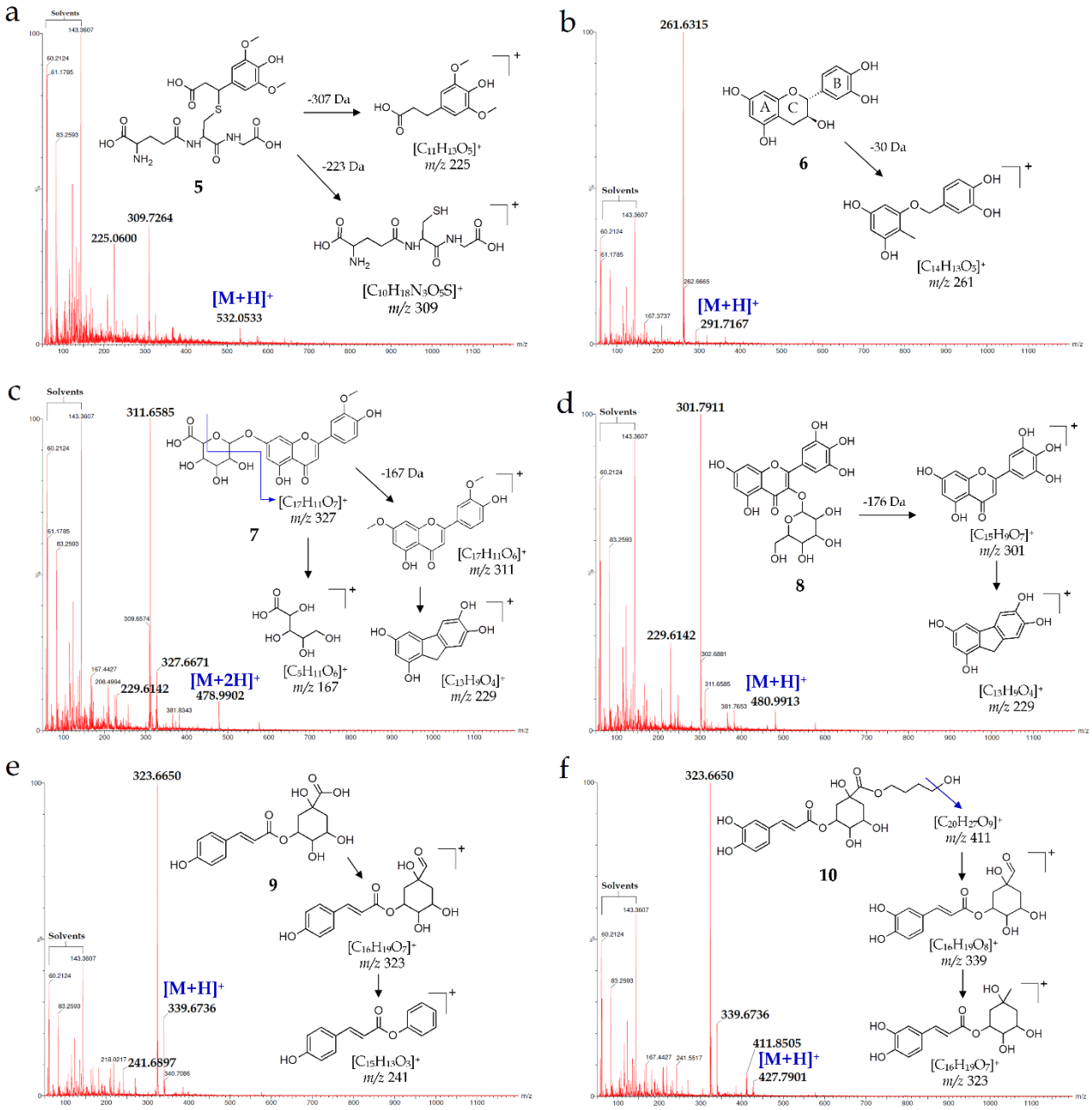
As part of the nutrition components in the *B. hispida* fruit, a peptide was also detected at a retention time of 5.16 minutes with a protonated molecular ion at m/z 532.05 $[M+H]^+$. Two fragment ions were observed at m/z 309 for a protonated glutathione and m/z 225 for a protonated sinapic acid (**Figure 4a**). The fragmentation is similar to those reported in the literature[20]. Hence, this compound was tentatively assigned as 3-S-glutathione-dihydrosinapic acid (**5**), and its presence as a natural product is first reported in the present study. The glutathione side in compound (**5**) might be a potential for antioxidant activity. Apart from the finding, a common peptide found in the Cucurbitaceae family is known as citrulline and has also been reported in the fruit of *B. hispida*[15]. However, this peptide was not detected in this study's 80% ethanol extract.

Some phenolics, including flavonoids also detected in the 80% ethanol extract of the edible part of *B. hispida* fruit. Gallic acid (**2**) was assigned to a peak at the retention time of 2.15 minutes, having a protonated molecular ion $[M+H]^+$ at m/z 171.44[21]. Furthermore, flavonoids (**6, 7, 8**) in the 80% ethanol extract were consecutively observed at the retention time of 5.16, 6.68, and 7.05 minutes, having molecular ions at m/z 291.71, m/z 478.99, and m/z 480.99, respectively. The molecular ion at m/z 291.71 was a protonated ion $[M+H]^+$, which is the base peak at m/z 261, showing a prominent fragment of catechin (**6**) after a loss of its hydroxyl group (3-OH) attached to the ring C of the structure and fission of that ring (**Figure 4b**). On the other hand, the molecular ion at m/z 478.99 was predicted as chrysoeriol-7-O-glucuronide (**7**) based on its base peak at m/z 311 after the elimination of a glucuronide group (167 Da). The presence of this group in the structure was further confirmed by a fragment ion at m/z 167 in the spectrum (**Figure 4c**). Furthermore, the molecular ion at m/z 480.99 had a base peak at m/z 301, indicating the fragment ion of myricetin after the elimination of the O-glucoside group (179 Da), for which myricetin-3-O-glucoside (**8**) is tentatively assigned for this peak (**Figure 4d**). Both compounds **7** and **8** had a fragment ion at m/z 229, which is predicted as a structural arrangement of flavone and flavonol skeletons, especially those that have 5, 7, 3', and 4'-hydroxy substitution, such as apigenin, myricetin, and quercetin. Previous studies have reported the occurrence of gallic acid (**2**) in the Cucurbitaceae family[22] and *B. hispida* from Malaysia[23]. Similarly, catechin (**6**), along with gallic acid (**2**), is also present in the *B. hispida* fresh juices from Romanian[24] and has been isolated from the dried flesh of *B. hispida* from Hainan, China[25]. As far as our search, compounds **7** and **8** have yet to be reported from *B. hispida*. However, various flavones and flavonols, as well as their glycosides, have recently been reported from the pericarp of *B. hispida* fruit also from China[26], which supported the findings of the present study.

In addition to flavonoids, two quinic acid derivatives (**9** and **10**) peaks were consecutively observed at the retention time of 7.74 and 7.91 minutes, respectively. The molecular ion of the first peak was at m/z 339.67, with a base peak at m/z 323 after a loss of a hydroxyl group. Further fragment ions of this peak were at m/z 241, 218, and 167, showing a pattern of 3-p-coumaroylquinic acid (**9**) (**Figure 4e**). This compound has recently been reported from the seed of *Cucumis sativus*[27]. Compound **10** was predicted to have a skeleton as in compound **9** since some fragmentation patterns are similar. The LC-MS/MS spectrum showed additional fragment ions at m/z 427 as a protonated molecular ion $[M+H]^+$ and m/z 411 for a loss of one hydroxyl group. The ion at m/z 411 has been reported for butyl chlorogenate[28], which further fragment ion at m/z 339 indicated an elimination of a butoxy side ($C_4H_8O^+$, 72 Da) from the main skeleton of chlorogenate (**Figure 4f**). Hence, the compound was tentatively identified as 4-hydroxybutyl-chlorogenate (**10**). The occurrence of chlorogenic acid has been reported in the Cucurbitaceae family[22]. However, to the best of our knowledge, the occurrence of 4-hydroxybutyl-chlorogenate (**10**) in the family has yet to be reported.

Two cucurbitanes (**14**, **18**) were observed in the LC-MS/MS spectrum at the retention time of 10.33 and 15.85 minutes, respectively. Compound **14** had a protonated molecular ion $[M+H]^+$ at m/z 443.79 with fragment ions at m/z 427 for a loss of methyl group attached to the side chain and m/z 327 for successive cleavage of that chain (**Figure 4g**). Further fragmentation of this compound (**Table II**) tentatively deduced it as cucurbita-5,23-diene-3β,25-diol (**14**), which has been reported from the seeds of *Sicana odorifera* (Cucurbitaceae)[29]. Compound **18** was suggested to have a protonated molecular ion $[M+H]^+$ at m/z 571.10 with a base peak at m/z 554, indicating a loss of a methyl group from the 3-methoxy group in ring A of the proposed structure. The fragment ion at m/z 441 also suggested the compound to have a cucurbita-5(10),6,23-

triene-3,25-diol 3β-form skeleton[30]. Hence, the compound was tentatively deduced as 25-O-acetyl-16,20-dihydroxy-3-methoxy-cucurbita-5(10),6,23-triene-3,11,12-trione (**18**). The plausible MS/MS fragmentation of compound **18** is displayed in **Figure 4h**.



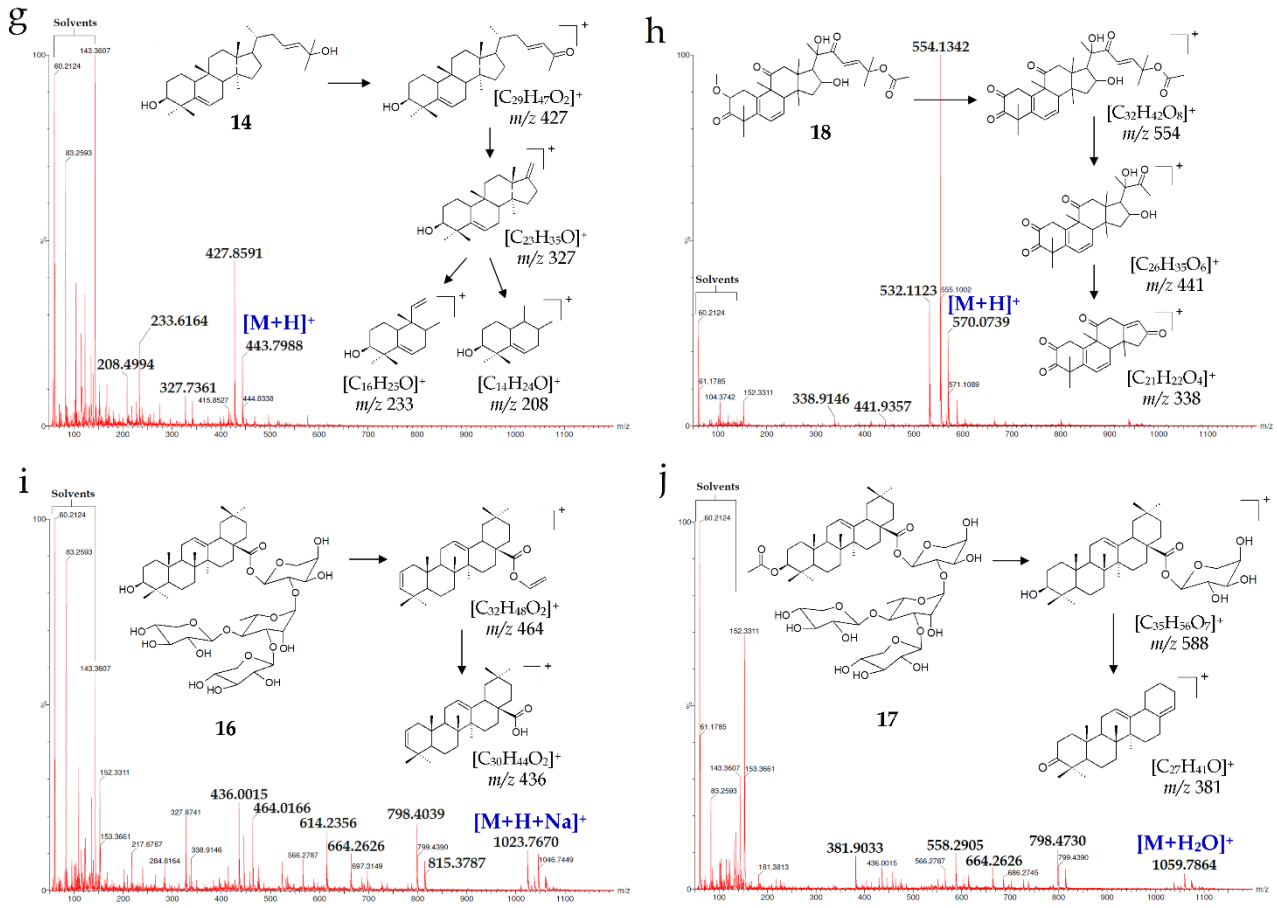


Figure 4. The plausible MS/MS fragmentation of compounds **5-10**, **14**, and **16- 18**.

Two peaks of oleananes with sugar moieties (**16**, **17**) were observed in the LC-MS/MS spectrum (**Figure 2**). The first peak appeared at the retention time of 14.19 minutes and suggested having a protonated and a sodium adduct ion $[M+H+Na]^+$ at m/z 1023.76. The fragment ions at m/z 815, 798, and 697 indicated the elimination of two xylopyranoses (2 x $C_5H_9O_5$, 298 Da) from the compound, followed by fragment ions at m/z 664, 614, and 464 for losing rhamnosyl and arabinopyranosyl sides from the oleanane skeleton. In addition, the fragment ion at m/z 436 indicated the oleanolic acid after losing its hydroxyl functionality at C-3 (**Figure 4i**). The compound was then tentatively deduced as oleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**16**), which has recently reported from the 70% ethanol extract of the sun-dried fruits of *B. hispida*[31]. Another peak was observed at the retention of 14.85 minutes and suggested to have a molecular ion with water adduct $[M+H_2O]^+$ at m/z 1059.78. Some fragmentations (m/z 798, 664, and 436) of this molecular ion were similar to those in compound **16**, while fragment ion at m/z 588 indicated the eliminations of three sugar moieties (xylopyranosyl-xylopyranosyl-rhamnosyl; $C_{16}H_{27}O_{12}$, 411 Da) and an acetyl group (43 Da) (**Figure 4j**). Hence, compound **17** was suggested as a derivative of compound **16**, for which 3β-O-acetyloleanolic acid 28-O-β-D-xylopyranosyl-[β-D-xylopyranosyl-(1→4)-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranoside (**17**) was tentatively assigned for the peak. Further isolation and more spectroscopic measurements are promising works to reveal the exact structure of this compound.

Total phenolic content, total flavonoid content, and antioxidant activity

Phenolics and flavonoids have been correlated to antioxidant activity in plants and foods[32]. The 80% ethanol extract of the edible part of *B. hispida* fruit contained low amounts of phenolics and flavonoids (**Table IV**). Despite low amounts of phenolics and flavonoids, the extract showed promising antioxidant activity towards DPPH radicals assayed qualitatively on a TLC plate (**Figure 5a**) and quantitatively using spectrophotometric measurement (**Figure 5b**) with an SC_{50} value of 23.4 µg/mL. Studies have shown that good antioxidant activities do not always define by the high amount of phenolics and flavonoids[9,10] but might be affected by the interactions among them either to promote synergistic effects or to result in

antagonistic effects[33]. Three compounds (**2**, **6**, **8**) identified in the extract have been reported to exhibit potent antioxidant activities[34-36]. Meanwhile, according to our search, the antioxidant activity of compound **7** (a flavonoid) has not yet been reported. The interactions of these compounds might promote synergistic effects, which resulted in the DPPH radical scavenging activity in the extract. The activity was compared to ascorbic acid, gallic acid, and quercetin as the positive controls. The activity of the edible part of the fruit was also considered more potent when compared with the seed[37]. Hence, this edible part could be used as a good source of natural antioxidants for nutraceutical and pharmaceutical developments.

Table IV. TPC, TPC, and DPPH radical scavenging activity (values as mean ± SD, n = 3)

Sample	TPC (mg GAE/g of extract)	TFC (mg QE/g of extract)	% RSA (at 100 µg/mL)	SC_{50} (µg/mL)
80% ethanol extract	1.1 ± 0.1	1.0 ± 0.1	92.5 ± 0.7	23.4
Ascorbic acid	-	-	99.6 ± 2.2	2.8
Gallic acid	-	-	97.1 ± 0.9	3.8
Quercetin	-	-	99.5 ± 2.4	9.5

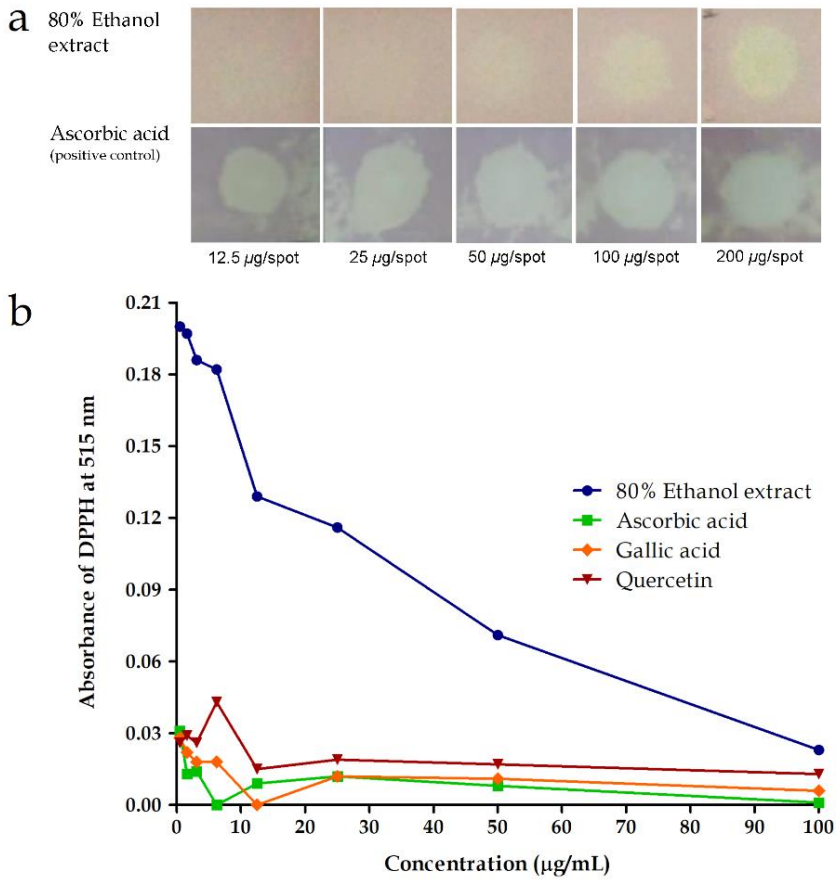


Figure 5. Qualitative (**a**) and quantitative (**b**) DPPH radical scavenging activity of 80% ethanol extract of the edible part of *B. hispida* fruit.

Antimicrobial activity

Some studies have shown the antimicrobial activity of the *B. hispida* fruit and its seeds toward bacteria[37,38]. From these studies, it is concluded that *B. hispida* displayed antibacterial activity at high doses. However, in the present study, a high dose (10,000 µg/well) of the 80% ethanol extract of the edible part of *B. hispida* fruit was found not to inhibit all the tested microbes, including the fungus *C. albicans* (**Table V**). Meanwhile, chloramphenicol and ketoconazole, as the positive controls, were consistently active in inhibiting the growths of the tested bacteria and fungus, respectively. Previous studies reported the antibacterial activity of *B. hispida* fruit and seed using water and 95% ethanol as solvents in the extraction[37,38]. The choice of solvents might affect the extraction of active compounds from the sample. In addition, the sugars (**1, 3**) might promote microbial growth as they act as nutrients. Thus, their presence might hinder the growth inhibition activity by active compounds in the extract. However, in the human gut, these sugars, especially raffinose (**3**), can act as a prebiotic that

promotes the activity and growth of good gut bacteria and reduce the abundance of pathogenic bacteria[39]. It might explain the traditional use of *B. hispida* fruit soup to treat typhoid fever. However, concerning the previous studies, further research is still needed to explore the antimicrobial compounds in *B. hispida*. For example, gallic acid (**2**), citric acid (**4**), and catechin (**6**) have been reported for antimicrobial activity[18,40]. However, considering the low amounts of phenolics and flavonoids in the extract, their activity might not be detectable.

Table V. Antimicrobial activity (values as mean ± SD, n = 3)

Sample	Concentration (µg/well)	Inhibition zone (mm)							
		S. typhi		*E. coli*		*S. aureus*		*C. albicans*	
		18 h	24 h	18 h	24 h	18 h	24 h	24 h	48 h
80% ethanol extract	10.000	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0
Chloramphenicol	20	19.77 ± 2.6	18.79 ± 2.7	24.99 ± 0.9	25.50 ± 1.4	18.97 ± 1.5	19.06 ± 1.5	-	-
Ketoconazole	0.5	-	-	-	-	-	-	22.75 ± 1.4	19.54 ± 0.9

CONCLUSION

The edible part of *B. hispida* fruit contains tannins, flavonoids, terpenoids, steroids, and saponins. Among them, eighteen compounds were tentatively identified, including sugars (**1, 3**), a simple phenolic (**2**), a tricarboxylic acid (**4**), a peptide (**5**), flavonoids (**6, 7, 8**), quinic acid derivatives (**9, 10**), phytosterols (**11, 12, 13**), triterpenoids (**14, 18**), and saponins (**15, 16, 17**). Despite low contents of phenolics and flavonoids, 80% ethanol extract of this part exhibited potent DPPH radical scavenging activity. The extract did not show antimicrobial activity towards all tested microbes. However, raffinose (**3**) as a potent prebiotic for human gut bacteria might explain the traditional use of *B. hispida* fruit in relieving typhoid fever. Hence, the study concluded that the edible part of *B. hispida* fruit could be used in the development of natural antioxidant agents and nutraceuticals.

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AUTHORS' CONTRIBUTION

Carla Wulandari Sabandar: the project leader responsible for research design, research management, results validation, manuscript writing and editing. **Harni Sartika Kamaruddin**: project team member who contributed ideas for research design and provided technical assistance for antimicrobial assay. **Reskiya Nur Insani**: a candidate for the Barchelor's degree of Pharmacy who conducted the experimental works, data collection, and data analysis. **Rana Triana Amin**: a fresh undergraduate student who helped the experimental work on antimicrobial assay and data collection as well as analysis. **Zulkifli**: a middler undergraduate student who helped in antioxidant assay and data analysis. **Tien**: a research collaborator who provided facilities for spectrophotometric measurements.

DATA AVAILABILITY

The supporting data of the article are accessible from the corresponding author upon reasonable request.

CONFLICT OF INTEREST

The authors have no conflict of interests to disclose. All authors are fully responsible for the content and writing of this article.

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Research Article

Antioxidant Activity of *n*-hexane and Etil Acetate Fractions of Bangkal (*Nauclea subdita* (Korth.) Steud.) Leaves

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Abstract

Bangkal (*Nauclea subdita* (Korth.) Steud.) is a tropical plant belonging to the Rubiaceae family, commonly found in South Kalimantan. This plant is one of the plants that has efficacy as a medicinal plant. This study aimed to quantitatively identify secondary metabolites and antioxidant activity in the *n*-hexane and ethyl acetate fractions of *N. subdita* leaves. The method of identification of secondary metabolites using the test tube. Antioxidant activity using the DPPH method based on IC_{50} value. The results of identifying secondary metabolites in the *n*-hexane fraction of *N. subdita* leaves contain alkaloids, flavonoids, steroids, and phenolic compounds, while the ethyl acetate fraction of *N. subdita* leaves contain alkaloids, flavonoids, steroids, tannins, saponins, and phenolics. The results of the antioxidant activity test of the *n*-hexane fraction and the ethyl acetate fraction of the leaves of *N. subdita* showed IC_{50} values of 229.61178±3.65919 and 54.54296±0.02236 ppm, respectively. Based on the IC_{50} value, the *n*-hexane fraction of *N. subdita* leaves had weak antioxidant activity, and the ethyl acetate fraction of *N. subdita* leaves had strong antioxidant activity.

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INTRODUCTION

Medicinal plants can be defined as plants that are part, all parts, and or parts of plant exudates that can be used as drugs, medicinal ingredients, or medicinal ingredients[1]. Medicinal plants are known to have efficacy because they contain secondary metabolite compounds, such as phenolic compounds, alkaloids, flavonoids, and terpenoids. These secondary metabolites are believed to have efficacy as a medicine for a disease which can also be used to maintain a healthy body[2].

Antioxidants have essential functions in the health of the body, including inhibiting and neutralizing the oxidation reactions that involve free radicals[3]. Antioxidants are substances that function as free radical inhibitors; generally, antioxidants work by inhibiting the formation of radicals that have the potential to carry out autoxidation[4]. Antioxidants are commonly found in various parts of plants, such as roots, stems, leaves, flowers, fruits, skins, and seeds[5]. One of them is found in the bangkal (*Nauclea subdita* (Korth.) Steud)[6].

Nauclea subdita is a tropical plant belonging to the Rubiaceae family, which is generally found in swamps, lowlands, watersheds and rivers, and mountain forests[6]. People typically use this plant for such treatments by applying the leaves to boils for tumors, and the boiled water of the leaves is used to treat diarrhea and toothache. This plant also contains antioxidants and steroids to help the growth of cells in the skin, so many people from Banjar Tribe use it as a basic powder ingredient and call it *pupur bangkal*[7]. This is supported by research conducted by Wardhani and Akhyar[8], stating that phytochemical screening was carried out on extracts from the stem bark of the *N. subdita*, which contain compounds belonging to the alkaloids, flavonoids, saponins, and polyphenols, and quinones. Research on the leaves of *N. subdita* will contribute to developing the utilization of this plant, and the leaves are abundantly available and easy to harvest and process. Previous research on the antioxidant activity of leaf and stem bark extracts of *N. subdita* has IC_{50} values of 79.62 ppm and

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307.1496 ppm[8]. This activity needs to be investigated further by researching to identify the content of secondary metabolites and the antioxidant activity of the *n*-hexane and ethyl acetate fractions of *N. subdita* leaves as a basis for obtaining the active compounds responsible for their antioxidant activity.

MATERIALS AND METHODS

Materials

The tools used in this study included a blender (Panasonic), separating funnel (Schott Duran), fume hood (Local), refrigerator, oven (Finco), pro pipette, analytical balance (Pioneer), water bath (Memmert), UV lamp with wavelengths of 254 and 366 nm, and UV-Vis Spectrophotometer (Perkin Elmer). The materials used in this study included distilled water, Mayer's reagent, Dragendorff's reagent, Mg powder, concentrated HCl, Liebermann-Burchard reagent, 1% gelatin, 5% $FeCl_3$, chloroform, 10% H_2SO_4, DPPH, quercetin, thin-layer chromatography (TLC) plate silica gel GF_{254}, methanol p.a., *n*-hexane p.a., ethyl acetate p.a., and leaves of *N. subdita*.

Methods

Determination

The leaves of *N. Subdita* were taken from the Banua Banjarbaru Botanical Garden, Jalan Dharma Praja I, Trikora, Cempaka, South Kalimantan Provincial Government Office Area, Banjarbaru, South Kalimantan, Indonesia. Plant determination was carried out at the Technical Implementation Unit of the Regional Research and Development Agency of the Banua Banjarbaru Botanical Garden, South Kalimantan with voucher number of 050/03-LIT/KRB. Plant samples in stems, leaves, roots, and fruit were made into a herbarium, and further determination was carried out.

Sample extraction

The leaves of *N. Subdita* were collected, processed into simplicia, and ground into powder. The powder weighed as much as 300 g, was then put into a macerator, then added methanol solvent up to 1 cm above the surface of the powder while measuring the volume of the inserted solvent. After all the powder was submerged, the leaves were stirred gently, then covered using aluminum foil. Extraction was carried out for 7 x 24 hours by changing the solvent every 24 hours and stirring every 8 hours or three times per 24 hours of the extraction process. Stirring and maceration were performed to increase the extraction process's effectiveness. The maceration results were then filtered and evaporated to obtain a thick extract. The thick extract was stored in a tightly closed container[9].

Fractionation

Fractionation was performed using a separating funnel with the liquid-liquid method. Fractionation was carried out using *n*-hexane and ethyl acetate as solvents. The extract from the maceration was taken, weighed 10 g, then suspended with 25 mL of distilled water. The extract suspension was then put into a separating funnel, then added with *n*-hexane into the separating funnel. The separating funnel was shaken slowly, then allowed to stand until two layers formed: the water and solvent. The *n*-hexane layer was removed, and the water layer was put back into a separatory funnel to be refracted using *n*-hexane solvent until it was wholly marked with the mixed solvent becoming clear. The same method was repeated for fractionation using ethyl acetate. The obtained liquid fraction was then evaporated using a water bath at a temperature of 50°C to obtain a thick fraction with constant weight, and the results were expressed in percent yield[10].

Identification of secondary metabolites

Identifying secondary metabolites of *n*-hexane and ethyl acetate fractions of *N. Subdita* leaves was used to determine the content of alkaloids, flavonoids, steroids, tannins, saponins, and phenolics[11].

Alkaloids: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were put into different test tubes, then 5 drops of Mayer's reagent were added to the first test tube of each fraction, and 5 drops of Dragendorff's reagent were added into the second test tube of each fraction.

Flavonoids: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were put into different test tubes, then 1 mg of Mg powder was added to each test tube, then 5 drops of concentrated HCl were added.

Steroids: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were put into different test tubes, then 10 drops of Liebermann-Burchard reagent were added.

Tannins: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were put into different test tubes, then 5 drops of 1% gelatin solution were added.

Saponins: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were diluted with 10 mL of distilled water in different test tubes, then shaken vigorously for 10 seconds.

Phenolics: About 1 mL of *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves were put into different test tubes, then 5 drops of 1% $FeCl_3$ were added.

Thin-layer chromatography

The methanol extract, *n*-hexane, and ethyl acetate fraction of *N. Subdita* leaves were dissolved using methanol : chloroform (1 : 1) v/v. The TLC plate was prepared, then activated in an oven at 105°C for 15 minutes. Each extract and fraction was spotted on the TLC plate, then eluted using *n*-hexane : ethyl acetate eluent in a ratio of 8 : 2, 5 : 5, and 2 : 8 v/v. The TLC plate was then observed under UV light at 254 and 366 nm, and each spot's Rf value was calculated.

Qualitative antioxidant activity

The TLC plate eluted and observed before was sprayed using 0.1 mM DPPH and allowed to dry. TLC spots with antioxidant activity change color to yellow on a purple background[12], then the Rf value for each stain that appears on the TLC plate that has been sprayed was calculated.

Quantitative antioxidant activity

DPPH solution: As much as 4 g of DPPH powder was weighed and dissolved with methanol p.a using a 25 mL volumetric flask, added with methanol p.a to the limit mark, then shaken until homogeneous to obtain a 0.4 mM DPPH solution. The solution was stored in a dark glass bottle and away from light.

Determination of maximum wavelength: About 1 mL of 0.4 mM DPPH solution was added with 4 mL of methanol p.a, then vortex for 1 minute. The solution was incubated in a dark room for 30 minutes. Determination of the maximum absorption wavelength of the DPPH solution was carried out using a UV-Vis spectrophotometer at a wavelength (λ) of 450-550 nm.

Determination of operating time: About 1 mL of 0.4 mM DPPH solution was put in a test tube with aluminum foil, then added with 4 mL of quercetin solution. The mixture was vortex for a minute, then the absorbance was read using a UV-Vis spectrophotometer every 2 minutes for 40 minutes, at the maximum wavelength of DPPH.

Determination of IC_{50} of quercetin: About 5 mg of quercetin was dissolved with methanol p.a, then put into a 50 mL volumetric flask, added methanol p.a to the limit mark, then shaken until homogeneous to obtain 100 ppm mother liquor. The grade series solution is prepared with a concentration of 1, 2, 4, 8, 10, and 12 ppm of 100 ppm quercetin mother liquor using a 10 mL volumetric flask. Each of the concentration series solutions was taken as much as 4 mL and then put into a test tube, added with 1 mL of 0.4 mM DPPH solution, vortex for a minute, then left in a dark room according to the operating time that has been obtained. After that, the absorbance of each solution was read using a UV-Vis spectrophotometer at the maximum wavelength of DPPH received.

Determination of IC_{50} of n-hexane and the ethyl acetate fractions of N. subdita leaves: As much as 25 mg of *n*-hexane thick fraction was dissolved with methanol p.a, put into a 50 mL volumetric flask, added methanol p.a to the limit mark, then shaken until homogeneous to obtain 500 ppm mother liquor. The 500 ppm solution was then made into a series of solutions with a concentration of 50, 100, 150, 200, 250, 300, and 350 ppm using a 10 mL volumetric flask. For ethyl acetate fraction, as much as 5 mg of n-hexane thick fraction was dissolved with methanol p.a, put into a 50 mL volumetric flask, added methanol p.a to the limit mark, then shaken until homogeneous to obtain 100 ppm mother liquor. The 100 ppm solution was then made into a series of solutions with a concentration of 10, 20, 30, 40, 50, 60, and 70 ppm using a 10 mL volumetric flask. As much as 4 mL from each concentration series solution was taken into a test tube, then 1 mL of 0.4 mM DPPH solution was added, vortex each tube for a minute, then allowing it to stand according to the operating time obtained. After that, the absorbance of each concentration was read using a UV-Vis spectrophotometer at the maximum wavelength of DPPH received.

Data analysis

The antioxidant activity of the *n*-hexane and ethyl acetate fractions of *N. Subdita* leaves in inhibiting DPPH radicals could be seen from the calculation results of the % inhibition value of the compounds tested. Determination of the IC_{50} value was accomplished by making a linear regression between the concentration of the test solution (*x*-axis) and the percentage of inhibition (*y*-axis), as shown in **Equation 1**[13].

$$IC50: \frac{(50-a)}{b} \quad [1]$$

RESULTS AND DISCUSSION

Extraction

Extraction was carried out using the maceration method and methanol as a solvent. The methanol extract of *N. subdita* leaves obtained was 49.13 g from 300 g of simplicia powder, with a percentage yield of 19.86%. Extraction separates a substance from its mixture by dividing it between two immiscible solvents to take the solute from one solvent to another[14]. Extracts obtained were dry, viscous, or liquid preparations made by extracting vegetable or animal simplicia according to a suitable method, outside the influence of direct sunlight[15].

Fractionation

Fractionation is carried out after obtaining the methanol extract of *N. subdita* leaves. The solvents used are *n*-hexane and ethyl acetate because they have different levels of polarity, *n*-hexane solvent has non-polar properties, and ethyl acetate solvent has semi-polar[16]. The results of the % yield of the fraction of *N. subdita* leaves obtained in this study are 10.8% for the *n*-hexane and 9.4% for the ethyl acetate fraction. The percentage yield shows that the *n*-hexane fraction is more in number than the ethyl acetate fraction. The difference in the percentage yield of the *n*-hexane and ethyl acetate fractions is caused by the difference in the number of compounds attracted to each solvent according to the level of polarity. Fractionation is separating compounds based on their level of polarity[17]. The principle of separation in the fractionation process is based on the difference in polarity level and a specific gravity between the two fractions[18].

Identification of secondary metabolites

Secondary metabolites are identified using the tube method to determine the secondary metabolites contained in the *N. subdita* leaves resulting from a metabolic process. The compounds tested included alkaloids, flavonoids, steroids, tannins, saponins, and phenolics. The results from the *n*-hexane and the ethyl acetate fractions of *N. subdita* leaves are shown in **Table I**. The identification test in the *n*-hexane fraction is positive for alkaloids, flavonoids, steroids, and phenolics, while the tannins and saponins are negative. Meanwhile, the test of ethyl acetate fraction is positive for alkaloids, flavonoids, steroids, tannins, saponins, and phenolics. Saponin compounds are active compounds that are polar. Therefore, the ethyl acetate fraction has positive results for tannins and saponins because the ethyl acetate solvent has semi-polar properties, which can also attract polar compounds[19]. Tannin compounds can be extracted using semi-polar solvents such as ethyl acetate[20]. Therefore, the test results for tannins and saponins in the *n*-hexane fraction, a non-polar solvent, are negative.

Table I. Secondary metabolites of the n-hexane and the ethyl acetate fractions of *N. subdita* leaves

Secondary metabolites	Sample	
	n-hexane fraction	Ethyl acetate fraction
Alkaloids	+	+
Flavonoids	+	+
Steroids	+	+
Tannins	-	+
Saponins	-	+
Phenolics	+	+

Note: (+): Present; (-): Absent

Thin-layer chromatography

A test of TLC is carried out to ensure that the fractionation process that has been carried out has separated the mixture of compounds in the extract entirely based on the level of polarity. The samples tested using the TLC method are methanol extract, *n*-hexane, and ethyl acetate fractions of *N. subdita* leaves. The results of these readings obtained that the TLC plate is fluorescent, and the spots are dark in UV light at 254 nm, while at UV light at 366 nm, the TLC plate is dark in color, and the spots are fluoresced[21]. The TLC test results are the chromatograms observed under UV light at 254 and 366 nm, and the Rf values are calculated as presented in **Table II**.

The appearance of the TLC stain under a UV lamp at 254 nm is a dark stain on the fluorescent plate. This happens because of the interaction between UV light and the fluorescence indicator on the scale[22]. The appearance of the TLC stain under a 366 nm UV lamp produces a dark-colored plate because the plate will not fluoresce under the light of that wavelength, while the resulting stain will fluoresce. This fluorescent stain occurs because of the interaction between UV light and stains with a chromophore group bound by auxochrome[23].

Table II. Rf from TLC of methanol extract, *n*-hexane, and ethyl acetate fractions of *N. subdita* leaves at UV light 254 and 366 nm

Mobile phase (*n*-hexane : ethyl acetate)	Sample	UV Wavelength (nm)	
		254	366
8 : 2	Methanol extract	1). 0.44	1). 0.08
		2). 0.6	2). 0.28
		3). 1.0	3). 0.44
			4). 0.62
	n-hexane fraction	1). 0.1	1). 0.08
		2). 0.18	2). 0.5
		3). 0.28	3). 0.68
		4). 0.44	4). 0.86
		5). 0.52	
		6). 0.64	
		7). 1.0	
	Ethyl acetate fraction	1). 0.24	1). 0.06
		2). 0.4	2). 0.26
		3). 0.6	3). 0.42
		4). 0.98	4). 0.62
5 : 5	Methanol extract	1). 0.12	1). 0.06
		2). 0.8	2). 0.14
		3). 0.88	3). 0.9
		4). 0.99	4). 0.98
	n-hexane fraction	1). 0.1	1). 0.05
		2). 0.16	2). 0.16
		3). 0.76	3). 0.46
		4). 0.86	4). 0.82
		5). 0.96	
	Ethyl acetate fraction	1). 0.06	1). 0.04
		2). 0.12	2). 0.16
		3). 0.18	3). 0.22
		4). 0.8	4). 0.38
		5). 0.9	5). 0.46
		6). 0.98	6). 0.82
			7). 0.9
			8). 0.98
2 : 8	Methanol extract	1). 0.06	1). 0.22
		2). 0.42	2). 0.54
		3). 0.96	
	n-hexane fraction	1). 0.26	1). 0.2
		2). 0.48	2). 0.56
		3). 0.92	3). 0.74
	Ethyl acetate fraction	1). 0.08	1). 0.22
		2). 0.18	2). 0.5
		3). 0.26	3). 0.58
		4). 0.98	4). 0.78
			5). 0.98

Qualitative antioxidant activity

A qualitative test of antioxidant activity is carried out using TLC. This test is carried out by spraying using a DPPH reagent. If the sample has the potential as an antioxidant, the stain on TLC will change color to pale yellow on a purple background[12]. Antioxidant compounds will donate hydrogen atoms to DPPH radicals to form a yellow color[26]. The results of the qualitative antioxidant test are shown in **Table III**.

Table III. Rf from TLC of methanol extract, *n*-hexane, and ethyl acetate fractions of *N. subdita* leaves by DPPH

Mobile phase (*n*-hexane : ethyl acetate)	Sample	Rf value
8 : 2	Methanol extract	1). 0.53
	n-hexane fraction	1). 0.69
		2). 0.86
		3). 0.94
	Ethyl acetate fraction	1). 0.1
		2). 0.52
5 : 5	Methanol extract	1). 0.18
	n-hexane fraction	1). 0.17
	Ethyl acetate fraction	1). 0.18
		2). 0.32
2 : 8	Methanol extract	1). 0.1
		2). 0.3
	n-hexane fraction	1). 0.08
	Ethyl acetate fraction	1). 0.12
		2). 0.27
		3). 0.46
		4). 0.6

Quantitative antioxidant activity

Determination of the maximum wavelength of DPPH is carried out to determine the wavelength with complete sensitivity. The purpose of determining the maximum wavelength is that the absorbance obtained is maximal, the difference in absorbance of each concentration series will be more significant because the sensitivity is higher, and the shape of the curve obtained will be linear[24]. This is indicated by the maximum or most significant absorbance[25]. The DPPH wavelength range is 515-520 nm[26]. The results obtained are at a wavelength of 515.30 nm, as shown in **Figure 1**.

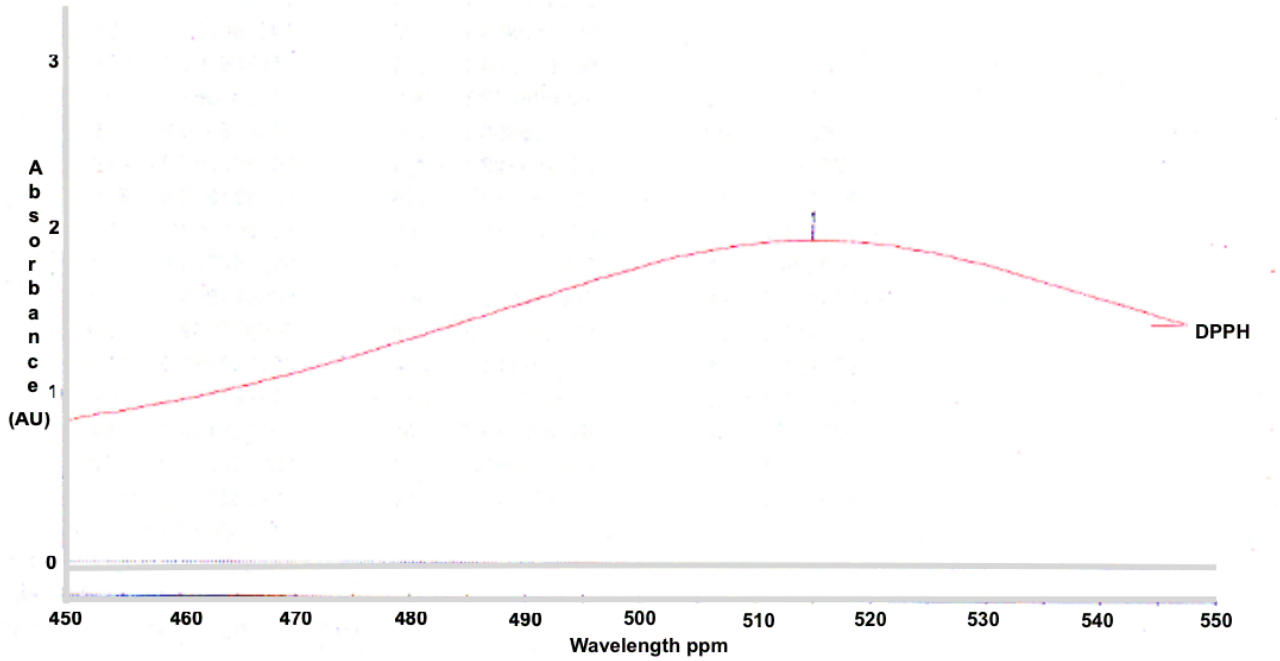

Figure 1. The maximum wavelength of DPPH.

Operational time is the measurement time taken when the solution absorbs a stable absorption light. The operating time determines the most appropriate time for the test solution to reduce DPPH radicals. In addition, the operating time also

shows that the reaction between the test solution and DPPH has stabilized, which can be indicated by the absence of a decrease in absorbance[27]. The results obtained in the stable reaction started from the 24th minute to the 40th minute (**Figure 2**) with a difference in absorbance value of 0.0006. This shows that the operating time obtained in this determination has different values but produces absorbance values that are uniform with each other, so it shows that DPPH has reacted stably with a standard solution of quercetin[28].

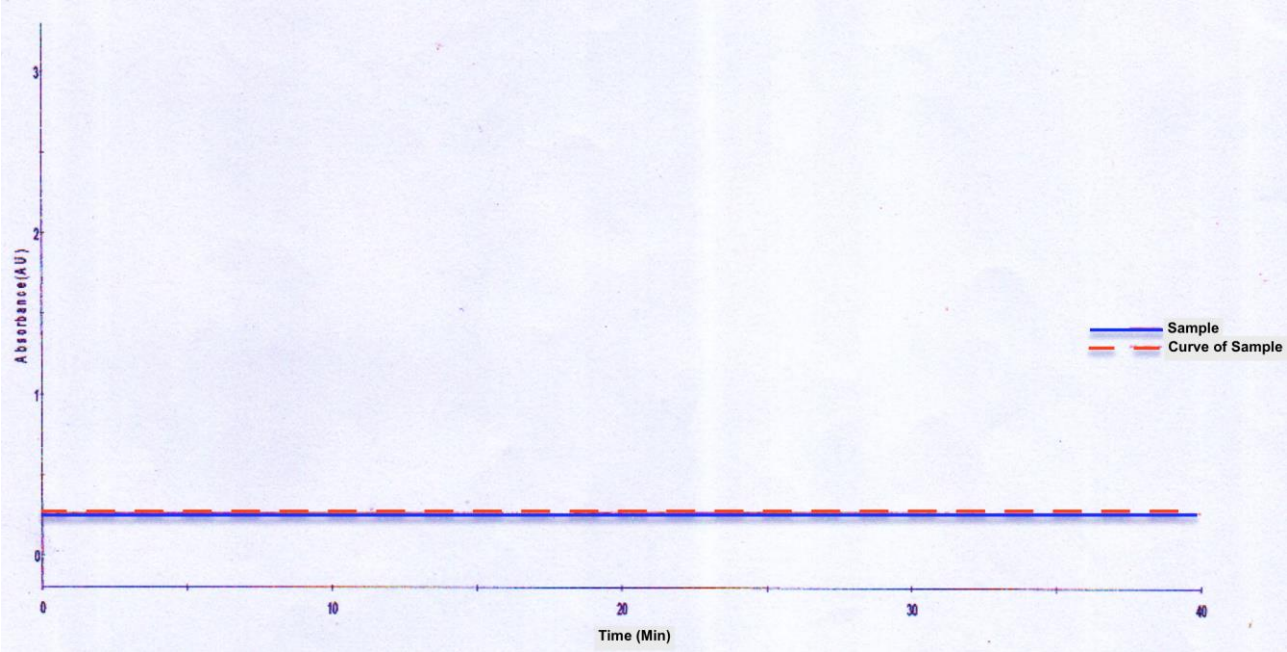

Figure 2. The determination of operating time.

Quercetin is the main flavonoid compound in the flavonol group and can be easily found in plants. In addition, quercetin is chosen because it has been shown to have potent antioxidant activity[29]. Quercetin will donate its proton to DPPH and become a radical compound. The unpaired electrons generated in the chemical structure of quercetin are localized into the aromatic system so that the quercetin revolutionary compound is relatively less reactive and has low energy[30]. Compared with other positive controls, ascorbic acid or gallic acid, quercetin can better stabilize DPPH radicals[31]. The antioxidant activity of the samples is tested by comparing their antioxidant activity with a positive control or comparison. The comparison or positive control used is quercetin in determining the IC_{50} value.

The results of the linear regression equation between the concentration of quercetin and the percentage of inhibition are $y = 5.9683x - 2.1812$ with a correlation coefficient (r) of 0.995 (**Figure 3**). The range of values for the r is 0.98, so the resulting data is by the provisions. The antioxidant activity value of the quercetin comparison solution is shown in **Table IV**. Comparing quercetin's antioxidant activity results obtained an average IC_{50} of 8.74306 ± 0.09144 ppm. The IC_{50} value obtained is included in the strong category with an IC_{50} value range of <50 ppm[26].

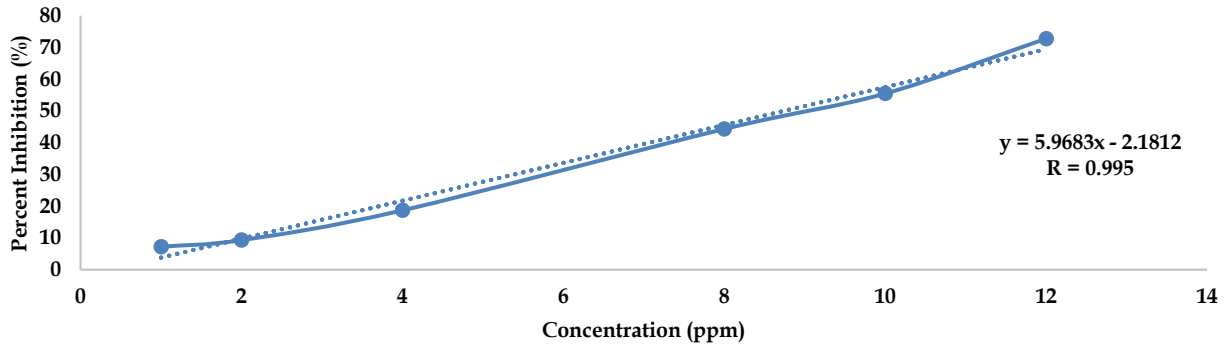

Figure 3. Linear regression equation of quercetin.

Table IV. The antioxidant activity of the quercetin

Concentration (ppm)	$\bar{x}$ Inhibition (%)	SD	RSD (%)	$\bar{x}$ IC_{50} ± SD (ppm)	RSD (%)
1	7.225	0.00269	0.156	8.74306 ± 0.09144	1.04571
2	9.300	0.00708	0.419		
4	18.672	0.00201	0.133		
8	44.302	0.00466	0.450		
10	55.476	0.02412	2.910		
12	72.765	0.00970	1.914		

Determination of the IC_{50} value of the *N. subdita* leaves *n*-hexane fraction is carried out by making test solutions in a series of concentrations of 50, 100, 150, 200, 250, 300, and 350 ppm, which are taken from the results of the dilution of the standard solution of 500 ppm. The regression equation (**Figure 4**) was obtained from the relationship between the *n*-hexane fraction concentration series of *N. subdita* leaves with the percent inhibition of $y = 0.1494x + 15.696$ with the r of 0.962. The IC_{50} value is inversely proportional to antioxidant activity, so the lower the concentration that can reduce DPPH radicals by 50%, the stronger the antioxidant activity[32]. The results of the antioxidant activity of the *n*-hexane fraction of *N. subdita* leaves obtained an average IC_{50} of 229.61178 ± 3.65919 ppm (**Table V**). The IC_{50} value obtained is included in the weak category, with an IC_{50} value range of >200 ppm[26].

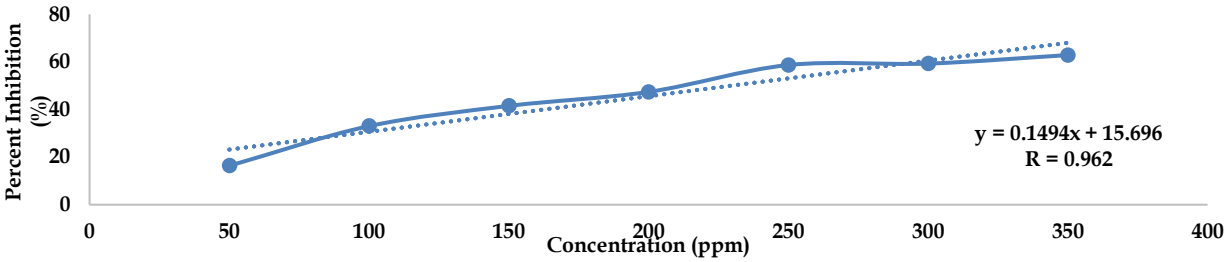

Figure 4. Linear regression equation of *n*-hexane fraction of *N. subdita* leaves.

Table V. The antioxidant activity of the *n*-hexane fraction of *N. subdita* leaves

Concentration (ppm)	$\bar{x}$ Inhibition (%)	SD	RSD (%)	$\bar{x}$ IC_{50} ± SD (ppm)	RSD (%)
50	16.356	0.00059	0.045	229.61178 ± 3.65919	1.59383
100	33.009	0.00050	0.048		
150	41.506	0.00035	0.038		
200	47.387	0.00010	0.012		
250	58.675	0.00059	0.092		
300	59.280	0.00036	0.057		
350	62.851	0.00052	0.090		

Determination of the IC_{50} value of the ethyl acetate fraction of *N. subdita* leaves was carried out by making a 100 ppm standard solution. Then a series of concentrations of 10, 20, 30, 40, 50, 60, and 70 ppm are made. The linear regression equation is obtained from the relationship between concentration and the percentage of inhibition of the ethyl acetate fraction of *N. subdita* leaves (**Figure 5**) of $y = 0.7529x + 8.9346$ with the r of 0.994. The results of the antioxidant activity of the ethyl acetate fraction of *N. subdita* leaves obtained an average IC_{50} of 54.54296 ± 0.02236 ppm, as shown in **Table VI**. The IC_{50} value obtained is included in the strong category, which has an IC_{50} value range of 50-100 ppm[26].

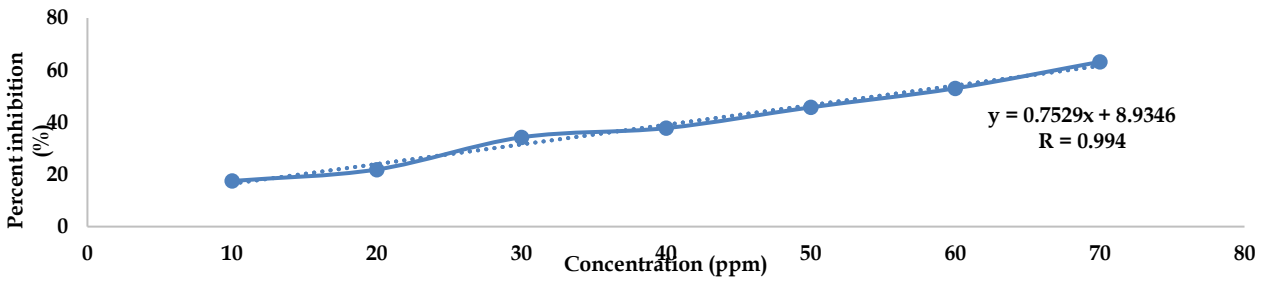

Figure 5. Linear regression equation of ethyl acetate fraction of *N. subdita* leaves.

Table VI. The antioxidant activity of the ethyl acetate fraction of *N. subdita* leaves

Concentration (ppm)	$\bar{x}$ Inhibition (%)	SD	RSD (%)	$\bar{x}$ IC_{50} ± SD (ppm)	RSD (%)
10	17.498	0.00029	0.022	54.54296 ± 0.02236	0.0499
20	21.901	0.00021	0.017		
30	34.235	0.00015	0.015		
40	37.768	0.00023	0.024		
50	45.739	0.00030	0.035		
60	53.028	0.00012	0.016		
70	63.182	0.00042	0.072		

CONCLUSION

Identification test of secondary metabolites in the *n*-hexane fraction of *N. subdita* leaves contain alkaloids, flavonoids, steroids, and phenolics, while the ethyl acetate fraction contains alkaloids, flavonoids, steroids, tannins, saponins, and phenolics. The *n*-hexane fraction of *N. subdita* leaves has weak antioxidant activity, and the ethyl acetate fraction has strong antioxidant activity.

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AUTHORS' CONTRIBUTION

Consept: **A.A., A.M., S.S**.; Design: **A.M., A.A., S.S**; Supervision: **S.S**.; Data collection and processing: **A.A., A.M., S.S**.; Resources: **S.S**.; Materials: **A.A., A.M., S.S**.; Analysis and interpretation: **A.A., A.M., S.S**.; Literature search: **A.A., A.M., S.S**.; Writing: **A.M., A.A., S.S**.; Critical review: **A.A., S.S**.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

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

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Research Article

Solubility and Scale-Up Potency of Norfloxacin-Urea Co-Crystal Prepared by Ultrasound-Assisted Slurry Co-Crystallization Method

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Co-crystal
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Urea

Abstract

Norfloxacin is an antimicrobial in treating urinary tract infections with low water solubility. This study aims to know the effect of norfloxacin-urea co-crystal formation on the solubility of norfloxacin and the potential for scale-up when prepared by ultrasound-assisted slurry co-crystallization method. Identification of the screening result of the norfloxacin-urea (1 : 1) co-crystal formation by a wet grinding method using an ethanol-acetone (1 : 1) solvent mixture was performed by powder X-ray diffractometer (PXRD). The ultrasound-assisted slurry co-crystallization method was used for co-crystal formation with five-fold the weight of norfloxacin and urea than the wet grinding method. The co-crystal product prepared by the ultrasound-assisted slurry co-crystallization method was observed for its crystal morphology and characterized by PXRD and differential scanning calorimeter (DSC). Solubility and dissolution tests in water and acetate buffer solution pH 4.0 were used to evaluate the physicochemical properties. Identification of co-crystal screening by PXRD revealed the formation of norfloxacin-urea co-crystal. The PXRD pattern of the norfloxacin-urea co-crystal product prepared by the ultrasound-assisted slurry co-crystallization method was similar to the wet grinding method. Norfloxacin-urea co-crystal has a different melting point and crystal morphology from pure norfloxacin and urea. The solubility and dissolution rate of norfloxacin-urea co-crystal was higher in water and not significantly different in acetate buffer solution pH 4.0 compared to pure norfloxacin. This study showed that the norfloxacin-urea co-crystal formation could enhance the solubility of norfloxacin in water and had the potential for scale-up when prepared using the ultrasound-assisted slurry co-crystallization method.

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INTRODUCTION

Norfloxacin is an analog of nalidixic acid, effectively treating urinary tract infections, such as gonorrhea and prostatitis[1]. This active pharmaceutical ingredient (API) has a broad antimicrobial activity to treat infections in humans and animals caused by *Escherichia coli*, *Citrobacter freundi*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, dan *Shigella*[2]. Norfloxacin is mostly given in oral solid dosage forms, such as tablets. Norfloxacin has low solubility and permeability, classified into class IV in the Biopharmaceutical Classification System (BCS)[3]. Several attempts have been made to improve the solubility of norfloxacin, among others, by forming co-crystals with nicotinic acid[3], resorcinol[4], and isonicotinamide[5].

Co-crystal are multicomponent crystals arranged stoichiometrically through weak bonds, such as hydrogen bonds between an active pharmaceutical ingredient (API) and another API or an API and a pharmaceutical excipient bound together in a crystal lattice[6,7]. The manufacture of co-crystal is an attractive strategy for the pharmaceutical industry to solve the physicochemical problems of APIs, such as solubility, stability, hygroscopicity, and mechanical properties[8,9].

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One of the limiting factors for the pharmaceutical industry in utilizing co-crystal is the difficulty in large production[10]. The manufacture of co-crystal using solution-based methods, such as solvent evaporation and cooling crystallization, although it can be done in large quantities in tanks, can be constrained by the process of crystallizing an active pharmaceutical ingredient and co-crystal former separately due to differences in the solubility of the two components. The co-crystal preparation by solid-based methods, such as dry grinding and wet grinding, produces co-crystals with less uniform size and defects[11,12]. The slurry is a solution-based method that can be used as an alternative to making large-scale co-crystals more efficiently. This method uses less solvent than solvent evaporation or cooling crystallization methods and produces uniform crystals without defects. In this method's preparation of the co-crystal, one or both pure compounds are suspended in a solvent to form a slurry[13]. The production of co-crystals using the slurry method can be assisted by ultrasonic waves to accelerate their formation, known as the ultrasound-assisted slurry co-crystallization or sonic slurry method[14,15].

The selection of a co-crystal former is a critical stage that can affect the success of co-crystal formation. Urea is a compound often used as a co-crystal former and can increase the solubility of active pharmaceutical ingredients, including agomelatine[16], bumetanide[17], catechin[18], and febuxostat[19]. The presence of two amide groups in urea has an excellent opportunity to form a co-crystal with norfloxacin, as well as the formation of norfloxacin-isonicotinamide co-crystal where hydrogen bonds occur between the amide group in isonicotinamide and the carboxylate group in norfloxacin[5].

Studies on co-crystal formation between norfloxacin and urea have yet to be reported, even though these two components can form co-crystals and increase the solubility of norfloxacin. The ultrasound-assisted slurry co-crystallization method can be an option in manufacturing norfloxacin-urea co-crystal on a large scale because it uses only a tiny amount of solvent and produces uniform crystals without defects. This study aims to determine the effect of norfloxacin-urea co-crystal formation on the solubility of norfloxacin and also to know its potential scale-up when prepared using the ultrasound-assisted slurry co-crystallization method.

MATERIALS AND METHODS

Materials

Norfloxacin was purchased from Beijing Mesochem Technology Co., Ltd (China), while urea was obtained from PT. Merck Indonesia. Sodium acetate, acetic acid, ethanol, and acetone were also purchased from PT. Merck Indonesia. The equipment used for the co-crystal preparation was a mortar grinder (Retsch RM 200) and Branson 3510-DTH ultrasonic. The types of equipment used for co-crystal characterization and evaluation consisted of a differential scanning calorimeter (Shimadzu DSC-60 plus), polarizing microscope (Olympus BX-53), powder X-ray diffractometer (Rigaku Miniflex), orbital shaker (IKA KS-260), water bath shaker (Lab Companion BS-11), Dissolution tester (ZRS6G), and ultraviolet spectrophotometer (Shimadzu UV-1800).

Methods

Screening of norfloxacin-urea co-crystal formation by wet grinding method

Screening of norfloxacin-urea co-crystal formation was carried out by the solvent-drop grinding method[20,21]. Norfloxacin and urea were weighed at 0.638 g (0.002 mol) and 0.120 g (0.002 mol), respectively, and put into a mortar grinder. After adding three drops of the ethanol-acetone (1 : 1) solvent mixture, the powder mixture was ground for ten minutes and left until all the solvent had evaporated. The grinding product was placed in a closed vial before being characterized by a powder X-ray diffractometer.

Characterization of the wet grinding product by powder X-ray diffractometer (PXRD)

The PXRD pattern was collected by irradiating approximately 500 mg of the norfloxacin-urea wet grinding product with an X-ray generated from Cu-Kα radiation at 2θ 5-45° using a Rigaku Miniflex diffractometer. Scans were performed at a speed of 10° per minute and a step-width of 0.02°. The scans were also conducted on each pure compound (norfloxacin and urea).

Preparation of norfloxacin-urea co-crystal by ultrasound-assisted slurry co-crystallization method

Norfloxacin-urea co-crystal was prepared using the ultrasound-assisted slurry co-crystallization method[14]. In the vial, 3.19 g (0.01 mol) of norfloxacin and 0.6 g (0.01 mol) of urea were dispersed in 2 mL ethanol-acetone (1 : 1) solvent mixture and

sonicated at a frequency of 42 kHz for 20 minutes using a Branson 3510-DTH ultrasonic. At the end of the sonication process, the suspension turned into a cake-like solid at the bottom of the vial. The solid was removed from the vial, and the crystal morphology was observed under a polarizing microscope. After the solid was dried, it was characterized by PXRD and differential scanning calorimeter (DSC) methods.

Crystal morphology observation by polarizing microscope

The solid resulting from the manufacture of norfloxacin-urea co-crystal by the ultrasound-assisted slurry co-crystallization method was placed on a slide and observed for crystal morphology using an Olympus BX-53 polarizing microscope. Crystal morphology was also observed in each recrystallized physical mixture of norfloxacin-urea (1 : 1), pure norfloxacin, and urea in an ethanol-acetone (1 : 1) solvent mixture. The recrystallization was carried out by placing 1-3 mg of each material on a glass object and dripping it with the solvent until dissolved and allowed to crystallize again.

PXRD patterns analysis of norfloxacin-urea co-crystal formation prepared by the ultrasound-assisted slurry co-crystallization method

The PXRD patterns analysis resulting from the manufacture of norfloxacin-urea co-crystal by the ultrasound-assisted slurry co-crystallization method was carried out according to the conditions as described in the procedure of screening the norfloxacin-urea co-crystal formation by the wet grinding method. Identification of co-crystal formation was carried out by comparing the PXRD pattern between the ultrasound-assisted slurry co-crystallization and wet grinding methods.

Thermal analysis of norfloxacin-urea prepared by the ultrasound-assisted slurry co-crystallization method

Thermal analysis of norfloxacin-urea co-crystal was conducted by a Shimadzu DSC-60 plus differential scanning calorimeter by placing 3-5 mg of sample in a tightly closed aluminum pan and heated at 10°C/minute while nitrogen gas flowed at 20 mL/minute for purging. The heating temperature range is 30-250°C. DSC analysis was also performed on pure norfloxacin and urea. The melting point is determined from the peak point of an endothermic transition.

Solubility test

The solvents used in the solubility test were water (room temperature) and acetate buffer solution pH 4.0 (37±0.5°C). The solubility test was carried out using the shake-flask method[22]. An orbital shaker was used for the solubility test in water, while a water bath shaker (Lab Companion BS-11) was used for the solubility test in an acetate buffer solution at pH 4.0. Norfloxacin-urea co-crystal was weighed equivalent to 100 mg of norfloxacin and put into each vial containing 5 mL of water and acetate buffer solution at pH 4.0. The vial containing the co-crystal dispersion in water was shaken for 24 hours using an orbital shaker (IKA-KS 260), while the co-crystal dispersion in acetate buffer solution pH 4.0 was shaken in a water bath shaker (Lab Companion BS-11). After completing the shaking, the samples were filtered, and the filtrate was analyzed for the norfloxacin solubility by ultraviolet spectrophotometer at 275 and 278 nm, respectively, for the samples tested in water and acetate buffer solution pH 4.0. The solubility tests were also carried out on pure norfloxacin.

Dissolution test

The dissolution test was carried out on pure norfloxacin and norfloxacin-urea co-crystal using a type 2 dissolution apparatus (paddle) with a stirring speed of 50 rpm (rotation per minute) at 37±0.5°C and 750 mL of acetate buffer solution pH 4.0 as a medium[23]. The dissolution test was also carried out in a water medium under the same conditions as the acetate buffer solution pH 4.0. As much as 5 mL samples were taken at 5, 10, 15, 20, and 30 minutes from the dissolution medium and were filtered. The percentage of norfloxacin dissolved in each sample filtrate was determined by ultraviolet spectrophotometer at 275 and 278 nm for the samples tested in water and acetate buffer solution pH 4.0, respectively.

RESULTS AND DISCUSSION

Screening of norfloxacin-urea co-crystal formation by wet grinding method

Screening for co-crystal formation is one of the steps in manufacturing co-crystals that must be carried out after selecting co-crystal forming materials[24]. Wet grinding, or liquid-assisted grinding, utilizes mechanical energy to induce the two components to form a co-crystal and a small amount of solvent can accelerate the induction process[25]. The advantages of co-crystal formation speed, efficiency in solvent use, and high success rate make this method suitable for the co-crystal formation screening stage[21]. The solubility of the two substances in the chosen solvent is significant to consider in the

preparation of co-crystal[26]. Urea is soluble in ethanol[27], while norfloxacin is slightly soluble in acetone[28]. So that in the preparation of the norfloxacin-urea co-crystal, an ethanol-acetone (1 : 1) solvent mixture was used as a solvent.

Characterization of the wet grinding product by powder X-ray diffractometer

The PXRD is a technique widely used to characterize the co-crystal formation by comparing the PXRD pattern of the product and its pure components. The PXRD patterns of norfloxacin-urea (1 : 1) wet grinding product, pure norfloxacin, and urea are shown in **Figure 1**. Norfloxacin has main peaks at 9.7°, 15.8°, 20.4°, 22.5°, 24.7°, and 27.6° of 2θ angles, while urea has major peaks at 2θ 22.2°, 22.5°, 24.7°, 31.8°, and 35.6° of 2θ angles. In the PXRD pattern of norfloxacin-urea (1 : 1) wet grinding product, the main peaks of norfloxacin and urea were not visible, but new peaks appear at angles 2θ 6.3°, 12.7°, 16.9°, 19.2°, 23.1°, and 25.8° showed by black arrows. The appearance of the new peaks and the disappearance of the peaks of pure norfloxacin and urea demonstrated that the wet grinding of the two components could change their crystal lattice due to the formation of hydrogen bonds between them. Thus, the difference in the PXRD pattern between the wet grinding product and its pure components reveals the formation of co-crystal[29,30].

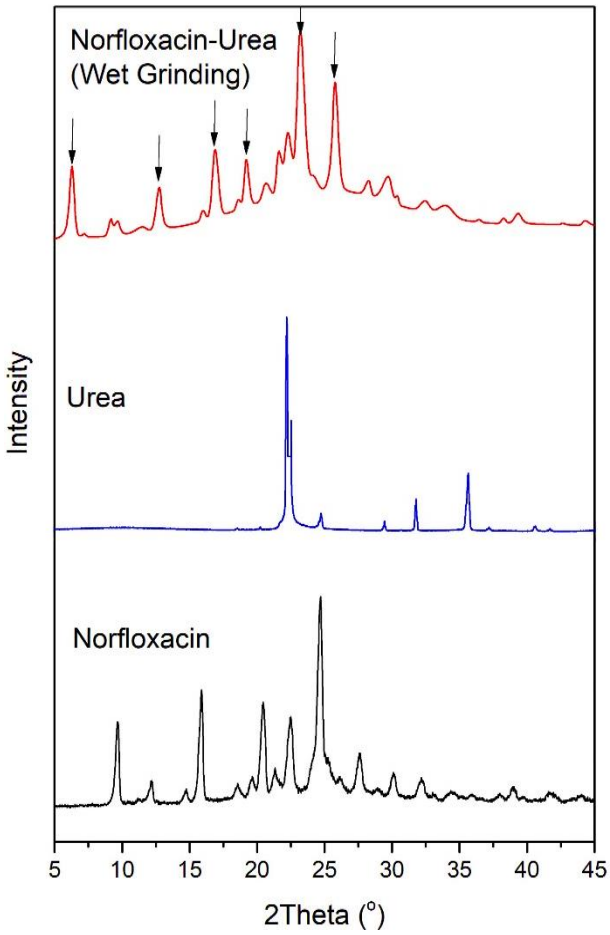

Figure 1. Powder X-ray diffraction patterns of norfloxacin-urea wet grinding product, pure norfloxacin, and urea.

Preparation of norfloxacin-urea co-crystal by ultrasound-assisted slurry co-crystallization method

The ultrasound-assisted slurry co-crystallization method was used to obtain more sufficient amounts of co-crystal for solubility and dissolution tests[21]. In this method, the weight of norfloxacin and urea was increased five-fold compared to the wet grinding method to produce more co-crystals in each manufacturing process. Early identification of the co-crystal product is needed to ensure the success of co-crystal formation by this method. The simple identification is by comparing the co-crystal morphology of the ultrasound-assisted slurry co-crystallization product with the recrystallization result of the physical mixture of the two pure compounds and each pure component. **Figure 2** shows that the ultrasound-assisted slurry co-crystallization product has the same crystal morphology as the recrystallization product of the norfloxacin-urea physical

mixture and different crystal morphology from the recrystallization product of pure norfloxacin and urea. This indicates that ultrasound-assisted slurry co-crystallization can also be used for norfloxacin-urea co-crystal preparation. The presence of ultrasonic vibrations and a small amount of solvent can induce the formation of co-crystal nuclei and then be followed by crystal growth[31]. However, characterization by PXRD and DSC methods was needed to ensure the formation of norfloxacin-urea co-crystal.

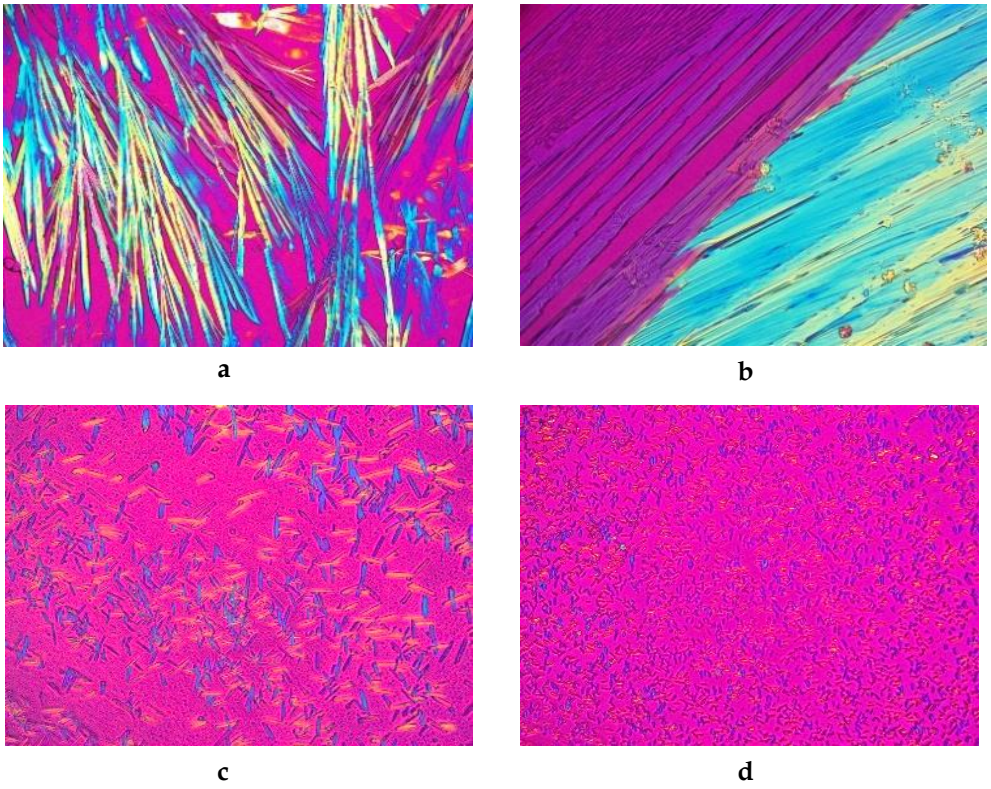

Figure 2. Crystal morphology of (**a**) norfloxacin, (**b**) urea, (**c**) physical mixture norfloxacin-urea (1 : 1) after recrystallized from ethanol-acetone (1 : 1) solvent, mixture, and (**d**) norfloxacin-urea co-crystal prepared by ultrasound-assisted slurry co-crystallization observed by polarizing microscope at a magnification of 200x.

PXRD patterns analysis of norfloxacin-urea co-crystal formation prepared by the ultrasound-assisted slurry co-crystallization method

The powder X-ray diffractometer can be used to characterize the different crystal structures or polymorphs of two or more materials by comparing the PXRD patterns of these materials[32,33]. Therefore, this technique can also be applied to identify the successful co-crystal formation of some methods by comparing the PXRD patterns. **Figure 3** shows a co-crystal product prepared by the ultrasound-assisted slurry co-crystallization method having a PXRD pattern similar to the PXRD pattern of co-crystal prepared by the wet grinding method. As in the PXRD pattern prepared by the wet grinding, the main peaks of norfloxacin and urea were also not visible in the ultrasound-assisted slurry co-crystallization product, and new peaks appeared at the same 2θ angle as the wet grinding product.

Thermal analysis of norfloxacin-urea co-crystal

The DSC thermogram in **Figure 4** showed the only endothermic transition at 187.3°C, corresponding to the norfloxacin-urea co-crystal melting point. An endothermic transition due to the melting of norfloxacin occurs at 215.2°C, a characteristic of norfloxacin Form A[34], while urea melted at 130.0°C. The DSC thermogram of a co-crystal is characterized by its melting point between or below the individual pure components. The results of this characterization using PXRD and DSC confirmed that the ultrasound-assisted slurry co-crystallization method could be used to manufacture norfloxacin-urea co-crystal in larger quantities than the wet grinding method, so it has the potential to be further developed on large scale.

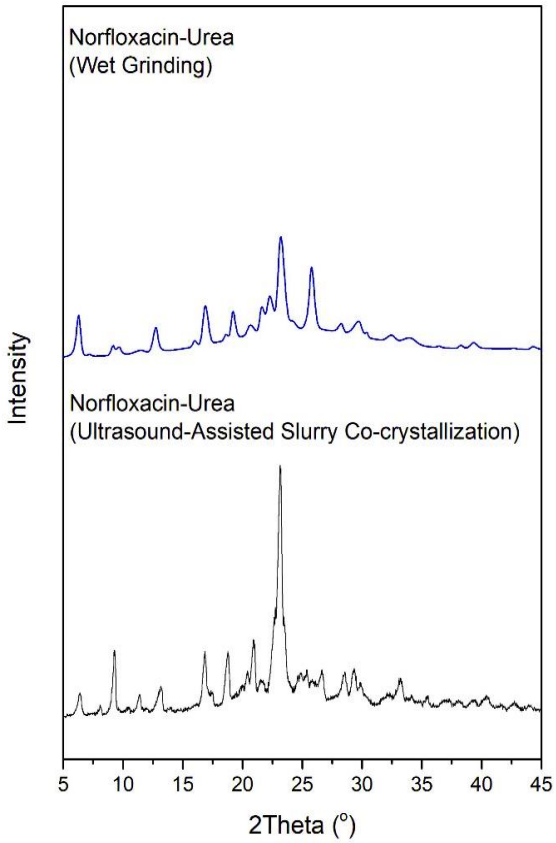

Figure 3. Powder X-ray diffraction patterns of norfloxacin-urea prepared by ultrasound assisted slurry co-crystallization and wet grinding methods.

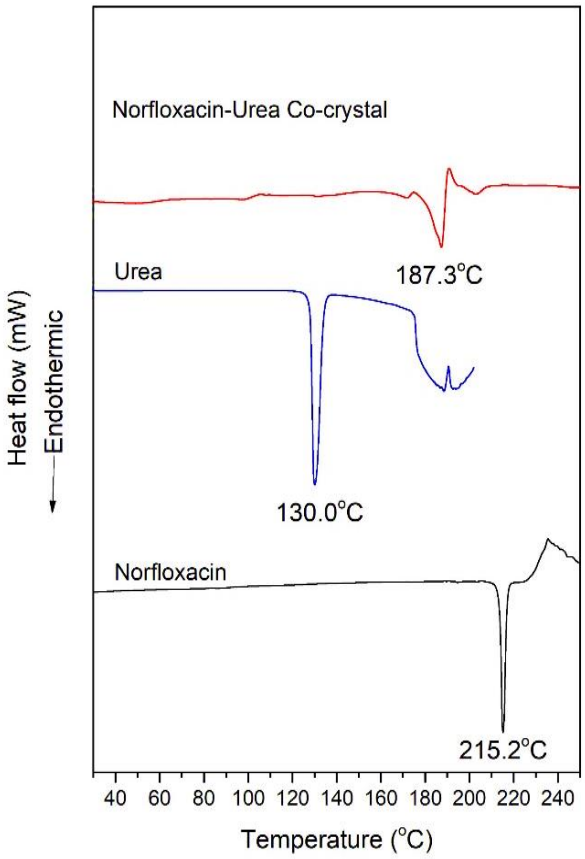

Figure 4. Differential scanning calorimetry thermograms of norfloxacin-urea co-crystal, pure norfloxacin, and urea.

Solubility test

Table I shows that the norfloxacin-urea co-crystal formation can increase the solubility of norfloxacin in water by as much as 1.9-folds compared to pure norfloxacin. The increase in solubility in the water is caused by the norfloxacin and urea molecules rearrangement to form hydrogen bonds or, in other words, due to changes in the crystal packing. The mechanism for increasing the drug solubility in the co-crystal is caused by the dissociation or re-breaking of weak hydrogen bonds between the drug and co-crystal former in an aqueous medium in a quick time (minutes or hours) so drug molecules become more easily wetted and dissolve[35]. Unlike in a water medium, the increasing solubility of norfloxacin in acetate buffer solution pH 4.0 was insignificant (1.1-folds) after the formation of norfloxacin-urea co-crystal. Norfloxacin is amphoteric, so its solubility depends on pH[3]. The solubility of norfloxacin in acetate buffer solution pH 4.0 was much higher than in water due to the protonation of the N atom in the piperazine ring[36]. Thus, the solubility of norfloxacin in the acetate buffer solution pH 4.0 was more due to ionization in an acidic environment than due to the hydrogen bonds formation, so there was no significant difference between the solubility of norfloxacin-urea co-crystal and pure norfloxacin in this medium.

Table I. Solubility of norfloxacin-urea co-crystal compared to pure norfloxacin (n=3)

Medium	Solubility (mg/mL)	
	Norfloxacin	Norfloxacin-urea co-crystal
Water	0.226 ± 0.007	0.430 ± 0.024
Acetate buffer solution pH 4.0	10.953 ± 0.701	12.085 ± 1.296

Dissolution test

Figure 5 shows the dissolution profiles of norfloxacin-urea co-crystal and pure norfloxacin in water and acetate buffer solution pH 4.0. The dissolution profile in the water medium showed that the percentage of dissolved norfloxacin from norfloxacin-urea co-crystal had reached more than 85% within 30 minutes, while that of pure norfloxacin only reached 56%. In contrast to the dissolution rate in water, the percentage of dissolved norfloxacin from both norfloxacin-urea co-crystals and pure norfloxacin reached more than 85% in acetate buffer solution pH 4.0. The difference in the dissolution rate profiles is related to the difference in the solubility of norfloxacin-urea and pure norfloxacin in a water medium and the similarity of their solubility in the acetate buffer solution pH 4.0.

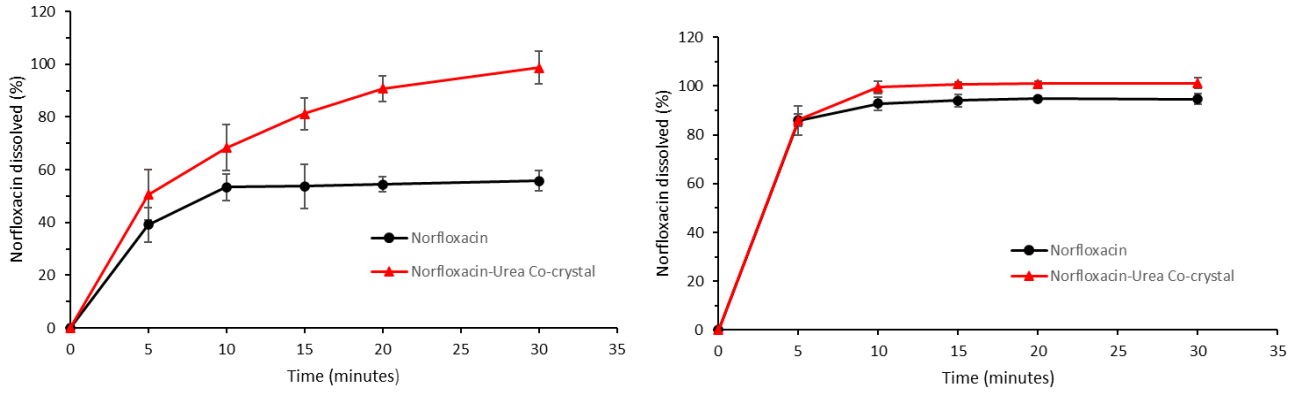

Figure 5. Dissolution profiles of norfloxacin-urea co-crystal and pure norfloxacin in the (**a**) water and (**b**) acetate buffer solution pH 4.0.

CONCLUSION

Norfloxacin-urea co-crystal was also successfully prepared by the ultrasound-assisted slurry co-crystallization method. The norfloxacin-urea co-crystal formation can enhance the solubility of norfloxacin in water and has the potential for scale-up when prepared by the ultrasound-assisted slurry co-crystallization method.

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AUTHORS' CONTRIBUTION

FA: initiated research, analyzed DSC, PXRD, and crystal morphology results, wrote manuscripts, **DS**: conducted co-crystal preparation, solubility test, and dissolution rate, **NAA**: provided advice on writing and analysis of solubility results. All authors have read and approved the publication of the manuscript.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

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

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Research Article

In-Silico Design and Evaluation of the Anti-Wolbachia Potential of Boron-Pleuromutilins

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Boron-pleuromutilins
Filariasis
Molecular docking
Pharmacokinetics
Wolbachia

Abstract

Filariasis (Lymphatic filariasis and Onchocerciasis) is a common neglected tropical disease caused by parasitic nematodes called filarial worms, which often host the *Wolbachia* bacteria. A good treatment approach seeks *Wolbachia* as a drug target. Here, a computer-aided design of some boron-pleuromutilin analogs was conducted using the ligand-based drug design approach while performing molecular docking investigation and pharmacokinetics analyses to evaluate their drug-likeness properties. The newly designed compounds (**49a**, **49b**, and **49c**) showed improved inhibitory activities (pEC_{50}) over those of the template and the clinically relevant pleuromutilins (retapamulin and lefamulin) in the order; **49b** (pEC_{50} = 9.0409) > **49c** (8.8175) > **49a** (8.5930) > template (**49**) (8.4222) > retapamulin (6.7403) > lefamulin (6.1369). Standard docking performed with OTU deubiquitinase (6W9O) revealed the order of binding energies; **49c** (-88.07 kcal/mol) > **49b** (-84.26 kcal/mol) > doxycycline (-83.70 kcal/mol) > template (-82.57 kcal/mol) > **49a** (-78.43 kcal/mol) > lefamulin (-76.83 kcal/mol) > retapamulin (-76.78 kcal/mol), with the new compounds all showing good pharmacological interactions with the receptor's amino acids. The new analogs were also predicted to be orally bioavailable with better pharmacokinetic profiles than the template, retapamulin, lefamulin, and doxycycline having no more than one violation of Lipinski's ROF. Therefore, the newly designed compounds could be considered potential anti-filarial drug candidates.

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INTRODUCTION

Lymphatic Filariasis (LF), also known as elephantiasis and onchocerciasis (river blindness), are common Neglected Tropical Diseases (NTD) caused by some parasitic nematode worms[1]. Filarial worms such as *Wuchereria bancrofti*, *Brugia timori*, and *Brugia malayi* are the causative organisms for LF and are usually transmitted by the *Culex* mosquitoes. Onchocerciasis, on the hand, is caused by *Onchocerca volvulus* being transmitted from one person to another by blood-feeding blackflies[2]. Elephantiasis alone is responsible for not less than 2.8 million disabilities globally, while River blindness is the world's second leading infectious cause of blindness[3].

The global program intended to eliminate these filarial diseases started far back through the Mass Drug Administration (MDA) of the combination therapy; ivermectin, albendazole, and diethylcarbamazine, either as a dual (annual to bi-annual) or triple-drug (once every three years) treatment[3,4]. However, it became unlikely that the MDA regimen would be enough to eliminate filariasis in all endemic areas, majorly due to their inability to kill the macro-filarial[5]. Given the current scenario, a macro-filaricidal agent is required to kill adult worms and drastically reduce both diseases' elimination periods[6]. Fortunately, one unique characteristic of these filarial worms is their symbiotic co-existence with a known bacterium called

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Wolbachia[7]. In the search for new anti-filarial drugs, some researchers have chosen *Wolbachia* as an anti-filarial drug target. Previous research has shown that eliminating *Wolbachia* from the host filarial nematodes leads to anti-filarial effects by reducing adult worm's lifespan[8,9]. Although the anti-bacteria drug doxycycline has been used clinically for the treatment of filarial diseases over the years, the treatment method is not efficient enough for use through MDA owing to its requirement for extended treatment periods (4-6 weeks) as well as contraindications in pregnancy and children[9]. Therefore, advances in developing new anti-*Wolbachia* agents with short treatment periods and reduced complications are necessary.

Ubiquitination is a biological process that significantly regulates several cellular processes in living cells, especially eukaryotes[10]. Manipulation of the host ubiquitin signaling is often observed amongst bacterial and viral pathogens, including *Wolbachia*. This manipulation is possible because most pathogens possess deubiquitinases that enable them to subvert host signaling[11]. As a result, the viability of bacteria within the host may be seriously affected by deubiquitinase inactivation. A common deubiquitinase is Ovarian Tumor (OTU) deubiquitinase[11,12]. Pleuromutilin was first reported in 1951 from the basidiomycetes *Pleurotus mutilis* (FR.) Sacc and *Pleurotus passeckerianus* Pilat[13]. Pleuromutilin and its analogs are antibacterial drugs that are inhibitors of protein synthesis in bacteria. Examples of antibiotics in this class include retapamulin, valnemulin, and tiamulin[14]. In the mid-1970s, much work was reported on using pleuromutilins as antibiotics for veterinary purposes only[13]. Since then, several works have been undertaken to develop derivatives of the base structure for human use. Retapamulin became the first approved pleuromutilin antibiotic for human use, approved by the FDA in 2007[15]. Pleuromutilins have generally been reported to show potency against Gram-positive and some fastidious Gram-negative organisms[15]. In 2021, Ugbe *et al*.[16] carried out the activity modeling, molecular docking, and pharmacokinetic studies of some boron-pleuromutilin derivatives as anti-*Wolbachia* agents with the potential for the treatment of LF and onchocerciasis.

Computer-aided modeling approaches such as QSAR modeling, molecular docking, drug-likeness properties prediction, molecular dynamics (MD) simulation, homology modeling, and others play a crucial role in drug discovery owing to their advantages over the conventional methods in terms of timeliness, cost-effectiveness, and reliability[17,18]. In the present study, therefore, OTU deubiquitinase from *Wolbachia pipientis* wMel with PDB ID 6W9O was adopted as the therapeutic protein target for the newly designed boron-pleuromutilin analogs. The crystal structure of OTU deubiquitinase determined by X-ray diffraction and expressed in *Escherichia coli* was obtained from the RCSB protein data bank and used in this study[11]. This work focuses on the ligand-based design of some boron-pleuromutilin analogs as novel *Wolbachia* inhibitors while subjecting them to molecular docking investigation, oral bioavailability test, and ADMET properties prediction to evaluate their pharmacological and drug-likeness properties.

MATERIALS AND METHODS

Materials

The hardware used was an HP laptop computer with the following specifications: Processor (Intel® Core™ i5-4210U CPU @1.70GHz 2.40 GHz), Installed RAM (8.00 GB), System Type (64-bit operating system, x64-based processor), Edition (Windows 10 Home Single Language), Version 21H2. Software used includes ChemDraw Ultra v. 12.0.2, Spartan '14 v. 1.1.4, Biovia Discovery Studio Visualizer v. 16.1.0.15350, and Generic Evolutionary Method for molecular docking (iGEMDOCK). The online web servers; http://www.swissadme.ch/index.php and http://biosig.unimelb.edu.au/pkcsm were used for the pharmacokinetics properties prediction[19,20].

Methods

Design template

From the results of our previous work on the anti-*Wolbachia* activity modeling of 52 boron-pleuromutilin derivatives[16], compound **49** (**Figure 1**) with a predicted activity (pEC_{50}) value of 8.4220 and a relatively better pharmacokinetic profile was identified as the template for designing improved derivatives. The QSAR model equation and its associated molecular descriptors, as obtained from our previous study[16], were presented in **Equation 1** and **Table I**, respectively.

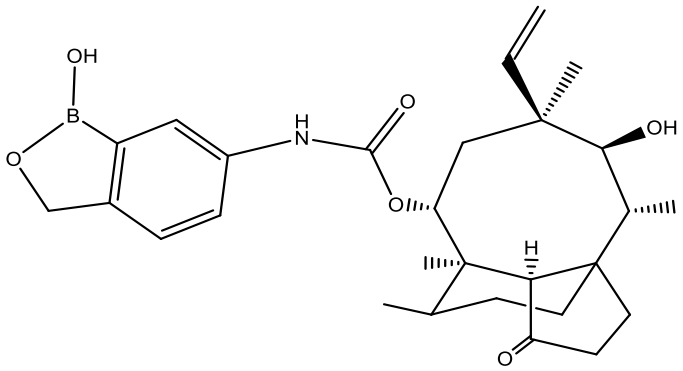


Figure 1. Two-dimensional structure of the template molecule (**49**).

$$pEC_{50} = 18.608123445 \times SpMax3\_Bhm + 38.931870819 \times SpMin3\_Bhs + 1.297730563 \times minHBint5 - 1.288520115 \times mindO - 2.058961109 \times MLFER\_BO + 0.199866243 \times RDF75v - 0.664720197 \times L3i - 107.380579189 \quad \textbf{[1]}$$

Table I. The QSAR model equation and the associated molecular descriptors

S/No	Symbol	Descriptor	Class	Mean Effect
1	SpMax3_Bhm	Largest absolute eigenvalue of Burden modified matrix - n 3 / weighted by relative mass	2D	0.5865
2	SpMin3_Bhs	Smallest absolute eigen value of Burden-modified matrix—n 3/weighted by the relative I-state	2D	0.5920
3	minHBint5	Minimum E-State descriptors of strength for potential Hydrogen Bonds of path length 5	2D	-0.0011
4	mindO	Minimum atom-type E-State: = O	2D	-0.1479
5	MLFER_BO	Overall or summation solute hydrogen bond basicity	2D	-0.0444
6	RDF75v	Radial distribution function - 075 / weighted by relative van der Waals volumes	3D	0.0279
7	L3i	2nd component size directional WHIM index/ weighted by relative Sanderson electronegativities	3D	-0.0130

Drug design

Three boron-pleuromutilin analogs were designed using the ligand-based design approach by substituting, adding, and inserting substituent(s) into the template (**49**). The various modifications were made at positions 3, 4, and 12 of the structural template (**Figure 2**). Compound **49a** was designed by modifying the 12-position of the pleuromutilin core by introducing methyl group (CH_3-) as R_1. Also, substituting hydrogen at position 4 of the benzoxaborole group with a methyl group (R_2) and introducing a methylene group (-CH_2) as R_1 resulted in **49b**. Inserting a methyl group (R_3) at position 3 of the benzoxaborole group while also keeping R_1 as methyl group produced compound **49c**.

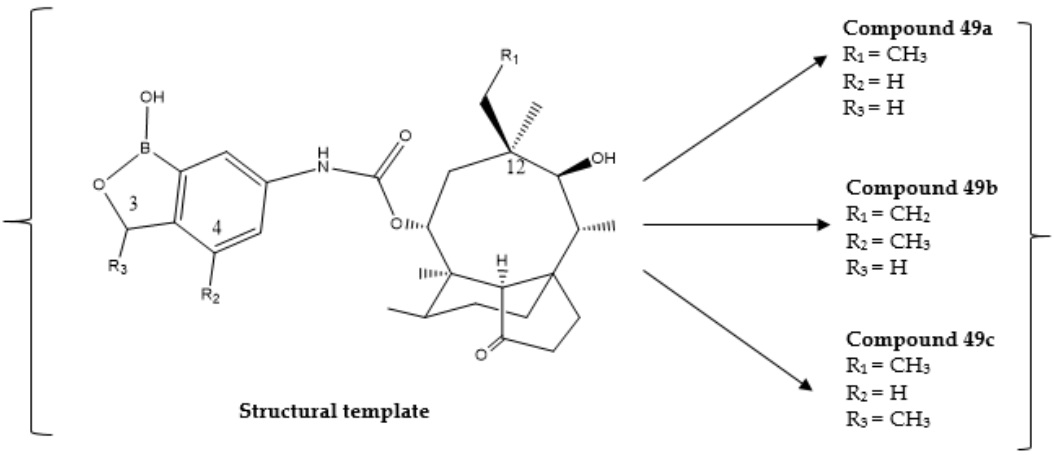


Figure 2. The design schemes.

Preparation of the ligands and descriptors calculation

The molecular structures of the template (**49**), newly designed, and the reference compounds (lefamulin, retapamulin, and doxycycline) were first drawn using ChemDraw Ultra, saved as MDL mol file format, and after that, fed separately into the Spartan software for energy minimization and geometry optimization. The structures were then pre-optimized using Molecular Mechanics Force Field (MMFF) before the optimization using Density Functional Theory (DFT) with a B3LYP/6-31G basis set[21-23]. The resulting stable conformations of the various analogs were saved in MDL SD and PDB file formats for subsequent analyses involving descriptor calculation and molecular docking study, respectively. The resulting data in SD file format were then fed into the Pharmaceutical Data Exploration Laboratory (PaDEL)-descriptor software to generate the descriptor pool from where the values of the seven descriptors were extracted16.

Preparation of the protein receptor and molecular docking investigation

The crystal structure of OTU deubiquitinase (PDB ID 6W9O) was obtained from the RCSB Protein Data Bank in PDB file format and then prepared separately using the Biovia Discovery Studio Visualizer by excluding water molecules and co-crystallized ligands found within the protein structures[20]. The receptor's Chain A was utilized. A Molecular docking investigation was performed between the receptor and the prepared ligands using the iGEMDOCK tool. iGEMDOCK is a program for computing a ligand conformation and orientation relative to the target protein's active sites. Here, the blind docking approach was used with the docking accuracy parameter settings for standard docking. This setting specified a population size of 200, a number of generations equal to 70, and a number of solutions equal to 2. GEMDOCK is an automatic system that generates all related docking variables, such as atom formal charge, atom type, and the ligand-binding site of a protein[24]. The resulting protein-ligand interaction profiles and docked poses were analyzed using the iGEMDOCK's post-screening analysis tool and the Biovia Discovery Studio Visualizer[25,26].

Prediction of pharmacokinetic properties

Drug-likeness and ADMET properties prediction are necessary for the initial stage of drug discovery because only molecules with good pharmacokinetic profiles make the pre-clinical phase of drug research[21]. The present study predicted the pharmacokinetic properties of the lead molecule, the newly designed, and the reference compounds by employing http://www.swissadme.ch/index.php and http://biosig.unimelb.edu.au/pkcsm for drug-likeness and ADMET profiling respectively. Lipinski's 'rule of five' (ROF), a widely used criterion for oral bioavailability, was used to assess the newly designed compounds for oral bioavailability[16,18].

RESULTS AND DISCUSSION

Drug design and activity/affinity prediction

Three new analogs of boron-pleuromutilin (**49a**, **49b**, and **49c**) were designed by the Ligand-based drug design method. The QSAR model (**Equation 1**) was used to predict the binding activities of the various compounds, while the binding energy of interactions was obtained from the docking simulation. The molecular structures, predicted bioactivities (pEC_{50}), and binding energies of the template, newly designed compounds, and the two clinically relevant pleuromutilins, as well as the standard drug (doxycycline), were presented in **Table II**, while the calculated values of the various descriptors were shown in **Table III**.

The binding activities of the various compounds were predicted adequately by the QSAR model equation (**Equation 1**). The predicted pEC_{50} of all the newly designed compounds were greater than those of the Template and the reference compounds in the order; **49b** (pEC50 = 9.0409) > **49c** (8.8175) > **49a** (8.5930) > template (**49**) (8.4222) > retapamulin (6.7403) > lefamulin (6.1369). The contribution of each descriptor to the inhibitory activities of the various compounds is expressed by the value of its Mean Effect (ME). From **Table I**, SpMin3_Bhs was reported as having the largest positive ME value of 0.592016. SpMin3_Bhs is the smallest absolute eigenvalue of Burden modified matrix – n 3/ weighted by relative I-state. As such, introducing an electronegative atom or electron withdrawing group is said to decrease the value of its coefficient, which in turn decreases the molecules' anti-proliferative activities[27]. This implies that an electron-donating group will increase the molecule's inhibitory activity. The newly designed compounds' relatively higher predicted pEC_{50} values may

be attributed to introducing of the methyl moiety, an electron-donating group that releases electrons to the ring system through a positive inductive effect. Therefore, this provides a reasonable explanation for their high anti-*Wolbachia* activity.

Table II. Molecular structures, predicted pEC_{50}, and binding energies of the template, newly designed, and reference compounds

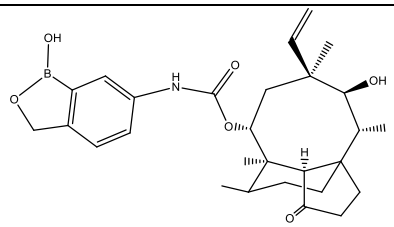
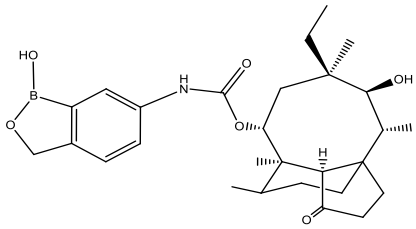
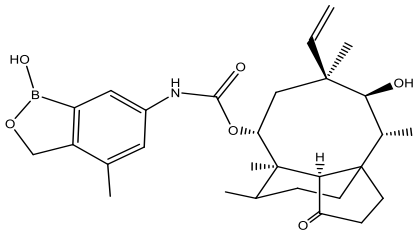
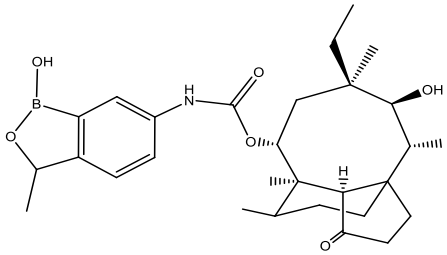
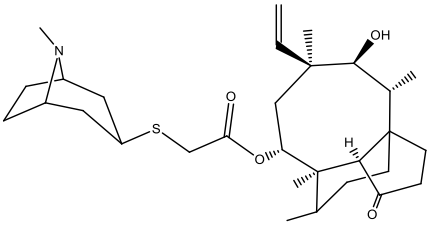
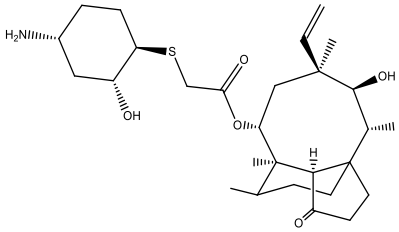
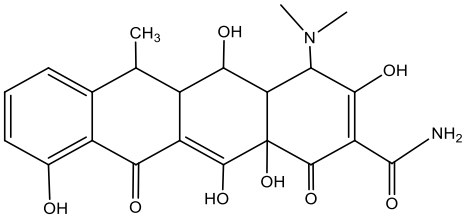
Compound ID	Molecular structure	Predicted pEC_{50}	Binding energy
Template (**49**)		8.422169	-82.57
49a		8.592983	-78.43
49b		9.040934	-84.26
49c		8.817465	-88.07
Retapamulin		6.740265	-76.78
Lefamulin		6.136877	-76.83
Doxycycline		-	-83.70

Table III. Calculated descriptors for the template, newly designed, and reference compounds

Comp ID	SpMax3_Bhm	SpMin3_Bhs	minHBint5	mindO	MLFER_BO	RDF75v	L3i
Template (**49**)	3.615	1.7597	0.0712	13.287	1.893	12.627	2.365
49a	3.6018	1.7625	-0.0887	13.313	1.857	14.524	2.2208
49b	3.6152	1.7597	0.02824	13.399	1.881	16.619	2.3738
49c	3.6023	1.7833	-0.1211	13.378	1.877	13.364	2.5185
Retapamulin	3.6543	1.7172	-0.1047	13.47	2.223	14.381	2.3145
Lefamulin	3.6147	1.7107	-0.5427	13.302	2.365	19.119	2.1869

Molecular docking study

A molecular docking simulation was conducted between the receptor, OTU deubiquitinase (6W9O), and the various compounds using the iGEMDOCK molecular docking tool to provide insight into the mode of binding interactions at the ligand-receptor interface and the associated binding energies. The results (binding energies) of the docking investigation were included in **Table II**, while **Table IV** and **Figures 3** to **9** showed the predicted pharmacological interaction profiles of the various compounds with the target protein (6W9O). Binding energies of the receptor-ligand interactions are necessary to describe how well ligands fit into active sites of the target protein to form the most energetically stable drug-receptor complex[28]. The receptor-ligand binding process is spontaneous, indicated by the negative values of the binding energy change[28]. Hence, the potential drug candidate's chances to initiate protein biochemical action/reaction increase as the binding energy becomes more negative[26].

Table IV. Predicted pharmacological interaction profiles of the various compounds with the protein target (6W9O)

Comp ID	Hydrogen bond interactions			Hydrophobic interactions
	Amino acid	Type	Distance (Å)	
Template	ARG-122	Conventional	2.19, 2.67, 2.69	TRP-123 (π-π stacked), π-alkyl (TYR-113, ARG-122), ARG-122 (steric bump), ASN-112 (Acceptor-acceptor clash)
	TRP-123	Conventional	3.28	
	TRP-123	π-donor	2.33	
49a	TYR-171	π-donor	3.88	TYR-171 (π-π T shaped), Alkyl (ARG-83, LYS-140, LEU-159)
	ASN-172	Carbon-hydrogen	2.91	
	LYS-187	Conventional	2.81	
49b	SER-163	Conventional	3.90	LYS-166 (Alkyl)
	TYR-171	Conventional	3.72	
49c	SER-155	Carbon-hydrogen	3.36	HIS-157 (π-π T shaped), HIS-157 (π-Sigma), π-alkyl (ALA-168, TYR-176), Alkyl (PRO-39, LYS-173, VAL-174)
	HIS-157	Conventional	4.89	
	VAL-174	Conventional	3.34, 4.99	
Retapamulin	HIS-157	Carbon-hydrogen	4.17	HIS-157 (π-sigma), π-alkyl (TYR-176, HIS-157), Alkyl (LEU-156, VAL-174, TYR-176, LYS-173, ALA-168), HIS-157 (Miscellaneous)
	ALA-168	Carbon-hydrogen	5.68	
	VAL-174	Conventional	3.93	
Lefamulin	LYS-140	Conventional	5.78, 5.84	Alkyl (LEU-159, LYS-166, LYS-140)
	ASP-175	Carbon-hydrogen	5.07	
	ASP-178	Conventional	4.23	
Doxycycline	CYS-134	Conventional	3.81	LYS-138 (Alkyl), LYS-140 (π-alkyl)
	GLU-135	Carbon-hydrogen	3.95	
	LYS-138	Conventional	3.97	
	VAL-139	Conventional	5.56	
	ASN-162	Conventional	3.53, 5.26, 5.39	

Analyses of the docking results in **Table II** revealed that compounds **49b** and **49c** showed relatively higher binding energies than the template, reference pleuromutilins, and reference drug doxycycline in the order; **49c** (-88.07 kcal/mol) > **49b** (-84.26 kcal/mol) > doxycycline (-83.70 kcal/mol) > template (-82.57 kcal/mol) > **49a** (-78.43 kcal/mol) > lefamulin (-76.83 kcal/mol) > retapamulin (-76.78 kcal/mol). **Table IV** and **Figures 3** to **9** showed the predicted pharmacological interaction profiles of the various compounds with the target protein. In general, as seen in **Table IV**, the interactions between these molecules (ligands) and the protein's amino acid residues were characterized by hydrogen bonding (H-bonding), hydrophobic interactions, and Van der Waals interactions, which are highly desirable for the reversibility of drug-receptor binding. H-bonds, alongside hydrophobic interactions, play a vital role in docked complex stabilization and enhancing the binding affinity of the ligand at the ligand-receptor interface[29]. In addition, H-bond plays a crucial role in determining the specificity of ligand binding[30]. The binding profile of the lead molecule (**Figure 3**) contained some unfavorable interactions, steric bump with ARG-22 at an interaction distance of 4.82Å, and acceptor-acceptor clash with ASN-112 at 4.67Å. All the interactions observed in the binding profiles of the newly designed and the reference compounds were favorable. The new

analogs could not compete favorably with the reference drug doxycycline regarding hydrogen bonding interactions but clearly showed more hydrophobic interactions than doxycycline. Therefore, the newly designed analogs have demonstrated adequate binding interactions with the target protein (OTU deubiquitinase), indicating their potential to arrest the protein receptor, an important factor that governs several activities essential for the viability of the bacteria (*Wolbachia*).

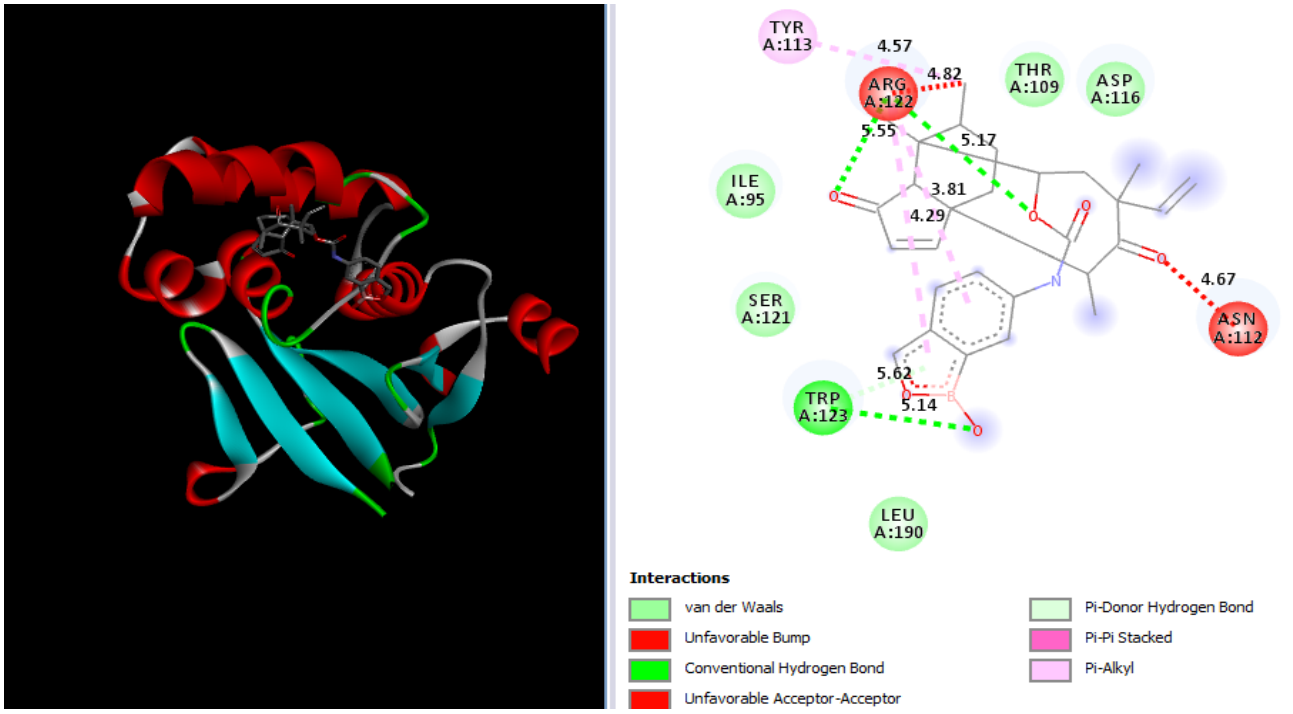


Figure 3. Binding interactions between 6W9O and the template (**49**).

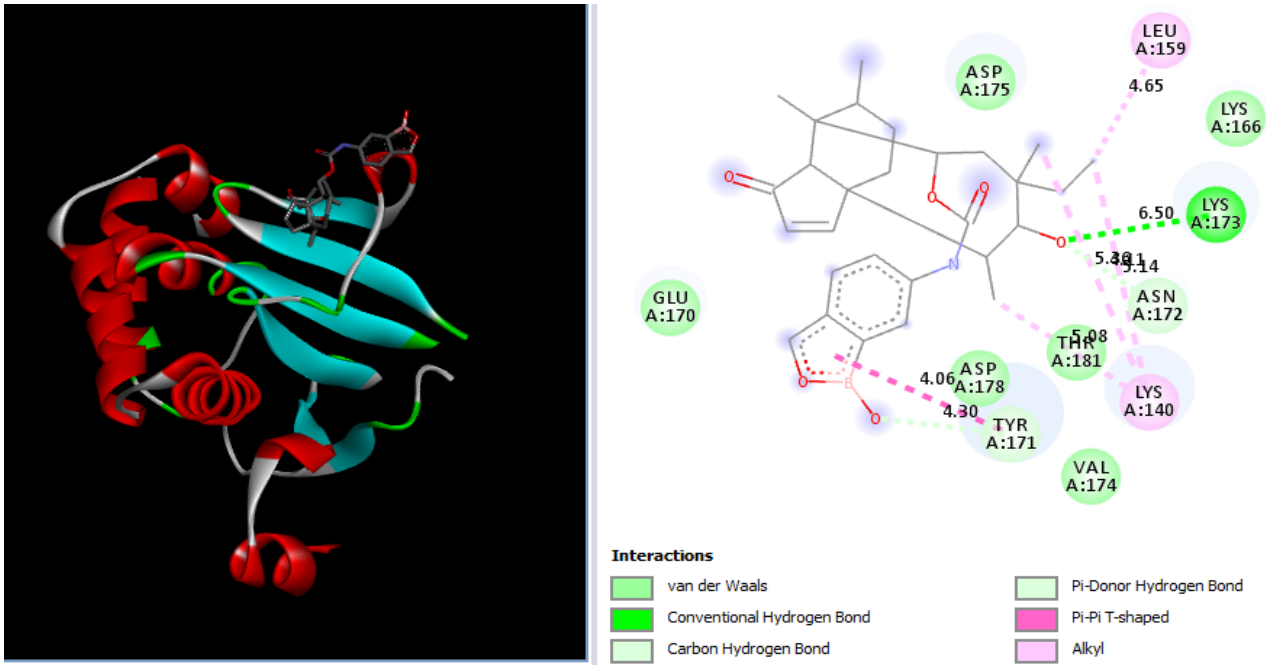


Figure 4. Binding interactions between 6W9O and compound **49a**.

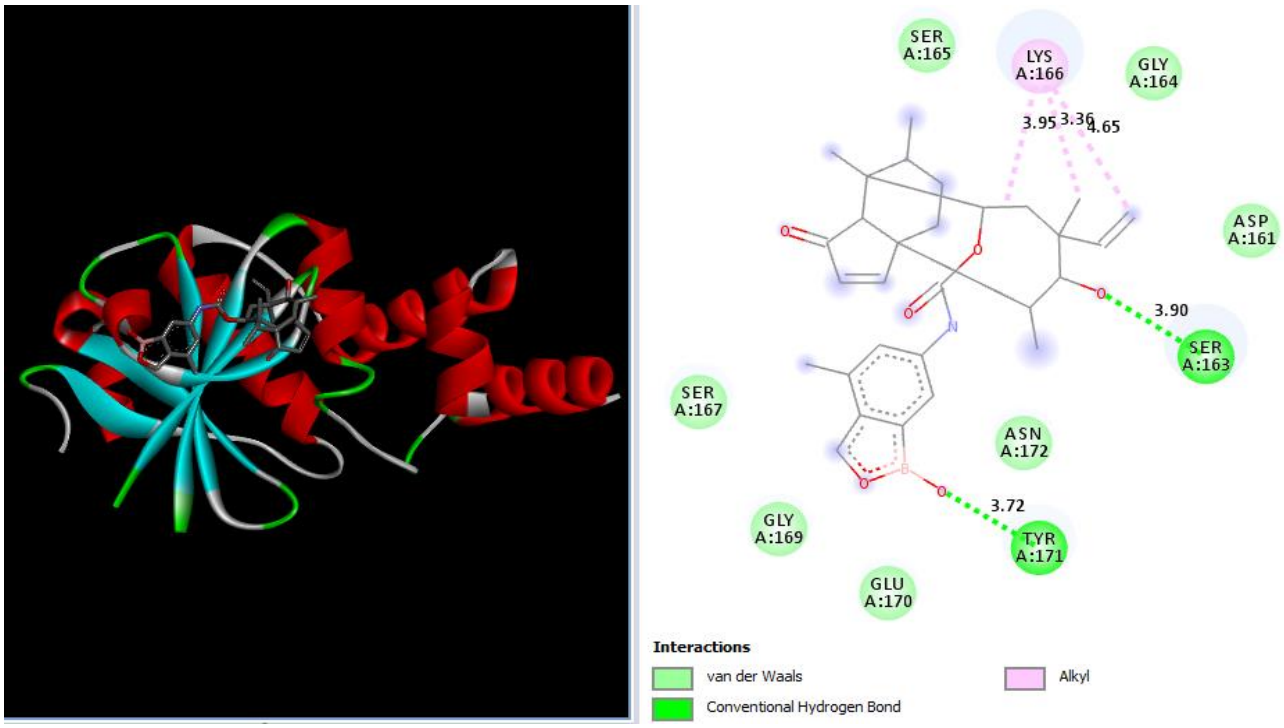


Figure 5. Binding interactions between 6W9O and compound **49b**.

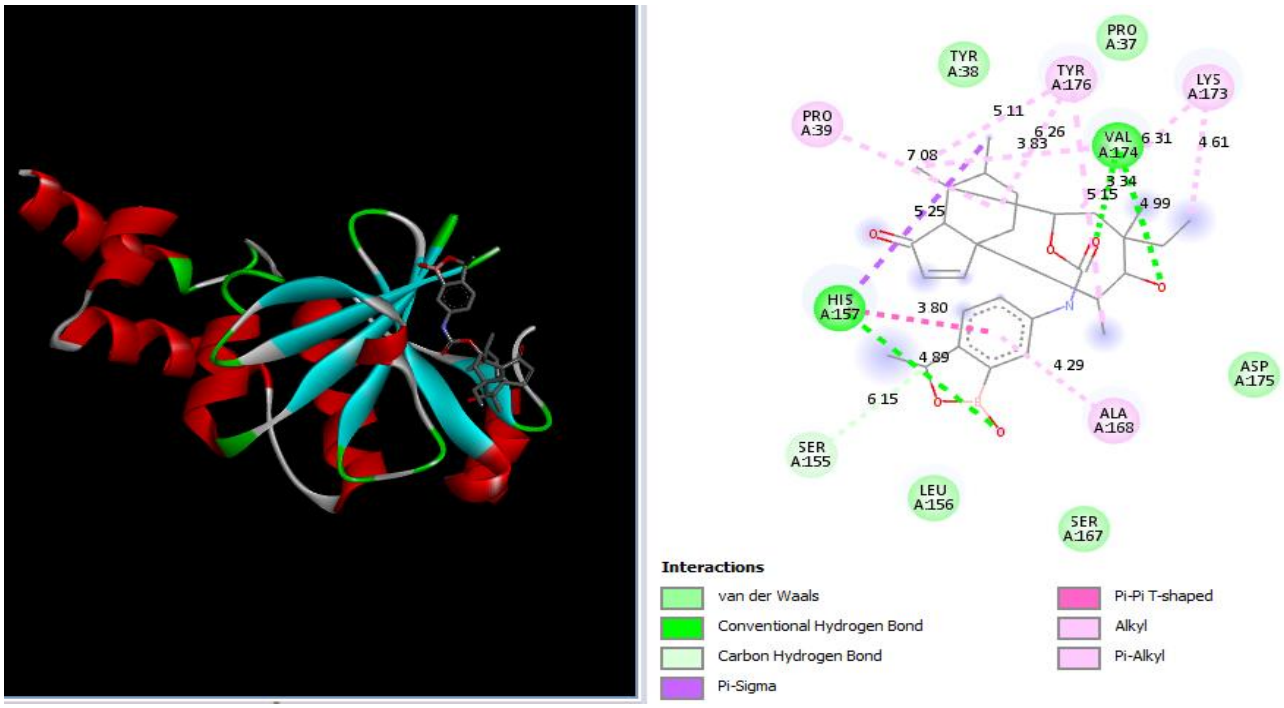


Figure 6. Binding interactions between 6W9O and compound **49c**.

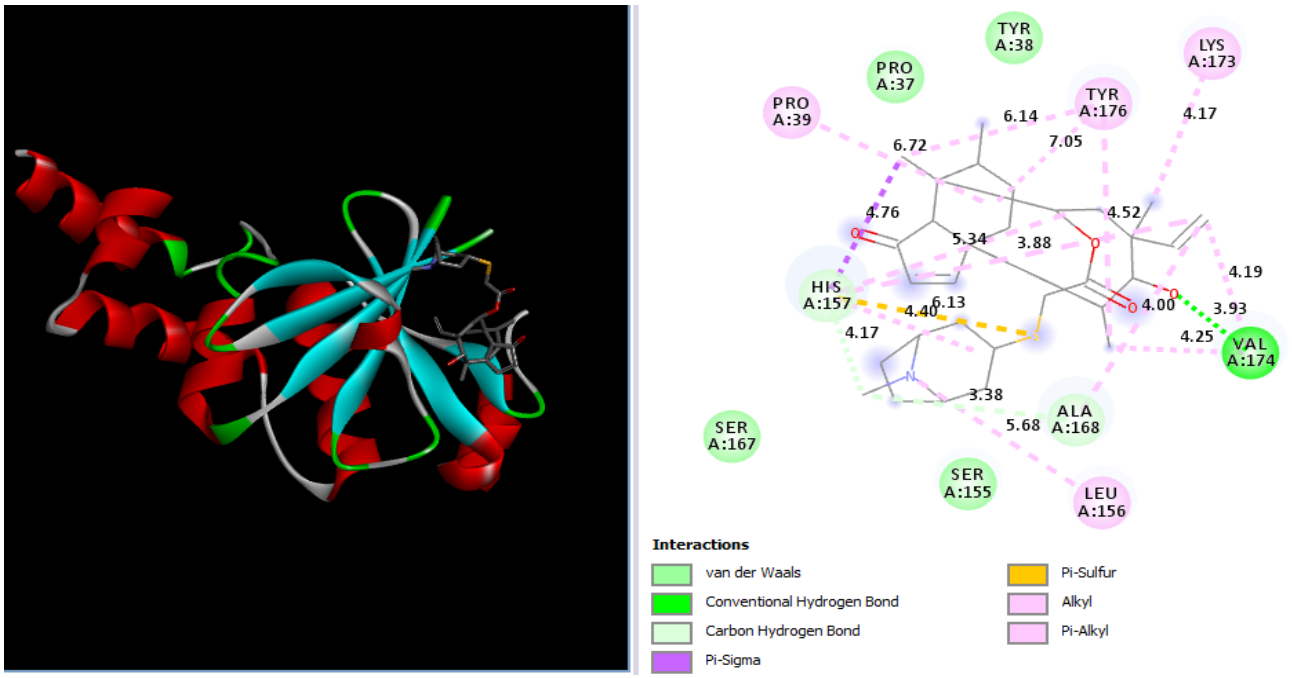


Figure 7. Binding interactions between 6W9O and retapamulin.

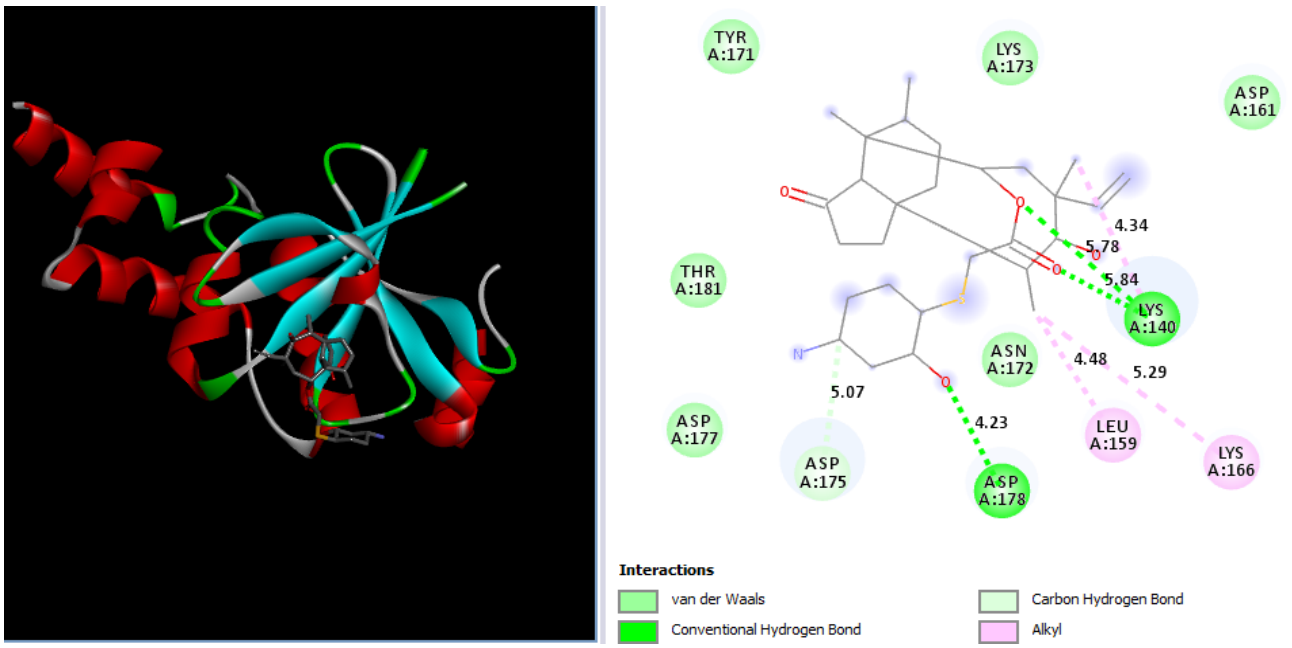


Figure 8. Binding interactions between 6W9O and lefamulin.

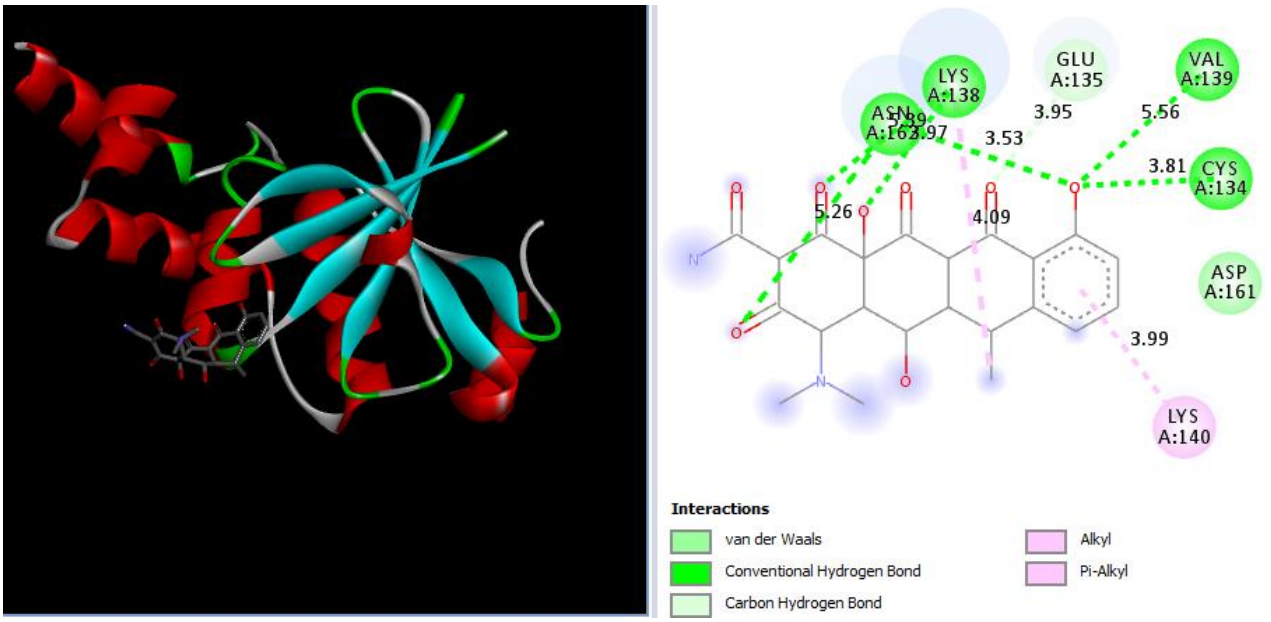


Figure 9. Binding interactions between 6W9O and doxycycline.

Evaluation of pharmacokinetic properties

Drug-likeness analysis and ADMET study were conducted on the three compounds (**49a**, **49b**, and **49c**) to evaluate their oral-bioavailability and compared identical with those of the lead compound and the reference drugs (retapamulin, lefamulin, and doxycycline). The results of both investigations are presented in **Tables V** and **VI**. Furthermore, **Figure 10** shows the oral bioavailability radar of the newly designed molecules, the template, the two clinically relevant pleuromutilins, and doxycycline.

Lipinski's rule for oral-bioavailability states that a drug molecule is more likely to have poor absorption or permeation when it has Hydrogen Bond Donors (HBD) of greater than 5, Hydrogen Bond Acceptors (HBA) > 10, Molecular Weight (MW) > 500, and lipophilicity (MLOGP > 4.15 or WLOGP > 5)[31]. Usually, molecules that obey at least three of the four requirements are said to be orally bioavailable[21]. **Table V** shows that all the molecules obeyed Lipinski's ROF since they satisfied at least three of the four requirements with **49a**, and the template showed no violation. Also, the reported Topological Polar Surface Area (TPSA) values for all molecules are less than 140 $Å^2$ except for doxycycline with a TPSA of 181.62 $Å^2$, indicating the likelihood of doxycycline being poorly absorbed. The synthetic accessibility scores of the newly designed molecules range from 6.66 to 6.83, indicating fewer rigors in their laboratory synthesis compared to retapamulin and lefamulin, with 7.51 and 6.95, respectively. However, the reference drug doxycycline with 5.15 is predicted to have a relatively more accessible synthetic pathway. The predicted ADMET properties in **Table VI** showed good intestinal absorption of more than 90% for all newly designed compounds, which are well clear of the 30% threshold value, and compare well with those of the template (93.385%), retapamulin (94.196%), lefamulin (81.211%), and doxycycline (31.193%, very poor).

All the molecules presented are substrates of P-glycoprotein, which act as a biological barrier by extruding toxins and xenobiotics, including drugs, out of cells. Interestingly, they are also inhibitors of both P-glycoprotein I and II except doxycycline, a shred of evidence that the molecules may mediate well to reach their target sites without being isolated by the P-glycoprotein. Additionally, the newly designed compounds and the template are substrates and inhibitors of Cytochrome P450 (CYP-3A4), an essential enzyme for drug metabolism in the body, which means a well-regulated (optimal) metabolic process for the molecules in the body. Retapamulin and lefamulin, on the other hand, are substrates of this enzyme only, while doxycycline is neither substrate nor inhibitor. Furthermore, all the molecules presented showed Blood Brain Barrier (BBB) permeability (Log BB) of less than 0.3, indicating they do not readily permeate through the blood-brain barrier. Also, the molecules all showed poor Central Nervous System (CNS) permeability since Log PS < -2. The total clearance for a drug molecule in the body for these molecules is within the accepted range, while they showed no AMES toxicity, indicating that the molecules are non-mutagenic and, as such, are non-carcinogenic[32,33]. From **Figure 10**, the colored zone represents the suitable physicochemical space for oral bioavailability, in which the template and the new analogs fit slightly better than the reference compounds. Based on the predicted parameters, the newly designed molecules are said to

relatively possess better pharmacokinetic profile than the reference compounds being that they showed high intestinal absorption, no more than 1 ROF violation, lower synthetic accessibility score, substrates and inhibitors of CYP-3A4, and no AMES toxicity.

Table V. Predicted drug-likeness properties of the newly designed compounds and reference drugs

Comp ID	MW (g/mol)	TPSA (Å²)	MLOGP	HBD	HBA	RO5 Violation	SA
Template	495.42	105.09	2.46	3	6	0	6.59
49a	497.43	105.09	2.54	3	6	0	6.66
49b	509.44	105.09	2.65	3	6	1	6.77
49c	511.46	105.09	2.74	3	6	1	6.83
Retapamulin	517.76	92.14	4.04	1	5	1	7.51
Lefamulin	507.73	135.15	2.84	3	6	1	6.95
Doxycycline	444.43	181.62	2.08	6	9	1	5.15

Note: SA – Synthetic accessibility

Table VI. Predicted ADMET properties of the newly designed compounds and reference drugs

ID	Absorption				Distribution		Metabolism		Excretion	Toxicity
	Intestinal absorption (%)	P-glycoprotein Substrate	P-glycoprotein Inhibitor I	P-glycoprotein Inhibitor II	BBB Permeability Log BB	CNS Permeability Log PS	CYP-3A4 Substrate	CYP-3A4 Inhibitor	Total clearance	AMES toxicity
Tem	93.385	Yes	Yes	Yes	-0.785	-2.849	Yes	Yes	0.163	No
49a	94.238	Yes	Yes	Yes	-0.817	-2.82	Yes	Yes	0.134	No
49b	93.859	Yes	Yes	Yes	-0.78	-2.803	Yes	Yes	0.111	No
49c	92.621	Yes	Yes	Yes	-0.846	-2.71	Yes	Yes	0.064	No
Ret	94.196	Yes	Yes	Yes	-0.736	-2.844	Yes	No	0.491	No
Lef	81.211	Yes	Yes	Yes	-0.986	-3.331	Yes	No	0.433	No
Dox	31.193	Yes	No	No	-1.763	-3.829	No	No	0.241	No

Note: Tem – template; Ret – retapamulin; Lef – lefamulin; Dox - doxycycline

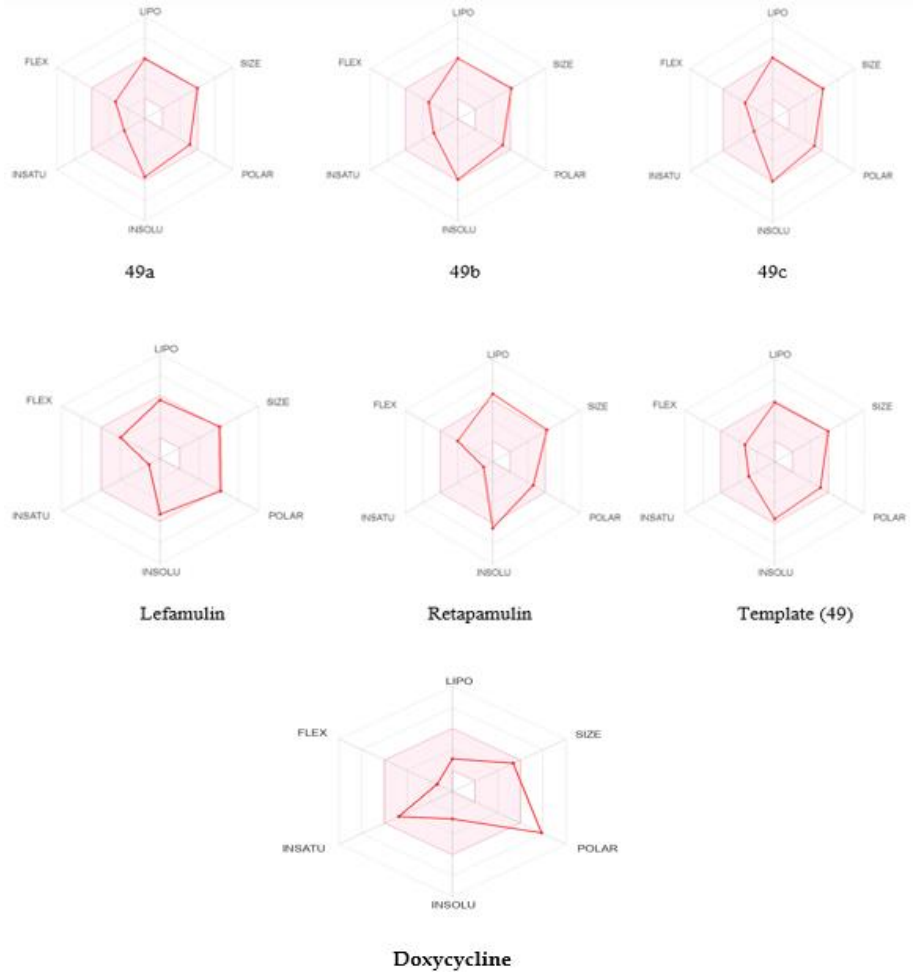


Figure 10. Oral bioavailability radar of the newly designed compounds, template, lefamulin, retapamulin, and doxycycline.

CONCLUSION

Computer-aided design of three boron-pleuromutilin analogs (**49a**, **49b**, and **49c**) as *Wolbachia* inhibitors were carried out using compound **49** as the template while also performing molecular docking study and pharmacokinetic analysis to evaluate their pharmacological and drug-likeness properties. The newly designed compounds had improved inhibitory activities (pEC_{50}) than those of the template and the two clinically relevant pleuromutilin compounds (lefamulin and retapamulin). The binding energies of interactions of the newly designed compounds were relatively higher while showing better pharmacokinetic profiles than the reference compounds because they showed higher human intestinal absorption, lower synthetic accessibility scores, substrates and inhibitors of CYP-3A4, and no AMES toxicity. As a result, this study has provided medicinal chemists with helpful information on the new derivatives as anti-filarial agents. More so, laboratory tests (*in vitro* and *in vivo*) could be carried out to validate the computational results.

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AUTHORS' CONTRIBUTION

All authors conceived and designed the study. **Fabian A. Ugbe** carried out the study and drafted the manuscript. **Gideon A. Shallangwa** conducted the technical editing. All authors read and approved the final manuscript.

DATA AVAILABILITY

All data related to this study are included herein.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Ointment Formulation of Tapak Dara (*Catharanthus roseus* (L.) G. Don) Flower Ethanol Extract and its Activity in Burn-Healing

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Keywords:
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Ointment
Tapak dara flower

Abstract

Treatment done on burn wounds is intended to provide local therapy to heal as quickly as possible. The content of secondary metabolites in the tapak dara (*Catharanthus roseus* (L.) G. Don) flower can help the healing process of burns, namely alkaloids, saponins, tannins, and flavonoids. Alkaloids act as antibacterial; saponins can trigger collagen formation; tannins as astringents that cause shrinkage of skin pores and stop minor bleeding in wounds; and flavonoids have anti-inflammatory effects. This study aimed to formulate an ointment of *C. roseus* flower ethanol extract and determine its physical characteristics such as organoleptic test, homogeneity, pH value, dispersion, and stability test of the preparation and examine the activity as a burn healer in white male rats. The research data were analyzed statistically using the ANOVA method, followed by the LSD test (least significant difference) to see how the ointment-containing extract reduced the diameter and percentage of the burn wounds. The results show that all ethanol extracts of *C. roseus* flower ointments met the requirements for its physical characteristic tests. It offers a good activity as a burn healer in white male rats. The most effective concentration is an ointment containing 15% of ethanol extract from *C. roseus* flower (F3 group), which shows a significant difference ($p \leq 0.05$) from the blank and the other group formula in burn wound healing.

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INTRODUCTION

Burns are skin tissue damage or loss due to contact with heat sources such as fire, hot water, chemicals, electricity, and radiation. The extent of tissue damage during burns impairs angiogenesis, collagen reorganization, and granulation tissue formation and induces free radical-mediated damage resulting in delayed tissue repair[1]. Burn recovery requires lengthy hospitalizations, expensive drugs, and prolonged rehabilitation periods[2,3]. Inappropriate burn care will lead to complications, infection, and bleeding. Burns that are not treated promptly will be inhabited by pathogenic bacteria that rapidly undergo exudation with the absorption of large amounts of water, protein, and electrolytes and often require skin grafting from other parts of the body to produce permanent wound closure[4,5].

The problem of burns is still a global problem that needs to be resolved[6]. Indonesia's potential, rich in medicinal plants, is expected to be able to answer these problems[7]. One of the medicinal plants commonly used empirically as a healer for wounds and burns is tapak dara (*Catharanthus roseus* (L.) G. Don). *Catharanthus roseus* is a plant from the Apocynaceae family originating from Central America and is generally grown as an ornamental plant. *Catharanthus roseus* is a type of herbal plant that can grow up to 1 meter, including perennial plants, or can live for approximately two years. The leaves are green, oval in shape, and the flowers have five trumpet-shaped crowns, the color of the flowers is white, pink, or white with a red

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spot in the middle[8]. The components of the active compounds found in *C. roseus* are phenolic acids, flavonoids, alkaloids, saponins, terpenoids, and tannins[9,10]. The purpose of this study was to determine the activity of the ethanol extract of the *C. roseus* flower, which had been formulated in the form of an ointment as a burning medicine, and to find out the best extract concentration capable of healing burns compared to the positive control.

MATERIALS AND METHODS

Materials

The tools used were digital scales, rotary evaporator, measuring cups, and pH meter (Hanna Instruments). The test animals used in this study were male white rats weighing 200 - 300 g. The sample and materials used in this research were *C. roseus* flower, adeps lanae, vaseline album, nipagin, vanilla oil, 70% ethanol, aquadest, lidocaine, and betadine ointment.

Methods

Plant collection and determination

Catharanthus roseus flowers were picked purposively from Medan Helvetia, Deli Serdang Regency, North Sumatra, then dried and processed into fine simplicia powder. Plant determination was carried out at the Herbarium Medanense, Center for Biology Research, Universitas Sumatera Utara, Medan, North Sumatra. The determination result stated that the plant used as the sample was the flower of *C. roseus*, with certificate number 5961/MEDA/2021.

Extraction

The fine simplicia powder was put into a maceration vessel, then immersed in 70% ethanol solvent. This immersion was carried out for five days. Stirring was done so that the solvent was immersed in all the simplicia powder. After five days, the soaking results were then filtered using filter paper. The filtrate was taken and accommodated. The residue was macerated again for two days, then collected and concentrated with a rotary evaporator at 45°C to obtain a thick extract[11].

Ointments formulation

Ointment formulations are presented in **Table I**. There were four formulas (0, 1, 2, and 3), with the difference being the concentration of *C. roseus* extract used. All ingredients were weighed according to the calculation. Nipagin was put into the mortar, French vanilla oil was added, then ground until homogeneous. Then, the vaseline album was added and ground homogeneously. Adeps lanae was added and grounded until homogeneous. The extract of *C. roseus* flower was added little by little and ground until homogeneous, then the remaining part of the vaseline album was grounded until homogeneous. The base formulation of the ointment without extract was made as a blank[12].

Table I. Ointment formulation

Ingredients	F0 (blank)	F1 (5%)	F2 (10%)	F3 (15%)
Catharanthus roseus flower extract (g)	-	5	10	15
Adeps lanae (g)	15	15	15	15
Nipagin (g)	0.05	0.05	0.05	0.05
Vanilla oil (mL)	0.05	0.05	0.05	0.05
Vaseline album ad (g)	100	100	100	100

Evaluation of ointment

Stability test: The stability test was performed using the cycling test method. The ointment preparation was stored at a temperature of ±4°C for 24 hours, then transferred to a temperature of ±40°C for 24 hours (1-cycle). Tests were carried out in the 1st to 6th cycle by observing organoleptic, homogeneity, pH, and dispersion[13].

Organoleptic test: The organoleptic examination of the ointment was observed visually, including the physical form, color, and odor. Organoleptic analysis was performed every cycle on the cycling test (6 cycles)[14].

Homogeneity test: The homogeneity test was carried out by weighing 1 g of the ointment preparation and then smeared on a slide and tightly closed with another slide; then, the homogeneity of the ointment was observed. Homogeneous ointments

were characterized by the absence of lumps and granules, an even structure, and a uniform color. Homogeneity analysis was performed every cycle on the cycling test (6 cycles)[15].

pH test: pH value was measured using a pH meter. First, the instrument was calibrated using the acid and aqueous buffer, then the electrode was rinsed with distilled water and wiped with a tissue. About 1 g of the ointment was dissolved in 100 mL of distilled water. Then the electrode was dipped into the solution until the pH meter showed a constant pH value. Analysis of the pH of the preparation was carried out before the preparation was tested for stability and every 1 cycle of the cycling test stability test[16].

Spreadability test: As much as 0.5 g of the ointment was placed on the center of the petri dish. Another choice of petri dish was placed on top of the gel and left for one minute. As much as 50 g and 100 g of load were put in; then, the diameter constant was measured. The analysis of the dispersion of the preparation was carried out before the stability test and after the 6 cycle cycling test stability test[17].

Burns on rats

This research has obtained research ethics approval from the Animal Research Ethics Committee, Universitas Sumatera Utara, with certificate number 0504/KEPH-FMIPA/2021. Method of applying burns was performed with 15 male white rats that were adapted for seven days. On the first day of the study, they were divided into five groups, each consisting of three rats. Each rat was marked or labeled on its tail using a waterproof marker according to its group. The hair in the area to be injured was shaved first and disinfected with 70% ethanol. Then, the mice were anesthetized using lidocaine. Administration of burns on the backs of rats was done using an iron coin plate heated on a blue fire for 3 minutes and then affixed to the back of the rat for 5 seconds until a second-degree burn was formed[18,19].

Calculation of burn diameter

Burns formed were measured using a caliper, then the diameter of the burn was calculated by **Equation 1**. After the burn diameter was obtained, the percentage of burn healing was calculated using **Equation 2**.

$$d = \frac{d_1+d_2+d_3+d_4}{4} \quad [1]$$

d: wound diameter

d_1, d_2, d_3, and d_4: wound diameter measured from various directions

$$P_x = \frac{d_1-d_x}{d_1} \times 100\% \quad [2]$$

P_x: percentage of healing day x

d_1: first-day wound diameter

d_x: wound diameter on day x

Data analysis

Burn healing time data were analyzed statistically using the ANOVA (One-way Analysis of Variant) method, followed by the LSD (least significant difference) test with a 95% confidence level.

RESULTS AND DISCUSSION

Extraction

The extraction method used is maceration, a simplicia extraction process with organic solvents carried out several times by stirring at room temperature[11]. A total of 200 g of *C. roseus* flower simplicia powder was soaked in 2,000 mL of 70% ethanol. The filtrate was then concentrated with a rotary evaporator to obtain a thick extract of 68.67 g. The yield obtained is 34.33%.

Evaluation of ointment

Evaluation of the ointment includes stability testing using the cycling test method (6 cycles) by observing organoleptic, homogeneity, pH, and dispersion[13]. Organoleptic stability testing includes the ointment's shape, color, and odor. The ointment was observed during the cycling test. The ointment has a semi-solid form which is characteristic of the ointment

itself. In the ointment base without extract, it has a yellowish-white color which, when combined with the extract of *C. roseus* flower, becomes brown. The higher the concentration level, the darker the brown color. The aroma of ointment smells of French vanilla due to adding fragrance to the modified formula to avoid the preparation from a rancid odor caused by adding extract. The organoleptic properties of all ointment formulas did not change after the cycling test treatment, which showed the stable properties of the preparation[14,15].

The homogeneity test results of the ointment during the cycling test obtained from the control and the formula using extracts with three different concentrations showed that all ointment preparations were homogeneous. The homogeneity of the ointment preparation is seen from the absence of substances that have not been mixed, so there is no homogeneity difference during the cycling test stability test[16]. The pH stability test was carried out on each ointment formula during the cycling test, in which the average pH obtained was 5.8-6.1. The pH test aims to determine the safety of the ointment preparation so as not to irritate the skin. The pH of the preparation is good according to the pH of the skin, which is 4.5-6.5. If the pH of the preparation is too acidic, it will irritate the skin, and if the pH of the preparation is too alkaline, it causes dry skin[17].

The dispersion results measurement obtained after the cycling test decreased compared to before the test. Although the dispersion results decreased after the test, each preparation was still within the range of good ointment spreadability between 5-7 cm. The dispersion test was carried out to ensure satisfactory drug administration. The wider the preparation is spread, the greater the diffusion coefficient, which results in increased drug diffusion[20].

Visual observation of burns and wound healing

Observations of burns were analyzed until 21 days on days 1, 7, 14, and 21 to see the physical changes that occurred in the treatment area[21,22]. The results of visual observations of burns in test animals can be seen in **Table II**.

Table II. Visual observation of burns

Formula	Rat	Description	Observation results (days)			
			1	7	14	21
F0 (blank)	1	Color	RB	DB	R	R
		Scab formed	-	-	✓	✓
		New skin formation	-	-	-	✓
	2	Color	RB	DB	C	R
		Scab formed	-	✓	✓	✓
		New skin formation	-	-	✓	✓
	3	Color	RB	DB	C	R
		Scab formed	-	-	✓	✓
		New skin formation	-	-	-	✓
F1 (5%)	1	Color	RB	DB	R	R
		Scab formed	-	-	✓	✓
		New skin formation	-	-	✓	✓
	2	Color	RB	DB	C	R
		Scab formed	-	-	✓	✓
		New skin formation	-	-	✓	✓
	3	Color	RB	DB	R	R
		Scab formed	-	-	✓	✓
		New skin formation	-	-	✓	✓
F2 (10%)	1	Color	RB	C	R	P
		Scab formed	-	-	✓	✓
		New skin formation	-	✓	✓	✓
	2	Color	RB	DB	R	P
		Scab formed	-	✓	✓	✓
		New skin formation	-	-	✓	✓
	3	Color	RB	DB	R	R
		Scab formed	-	✓	✓	✓
		New skin formation	-	-	✓	✓
F3 (15%)	1	Color	RB	C	P	W
		Scab formed	-	✓	✓	✓
		New skin formation	-	✓	✓	✓
	2	Color	RB	DB	R	W
		Scab formed	-	✓	✓	✓
		New skin formation	-	✓	✓	✓
	3	Color	RB	DB	R	P
		Scab formed	-	✓	✓	✓
		New skin formation	-	-	✓	✓

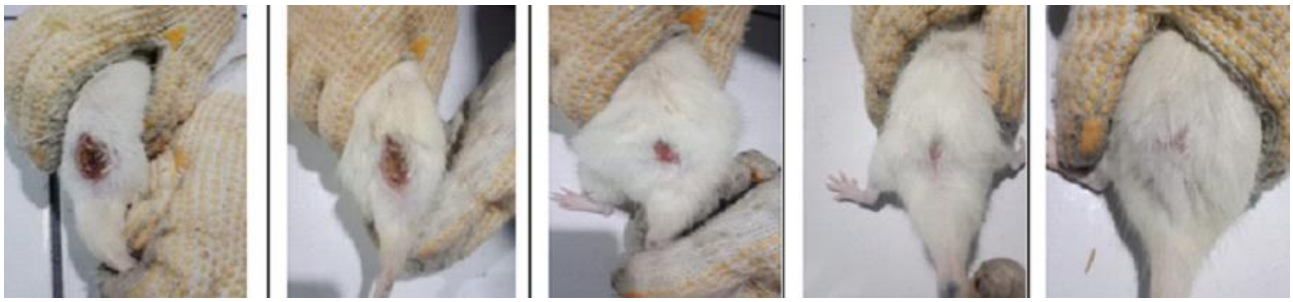
Positive control (C+)	1	Color	RB	C	P	W
		Scab formed	-	✓	✓	✓
		New skin formation	-	✓	✓	✓
	2	Color	RB	C	P	P
		Scab formed	-	✓	✓	✓
		New skin formation	-	✓	✓	✓
	3	Color	RB	C	P	W
		Scab formed	-	✓	✓	✓
		New skin formation	-	✓	✓	✓

Note: Brownish red (RB); Dark brown (DB); Chocolate (C); Red (R); Pink(P); White (W); Occur (✓); Not occur (-)

Measurement data on the average diameter of burns and the percentage of healing in the negative control, positive control, and test group with a concentration of 5%, 10%, and 15% on day 1 to 21 are presented visually in **Figures 1** and **2**, with calculations can be seen in **Tables III** and **IV**.

F0 **F1** **F2** **F3** **C+**

Figure 1. Day 1 observations.



F0 **F1** **F2** **F3** **C+**

Figure 2. Day 21 observations.

Table III. The average diameter of burns

Group	Average ± SD burn diameter (days)			
	1	7	14	21
F0	20.23 ± 0.15	19.03 ± 0.15[b]	18.10 ± 0.10[b]	14.60 ± 0.40[b]
F1	20.20 ± 0.10	18.13 ± 0.15[ab]	17.33 ± 0.15[ab]	9.86 ± 0.20[ab]
F2	20.23 ± 0.15	17.83 ± 0.20 [ab]	16.93 ± 0.15[ab]	7.20 ± 0.36[ab]
F3	20.30 ± 0.10	17.43 ± 0.15[a]	9.86 ± 0.30[a]	3.03 ± 0.68[a]
C+	20.26 ± 0.20	17.36 ± 0.20[a]	9.40 ± 0.55[a]	2.56 ± 0.76[a]

Note: [a]: Significantly different from negative control; [b]: Significantly different from positive control

Table IV. Burn wound healing percentage

Group	Average ± SD percentage of burn healing (%)		
	After 7 days	After 14 days	After 21 days
F0	5.93 ± 0.04[b]	10.54 ± 0.68[b]	27.83 ± 2.22[b]
F1	10.23 ± 0.33[ab]	14.19 ± 1.08[ab]	51.15 ± 0.86[ab]
F2	11.86 ± 0.84[ab]	16.31 ± 0.46[ab]	64.42 ± 1.67[ab]
F3	14.12 ± 1.17[a]	51.39 ± 1.67[a]	85.05 ± 3.43[a]
C+	14.31 ± 0.48[a]	53.60 ± 3.12[a]	87.32 ± 3.61[a]

Note: [a]: Significantly different from negative control; [b]: Significantly different from positive control

In this study, an ointment base was used as a negative control. This was done to ensure that the extract of *C. roseus* flower gave the effect of healing burns. Betadine ointment was used as a positive control because it is a pharmaceutical preparation in the form of an ointment that can also be used as a burn healer, readily available in the market. In comparison, the secondary metabolite compounds in the *C. roseus* flower that can help the healing process of burns are flavonoids, alkaloids, saponins, and tannins[23,24].

Flavonoids work as antibacterial by forming complex compounds against extracellular, which disrupt cell integrity. In addition, flavonoids also have anti-inflammatory effects that function as anti-inflammatory and can prevent stiffness and pain[25]. Alkaloids also have the ability as antibacterial; the mechanism is thought to be a way of disrupting the peptidoglycan component in bacterial cells so that the cell wall layer is not formed and causes the death of the cell[26]. Saponins can trigger the formation of collagen where the more collagen there is, the faster it will attract fibroblasts to the edges of the wound so that the fibroblasts will experience a phenotypic change to become myofibroblasts which accelerates the wound contraction process so that the size of the wound quickly decreases[27]. Tannins also play an essential role in the healing process of burns, which are helpful as astringents that cause shrinkage of skin pores, stop minor bleeding so that they can cover wounds, and prevent bleeding that usually occurs in wounds[28].

Figure 3 shows the healing of burns in test animals that occurred for 21 days. The group that was given a formula containing active compounds showed a better healing process than the wounds that were given blanks. The best activity is shown by Formula 3, whose results are close to the positive control.

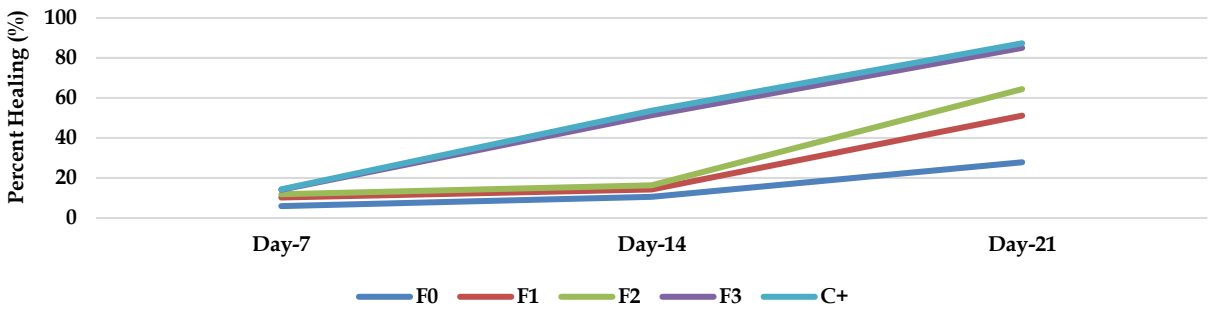


Figure 3. Healing wound percentage.

CONCLUSION

All the formulas for the ethanol extract of *C. roseus* ointment met the requirements for the evaluation of stability, organoleptic, homogeneity, pH, spreadability, and the best activity is shown by Formula 3 with 15% extract content.

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AUTHORS' CONTRIBUTION

L determined, designed the study, supervised, analyzed data, and partook in writing the original draft of the article. **TNKS** supervised the laboratory, managed the work, and partook in writing the original draft of the article. **RS** conducted the search, interpreted data, and partook in writing the original draft of the article. **IH** and **BI** individually revised the final article. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

DATA AVAILABILITY

The data that support this study are available from the corresponding author **L**, upon reasonable request.

CONFLICT OF INTEREST

All of the authors have no conflict of interest to declare.

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Research Article

Antioxidant Activity and Phytochemicals of Locally Consumed Plant Foods from Baguio City, Philippines

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Abstract

In the Philippines, Baguio City – known as the "City of Pines" – holds the country's major source of temperate climate vegetables. With increased dietary awareness, the consumption of plant foods rich in antioxidants has become relevant. Twenty-nine methanolic extracts from Baguio-produced plant foods were evaluated for antioxidant potential using DPPH, ferric reduction antioxidant power (FRAP), metal chelation, superoxide anion, nitric oxide, hydroxyl radical scavenging activities, MTT reduction, and phytochemical tests. *Fagopyrum tataricum* leaves, *Vaccinium myrtoides* fruit, and *Morus alba* fruit showed the most effective DPP radical, concentration-dependent reducing power, but low metal chelating activity. *Solanum tuberosum* tuber (22.86±63.26%) showed effective concentration-dependent chelating activity at 125 µg/mL. *Citrus aurantium* fruit (26.77±9.24%) and *Raphanus raphanistrum* root (41.13±0.11%) demonstrated an effective scavenging activity against superoxide anions at 45.5 µg/mL. Significant nitric oxide scavenging activity was observed in some fruits. *Brassica oleracea* Cab leaves (54.36 ± 2.38%) showed the highest inhibitory activity against hydroxyl radicals at 166.7 µg/mL. Phytochemical analyses showed that most plant samples revealed the presence of glycosides, terpenes/terpenoids, and steroids/phytosterols, while few contained phenolic and tannin components. These phytochemicals may explain the dual behavior as an antioxidant or a prooxidant observed. Thus, determining food antioxidant component types and their concentration is necessary to maximize the potential to scavenge oxidants.

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INTRODUCTION

Reactive Oxygen Species (ROS) are normal oxidant by-products of aerobic metabolism[1]. These reactive intermediates, which include superoxide anion (O_2•), the hydroxyl radical (OH•), and the singlet oxygen, are derived from the incomplete one-electron reduction of molecular oxygen[2]. Prolonged exposure to high ROS concentrations may lead to non-specific damage to proteins, lipids, and nucleic acids, often inducing irreversible functional alterations or complete destruction[3]. Antioxidants have gained importance for years due to their ability to neutralize the pathological effects of increased oxidative concentrations[4,5]. Though our bodies are equipped with innate antioxidant machinery, such as antioxidant enzymes and vitamins, these may not be enough to protect against the detrimental effects of excess free radicals. Consumption of foods rich in phytochemicals with strong antioxidant properties may help prevent and repair cellular damage to our bodies[5].

Natural antioxidants, particularly in fruits and vegetables, have gained increasing interest among consumers and the scientific community. Fruit and vegetable juices are rich sources of numerous phytochemicals, polyphenols, carotenoids, fiber, vitamins, and minerals[6,7]. Complex mixtures of these phytochemicals present in fruits and vegetables provide additive and synergistic effects on health promotion[8]. Thus, dietary intake of this food as a source of antioxidants is recommended.

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In the Philippines, Baguio City – known as the "City of Pines" – supplies the Philippines' daily requirements for highland vegetables[9]. Due to the temperate climate and wide land area, Baguio provided a conducive environment to cultivate quality fruits, vegetables, and other agricultural products. The Philippine Department of Agriculture stated that Baguio and neighboring provinces in Cordillera supply 80% of the highland vegetable requirement in major markets of Metro Manila and other lowland provinces[10]. Knowing the possible beneficial effects of plant foods cultivated from the country's primary agricultural source of prime vegetables, fruits, and crops is essential. Currently, limited studies are conducted on Baguio's commonly consumed plant foods. Thus, the study aims to determine the phytochemical constituents of some Baguio-derived vegetables and evaluate their antioxidant activities *in vitro*.

MATERIALS AND METHODS

Materials

Twenty-six plant samples were collected from local markets of Baguio (shown in **Table I**). Edible plant parts commonly consumed by locals were utilized in the study. All samples gathered were authenticated by the Institute of Biology Herbarium - University of the Philippines, Diliman. 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), sodium nitroprusside (SNP), sulfanilamide, naphthyl ethylenediamine dihydrochloride (NED), potassium ferricyanide [$K_3Fe(CN)_6$], quercetin, butylated hydroxytoluene (BHT), ethylene diaminetetraacetic acid (EDTA), ascorbic acid, and reduced nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich Co. St. Louis, Germany. All other chemicals used were of analytical grade.

Table I. List of plant foods used with sample shortened names and parts used

Plant sample	English name/local name	Parts used	Specimen No.
Beta vulgaris L.	Sugar beets/redbeet	Root	NPL-032
Daucus carota subsp. *Sativus* (Hoffm.) Arcang.	Carrots/karot	Root	NPL-033
Lactuca sativa L. (Iceberg)	Iceberg lettuce/letsugas iceberg	Leaves	NPL-034
Lactuca sativa L. (Romaine)	Romaine lettuce/letsugas iceberg	Leaves	NPL-035
Lactuca sativa L. (Green Ice)	Green ice lettuce/letsugas green ice	Leaves	NPL-036
Lactuca sativa L. (Deep Red)	Deep red lettuce/pulang letsugas	Leaves	NPL-037
Brassica oleracea L. (cabbage)	Cabbage/repolyo	Leaves	NPL-038
Brassica oleracea L. (red cabbage)	Red cabbage/pulang repolyo	Leaves	NPL-039
Brassica oleracea L. (broccoli)	Broccoli/brokoli	Flower buds	NPL-040
Brassica oleracea L. (cauliflower)	Cauliflower/koliplor	Flower buds	NPL-041
Brassica rapa L. (pechay baguio)	Chinese cabbage/petchay Baguio	Leaves	NPL-042
Brassica rapa L. (flowering pechay)	Chinese cabbage/petchay Baguio	Flower	NPL-043
Nasturtium officinale R.Br	Watercress/tonghoy	Leaves	NPL-044
Raphanus raphanistrum subsp. *sativus* (L.) Domin	Red radish /pulang labanos	Fruit	NPL-045
Sechium edule (Jacq.) Sw.	Chayotte/sayote	Fruit	NPL-046
Morus alba L.	Mulberry/moras	Fruit	NPL-049
Fagopyrum tataricum (L.) Gaertn.	Buckwheat/malabato	Leaves	NPL-050
Fragaria x ananassa (Duchesne ex Weston) Duchesne ex Rozier	Strawberry/presa	Fruit	NPL-051
Citrus x aurantium L.	Sweet orange/Sagada oranges	Fruit	NPL-052
Solanum melongena L.	Eggplant/talong	Fruit	NPL-053
Capsicum annuum L.	Bell pepper	Fruit	NPL-054
Solanum lycopersicum L.	Tomato/kamatis	Fruit	NPL-055
Solanum tuberosum L.	Potato/patatas	Tuber	NPL-057
Cucurbita maxima Duschesne	Squash/kalabasa	Fruit	NPL-058
Solanum nigrum L.	Deadly nightshade/lubi-lubi or amti	Leaves	NPL-059
Vaccinium myrtoides	Blueberry/ayusip	Fruit	NPL-060

Methods

Sample preparation and extraction

All collected leaves, fruits, and roots were washed with distilled water, chopped, and cut into smaller pieces. Samples were dried through air-drying and lyophilization. Air-drying was employed on all leaf samples. On the other hand, samples with high water content were freeze-dried using Martin Christ Freeze Dryer Beta 2-8 LSC. All dried samples were kept at 4°C in sealed containers until ready for extraction. The dried samples were extracted with methanol at room temperature for 24 hours. The extract was then separated from the residue by filtration. The residue was re-extracted twice. The methanol

extract was concentrated using a rotary evaporator (Buchi Rotavapor R-200) at 40°C. Extracts were kept in tightly sealed bottles at 4°C until use.

Phytochemical screening

The secondary metabolites were determined in each plant sample using various tests[11,12]. The presence of reducing sugars in all plant samples was detected using Molisch, Fehling's, and Benedicts tests; proteins using Ninhydrin and Biuret tests; alkaloids using Mayer's, Wagner's, Hager's, and Dragendorff's tests; glycosides using Modified Borntranger's and Keller Killiani tests; steroids using Liebermann-Burchard test; terpenes and terpenoids using Salkowski's test; quinones using sulfuric acid test; anthraquinones using hydrochloric acid test; flavonoids using alkaline reagent and Shinoda tests; polyphenols using ferric chloride test; tannins using ferric chloride and gelatin tests; and saponins using froth test.

Antioxidant assays

DPPH scavenging activity: As much as 10 µL of standard and test compounds at different concentrations were loaded into a 96-well microplate. Afterward, 140 µL of 6.85×10^{-5} M DPPH was added to each well. The microplate was incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm[13].

Ferric reduction antioxidant power (FRAP) assay: As much as 70 µL of standard and test compounds at different concentrations were mixed with 176.5 µL of 0.2 M sodium phosphate buffer (pH=7.4) and 176.5 µL of 1% [$K_3Fe(CN)_6$]. The mixture was incubated at 50°C for 20 minutes. After incubation, the reaction mixtures were acidified with 176.5 µL of 10% trichloroacetic acid and were centrifuged at 650x for 10 minutes. An aliquot of 273 µL of the supernatant was added to 273 µL of deionized water. Finally, 55 µL of 0.1% $FeCl_3$ was added to this solution. The absorbance was measured at 700 nm[14].

Metal chelating activity: In a 96-well microplate, different concentrations of the 20 µL methanol extracts were loaded, and 100 µL of 0.2 mmol/L $FeCl_2$ was added to each well. Afterward, 40 µL of 5 mmol/L ferrozine was added. The reaction mixture was incubated at room temperature for 10 minutes. The absorbance was measured at 562 nm[15].

Superoxide scavenging activity: As much as 10 µL of standard and test compounds at different concentrations were loaded into a 96-well microplate. Then, 100 µL 468 µM NADH, 100 µL 156 µM NBT, and 50 µL 60 µM PMS were added into each well. Five-minute incubation was done at room temperature. The absorbance was measured at 560 nm[16].

Nitric oxide scavenging assay: Sodium nitroprusside (10 mM, 2 mL) in phosphate buffer saline was incubated with test compounds in different concentrations at room temperature for 150 minutes. After 30 minutes, 0.5 mL of the incubated solution was added with 1 mL of Griess reagent (0.33% sulfanilamide in 20% glacial acetic acid, 0.5 mL, and 0.1% NED, 1 mL) and was incubated for 30 minutes at room temperature. The absorbance was measured at 546 nm[14].

MTT assay: Stock solutions of test compounds and extracts were prepared in DMSO (250-1000 µg/mL). The MTT (1 mg/mL) was dissolved in water. An aliquot of 190 µL of MTT solution in water and 10 µL of test compounds or extracts in DMSO were vortexed in a capped glass vial (2 mL) for 1 minute. As much as 200 µL DMSO was added, and the solution was vortexed again. The reaction mixture was then incubated at 37°C for six hours, 200 µL of the reaction mixture was pipetted to a 96-well cell culture plate, and the absorbance was measured at 570 nm[17].

Hydroxyl radical scavenging assay: The reaction mixture in a final volume of 1.0 mL contained 100 µL of 2-deoxy 2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 µL of the extract at various concentrations in buffer, 200 µL of 1.04 mM EDTA and 200 µM $FeCl_3$ (1 : 1, v/v), 100 µL of 1.0 mM hydrogen peroxide (H_2O_2), and 100 µL of 1.0 mM ascorbic acid. Test samples were kept at 37°C for an hour. The free radical damage imposed on the substrate, deoxyribose, was measured using the thiobarbituric acid test. As much as 1 mL of 1% thiobarbituric acid (TBA) and 1 mL of 2.8% trichloroacetic acid (TCA) was added to the test samples and incubated at 100°C for 20 minutes. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer[18].

Data analysis

Results are expressed as mean ± SD. The statistical analysis was performed using one-way ANOVA. The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

DPPH scavenging activity

One mechanism by which antioxidants inhibit oxidation is by quenching reactive species through hydrogen or electron donation[19]. The DPPH assay is one of the most popular and frequently employed methods among antioxidant assays. DPPH is a stable free radical with a deep purple color and a strong absorption around 517 nm[20]. Increased concentration of antioxidants may result in a lighter solution[21].

Methanol extracts of *F. tataricum*, *L. sativa* GI, *V. myrtoides*, and *M. alba* have exhibited good concentration-dependent inhibitory activity against DPP radicals. *Fagopyrum tataricum* showed the most effective DPP radical inhibition (74.53±1.59%) at 66.67 µg/mL. Relatively good inhibitions were also observed in *L. sativa* GI (69.86±1.59%), *V. myrtoides* (66.10±4.43%), and *M. alba* (63.25±0.05%) at the same concentration. In addition, all these plant samples had demonstrated >50% scavenging even at 33.33 µg/mL dose. Similar findings have been observed in other studies. *Fagopyrum tataricum* exhibited significant antioxidant activity with IC_{50} of 159.51±1.29 µg/mL[22]. *Vaccinium myrtoides* fruit exhibited the highest antioxidant activity as indicated by its 92.35±0.69% DPPH radical scavenging activity among Baguio fruit and fruit wines[9]. Moreover, ethanolic leaf extracts of *V. myrtoides* (20.85 µg/mL) from Ifugao showed higher DPP inhibition than ascorbic acid (21.56 µg/mL)[23]. Significant increase in scavenging activity was observed in *M. alba* with 3.48%, 8.28%, 16.63%, and 25.84% inhibitions between 100-1000 µg/mL[24].

Different DPP inhibitory activities were observed between *L. sativa* and *B. oleracea* sample varieties. The four varieties of *L. sativa* exhibited DPPH inhibition at 66.67 µg/mL in increasing order: *L. sativa* RL (10.79±2.74%), *L. sativa* I (17.99±2.36%), *L. sativa* DR (33.18±5.56%), and *L. sativa* GI (69.86±1.59%), respectively. Similar findings showed that red and darker green types of lettuce possess higher antioxidant activity than the green type of lettuce. Red coral lettuce possessed the lowest EC_{50} value, followed by green coral, iceberg, butterhead, and romaine with 303.56±11.3, 775.55±43.7, 3991.67±174.7, 4230.13±401.5, and 4485.41±784.4 µg/mL, respectively[25]. In addition, red varieties of lettuce were significantly higher than those of green lettuce[26]. Likewise, four varieties of *B. oleracea* showed different activities at the highest concentration. *B. oleracea* RCab was revealed to be the most active extract, and *B. oleracea* Cab (5.96±8.82%) was the least. Methanolic extracts of *B. oleracea* varieties exhibited varying DPP radical inhibitions in the order: red cabbage > green cabbage > broccoli > cauliflower[27].

Some plant samples showed moderate scavenging activities. Moderate scavenging activities of extracts were observed in the following plant samples in increasing order: *B. oleracea* RCab, *F. anannasa*, *L. sativa* DR, *B. vulgaris*, and *S. nigrum*, with inhibitions of 29.99±3.38%, 32.92±2.06%, 33.18±5.56%, 34.28±1.67% and 47.54±0.93% at 66.67 µg/mL, respectively. Methanol leaf extract of *S. nigrum* significantly inhibited DPP radicals in a concentration-dependent manner with IC_{50} of 165 µg/mL[28]. In contrast, low scavenging activities (DPPH inhibition <25%) were demonstrated by other extracts, even at the highest concentration. No DPPH inhibition was observed for *C. aurantium*. The overall results are presented in **Figure 1**.

FRAP assay

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this assay, antioxidant capacity measures the reduction of the ferric ion (Fe^{3+}) to the ferrous ion (Fe^{2+}) by donor electrons in the sample[29]. The activity is monitored by measuring the formation of Perls' Prussian blue complex at 700 nm[30]. Methanol extracts *of F. tataricum*, *L. sativa* GI, *V. myrtoides*, and *M. alba* have exhibited the highest concentration-dependent reducing ability among Baguio plant foods. *Fagopyrum tataricum* showed the most effective iron reduction (72.44±1.99%) at 38.2 µg/mL. Comparable reductions to that of *F. tataricum* were also observed in *L. sativa* GI (71.60±1.00%), *V. myrtoides* (71.80±1.80%), and *M. alba* (71.77±1.94%). As mentioned earlier, the plant possessed reducing abilities based on other studies. Maximum activity was exhibited by methanol extract of *F. tataricum*, greater than the gallic acid standard at 200 µg/mL[31]. Wild Blueberries (*Vaccinium* sp.) extract of some wild and cultivated Blueberries from Romania showed iron reduction, with Wild Type 1 (73.71±3.2 µM Fe^{2+}/g) being the most potent[32]. Mulberry variety (S-1708; *Morus* sp.) exhibited reducing activity of 4107.22 ± 97.6 µM/Fe(II) mg and 3540.60 µM/Fe(II) mg in methanol and ethanol solvents, respectively[33].

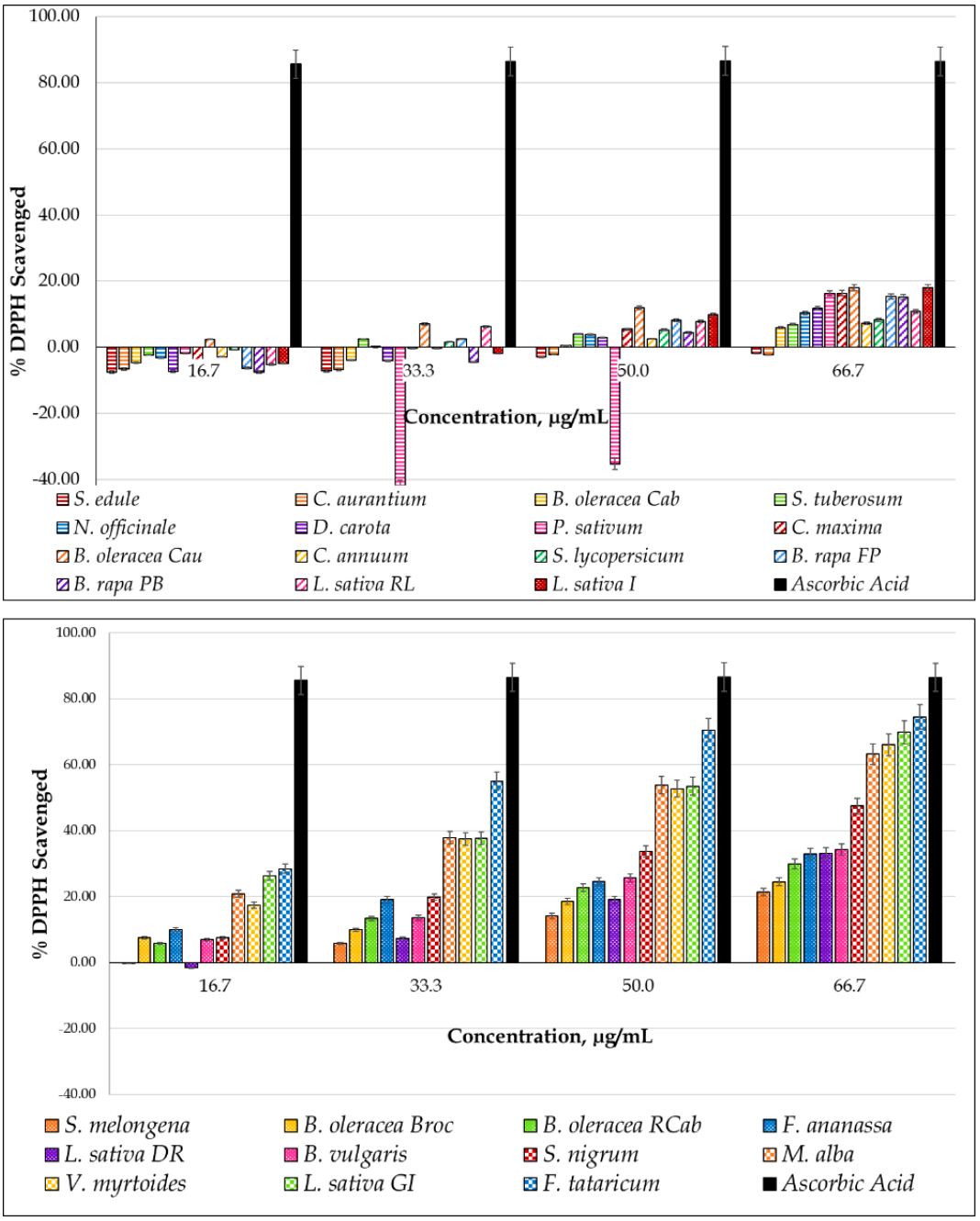

Figure 1. DPPH scavenging assay of Baguio plant foods.

Interestingly, other plant samples showed high reducing abilities. Relatively high reducing power of extracts was observed in the following plant samples in increasing order: *B. oleracea* RCab, *B. vulgaris*, *L. sativa* DR, and *B. rapa* FP, with an inhibition of 71.92±3.46%, 72.74±3.41%, 74.32±0.33% and 74.65±2.83% at 50.9 µg/mL, respectively. In contrast, low-reducing activities (iron reduction <25%) were demonstrated by other extracts, even at the highest concentration. Moreover, no iron reduction was observed for *C. maxima*. Reducing power between sample varieties of *L. sativa* was assessed. Varieties of *L. sativa* exhibited varying iron reduction at 50.9 µg/mL in increasing order: *L. sativa* I (37.06±8.80%), *L. sativa* RL (41.84±2.38%), *L. sativa* DR. (74.32±0.33%) and *L. sativa* GI (76.46±2.21%), respectively. Strong reducing capacity exhibited by plant samples may indicate greater antioxidant activity[34]. These results infer that dark-colored lettuces have higher FRAP values than the typical green varieties[35]. The overall results are presented in **Figure 2**.

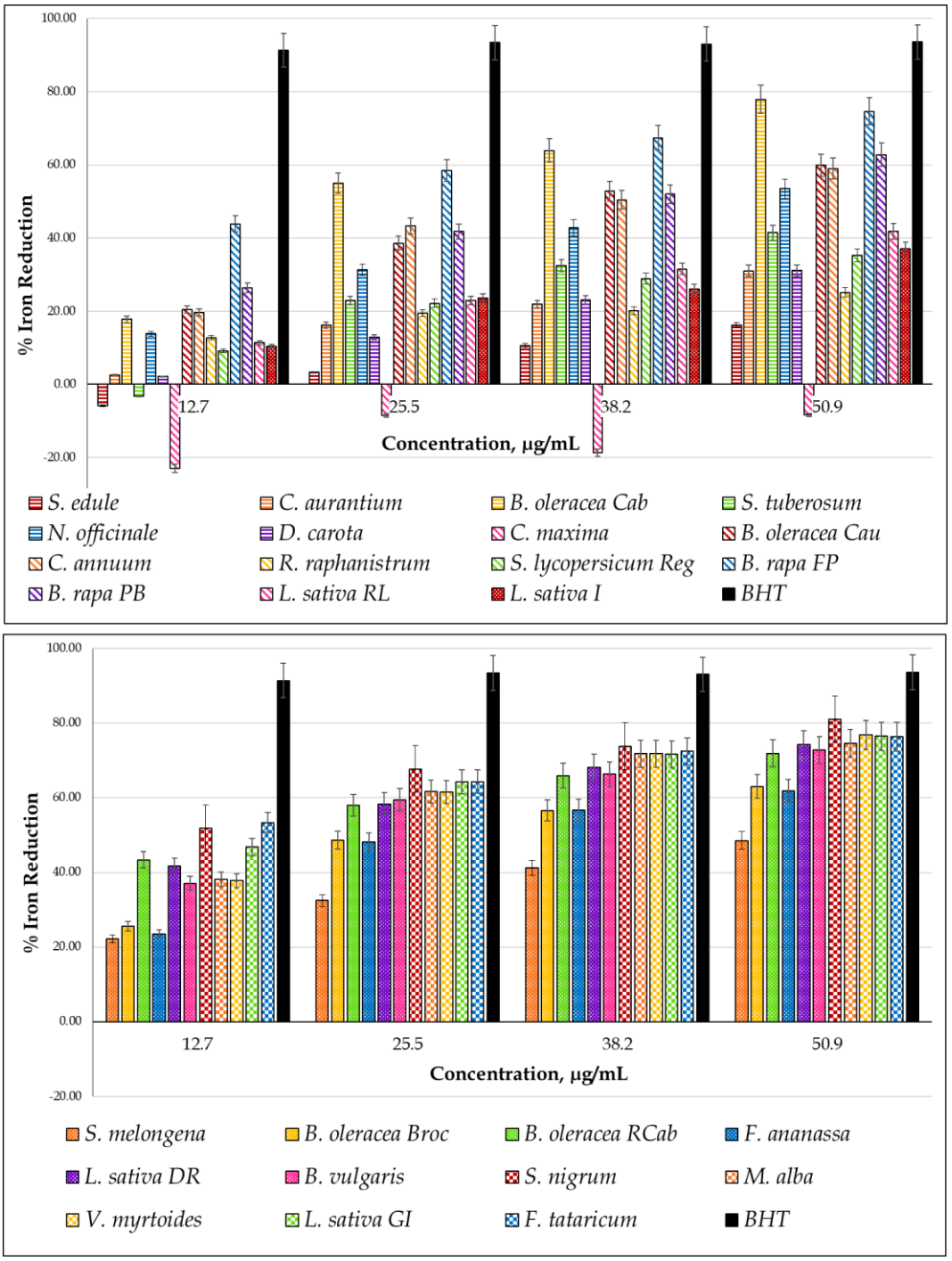

Figure 2. Reducing Power of Baguio plant foods.

Metal chelating activity

Metals, such as Fe and Cu, and ultra-trace elements, Co and Ni, act as cofactors that assist enzymes in catalyzing biochemical reactions efficiently[36]. Transition metals become toxic at elevated tissue concentrations. Excess transition metals can initiate hydroxyl radical production through the Fenton reaction ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH\bullet$)[37]. This process hastens the rate of lipid peroxidation by the continuous generation of peroxy radicals (LOO•) to form lipid hydroperoxides (LOOH) – the main primary products of lipid peroxidation[38]. Chelation therapy is the preferred medical treatment for reducing the toxic effects of metals[39]. The use of metal chelators can improve the symptoms of metal overload. Synthetic chelators have posed severe side effects; hence, using natural plant foods with chelating ability has been getting more attention[40].

In metal chelation, a decrease in the absorbance of the red-violet Fe^{2+}/ferrozine complex is monitored[41]. Low metal chelating activity was observed from Baguio plant foods. Most plant samples were <20%, even at the highest concentration. *S.*

tuberosum showed the highest effective concentration-dependent chelating activity with inhibitions 6.30±3.82%, 16.76±3.82%, and 22.86±63.26% at 62.5, 93.8, and 125 µg/mL, respectively. However, lower chelation was observed in Baguio-grown *S. tuberosum* compared with other studies. Benguet varieties, Igorota (EC_{50}= 11.0±3.2 µg/mL) and Ganza (EC_{50}= 14.7±3.2 µg/mL) exhibited effective chelating activity[42]. Purple-colored potatoes had a higher chelating effect than white and yellow potato varieties from Korea[43]. Conversely, no iron chelation was observed for *D. carota* and *C. annuum*. The overall results are presented in **Figure 3**.

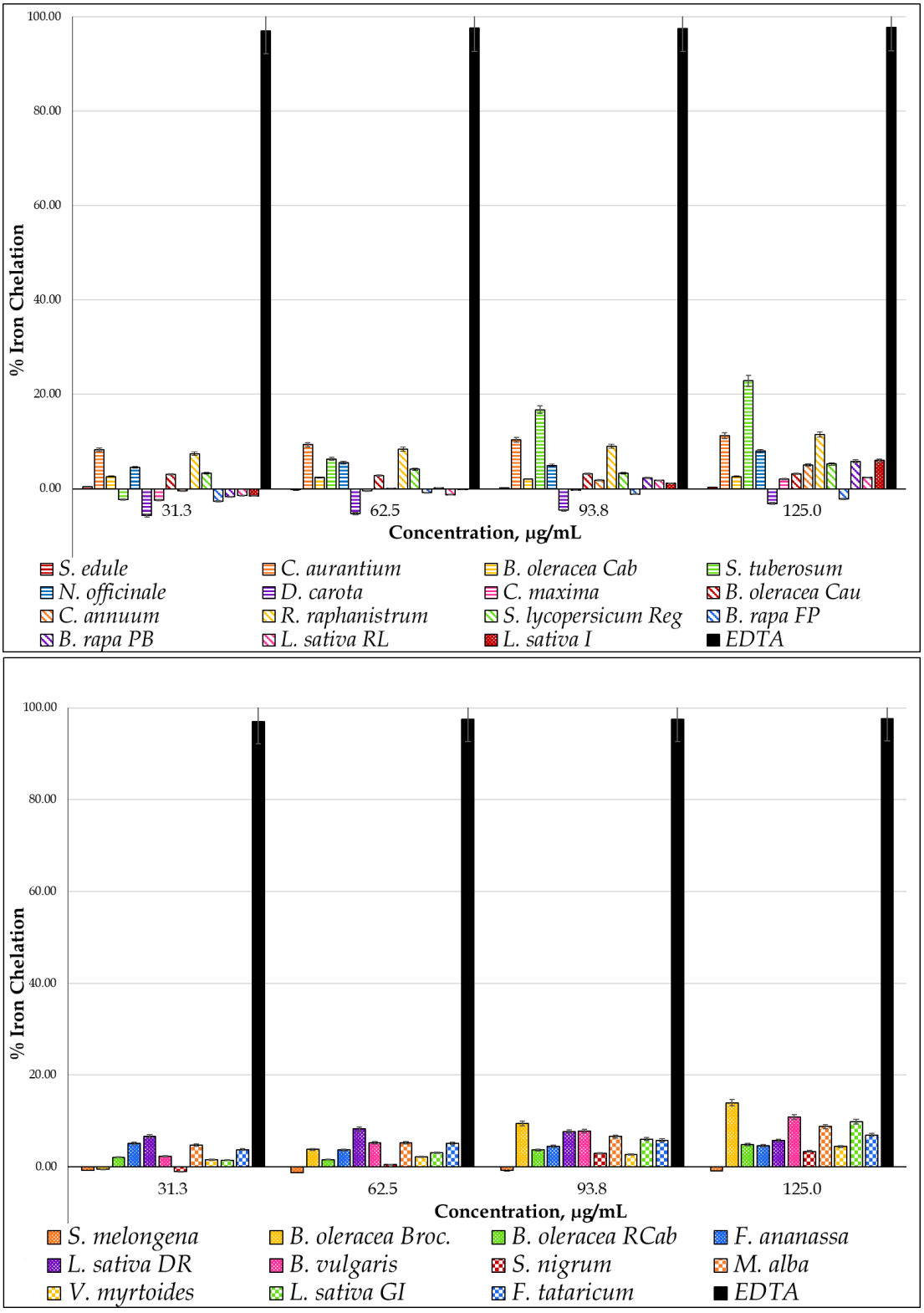

Figure 3. Metal chelating activity of Baguio plant foods.

Superoxide scavenging activity

Superoxide ($O_2^{\bullet-}$) is a free radical with a short biological lifespan produced by the one-electron reduction of molecular oxygen[44]. Superoxide anion initiates free radical formation of other reactive oxygen species in living systems[45]. It can also react with nitric oxide and form peroxynitrite[46]. Thus, knowing the right food which will provide good superoxide scavenging properties may help lower the risk of many degenerative diseases. The overall results are presented in **Figure 4**.

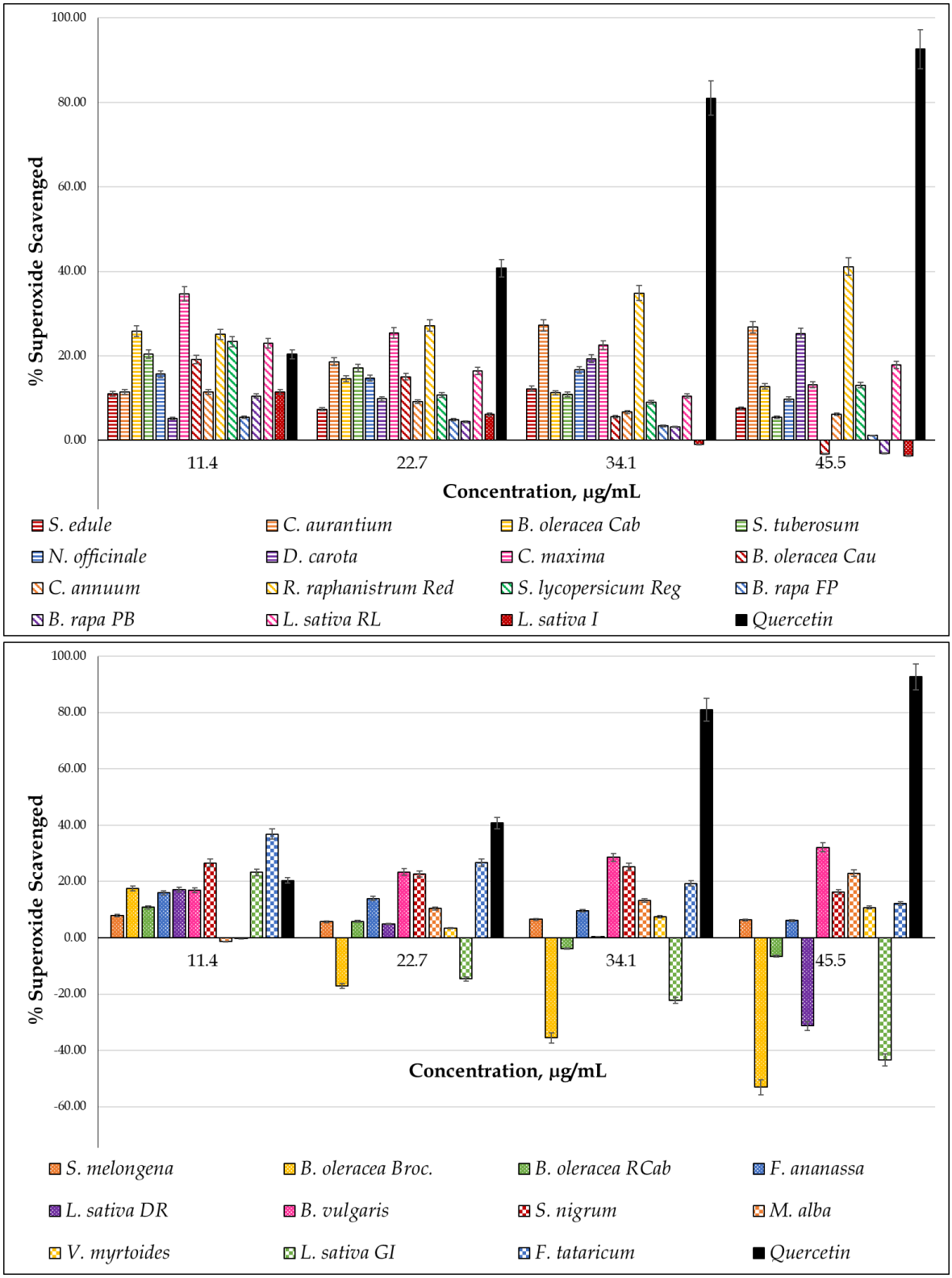

Figure 4. Superoxide scavenging activity of Baguio plant foods.

In this assay, a decrease in absorbance at 560 nm indicates the scavenging of superoxide radicals[47]. Methanol extracts of Baguio plant foods showed inhibitions of superoxide radicals. Some extracts showed the highest superoxide radical scavenging activity at the lowest concentration. Greater than 20% superoxide scavenging activity was observed at 11.4 µg/mL dose in the following plant samples in increasing order: *L. sativa* RL (22.94±2.51%), *L. sativa* GI (23.20±8.44%), *S. lycopersicum* Reg (23.35±2.70%), *B. oleracea* Cab (25.87±0.47%), *S. nigrum* (26.57±3.98%), *C. maxima* (34.61±2.21%), and *F. tataricum* (36.81±1.70%). *Citrus aurantium* and *R. raphanistrum* demonstrated an effective scavenging activity against superoxide anions. However, it was observed that as the dose of some extracts increases, their scavenging ability decreases. *Fagopyrum tataricum* showed a significant change in activity from the lowest concentration with 36.81±1.70% (at 11.4 µg/mL) superoxide scavenging capacity to a 12.15±0.46% (at 45.5 µg/mL). The same pattern of activity was demonstrated by *L. sativa* GI (23.20 to -43.41%), *S. lycopersicum* Reg (23.35 to 13.03%), *B. oleracea* Cab (25.87 to 12.73%), *S. nigrum* (26.57 to 16.26%) and *C. maxima* (34.61 to 13.15%). Similar findings were reported in some Quezon Province plant food extracts that exhibited a decrease in superoxide scavenging activity and stimulation of superoxide radical production as the dose was increased.5 *Ficus odorata*, an endemic medicinal plant in the Philippines, has shown a concentration-dependent stimulation of $O_2^{\bullet -}$[48]. With these reported results demonstrated the dual action, as an antioxidant and as a prooxidant, of some plant foods studied. Therefore, natural antioxidants have been shown to act as prooxidants under certain conditions[5].

Nitric oxide scavenging activity

Nitric oxide (NO) is an important cell signaling molecule. At lower concentrations, NO aids in angiogenesis[49]. Nitric oxide plays vital roles in humans, which include dilating blood vessels, raising blood supply and lowering blood pressure, regulating platelet aggregation, signaling molecules between neurons, and killing bacteria[50,51]. However, NO also possesses a dual role. As a free radical, nitric oxide readily reacts with other radical species and the metal centers of metalloproteins[52]. Moreover, when nitric oxide is near superoxide, peroxynitrite ($ONOO^-$) may spontaneously form[44]. In this assay, the Griess reagent can estimate nitric oxide formed[14]. Nitric oxide scavengers compete with oxygen, producing reduced nitric oxide. Most of the Baguio plant foods exhibited high nitric oxide scavenging ability. Greater than 50% superoxide scavenging activity was observed in *S. lycopersicum* Reg (75.43±0.71%), *B. vulgaris* (65.21±0.37%), *C. aurantium* (64.10±0.57%), *S. melongena* (62.20±0.84%), *V. myrtoides* (57.69±1.47%), *R. raphanistrum* (56.70±2.64%), *L. sativa* GI (56.02±1.01%), *S. tuberosum* (53.77±3.43%), *M. alba* (51.96±0.60%), *C. annuum* (50.84±0.88%), at 16.7 µg/mL dose. Observed activities were comparable to gallic acid (56.93±6.35%) at the same concentration. However, some of the Baguio extracts were seen to behave as prooxidants as the dose was increased to 66.7 µg/mL.

Lactuca sativa GI (Green Ice Lettuce) exhibited a significant change in NO scavenging ability from 56.02±1.00% (at 16.7 µg/mL) to -87.33±2.50% (at 66.7 µg/mL). The same pattern of activity was demonstrated by *B. oleracea* Broc (35.74 to -45.58%), *B. rapa* FP (11.73 to -27.58%), *S. nigrum* (29.51 to -69.25%), *L. sativa* RL (33.67 to -57.57%), *N. officinale* (47.09 to -47.27%), and *S. edule* (18.96 to -24.39%). The overall results are presented in **Figure 5**.

MTT assay

MTT was developed as an antioxidant assay utilizing the redox reaction to screen natural product extracts or purified compounds[17]. Methanol extracts of *S. tuberosum*, *B. oleracea* Cab, *B. vulgaris*, *S. nigrum*, and *L. sativa* GI have exhibited the highest concentration-dependent reducing ability among Baguio plant foods. *Brassica oleracea* Cab showed the most effective MTT reduction (48.89±0.431%) at 66.7 µg/mL. Good reductions were also observed in *B. vulgaris* (45.79±1.35%), *L. sativa* GI (43.73±0.81%), *S. nigrum* (41.47±2.04%), and *S. tuberosum* (31.97±1.18%) at the same concentration. In addition, all these plant samples had extended their potency showing >20% scavenging even at 16.7 µg/mL. In contrast, low-reducing activities (iron reduction <25%) were demonstrated by other extracts, even at the highest concentration. Moreover, no MTT reduction was observed for *S. melongena*, *F. annanasa*, *M. alba*, *V. myrtoides*, and *F. tataricum*. The overall results are presented in **Figure 6**.

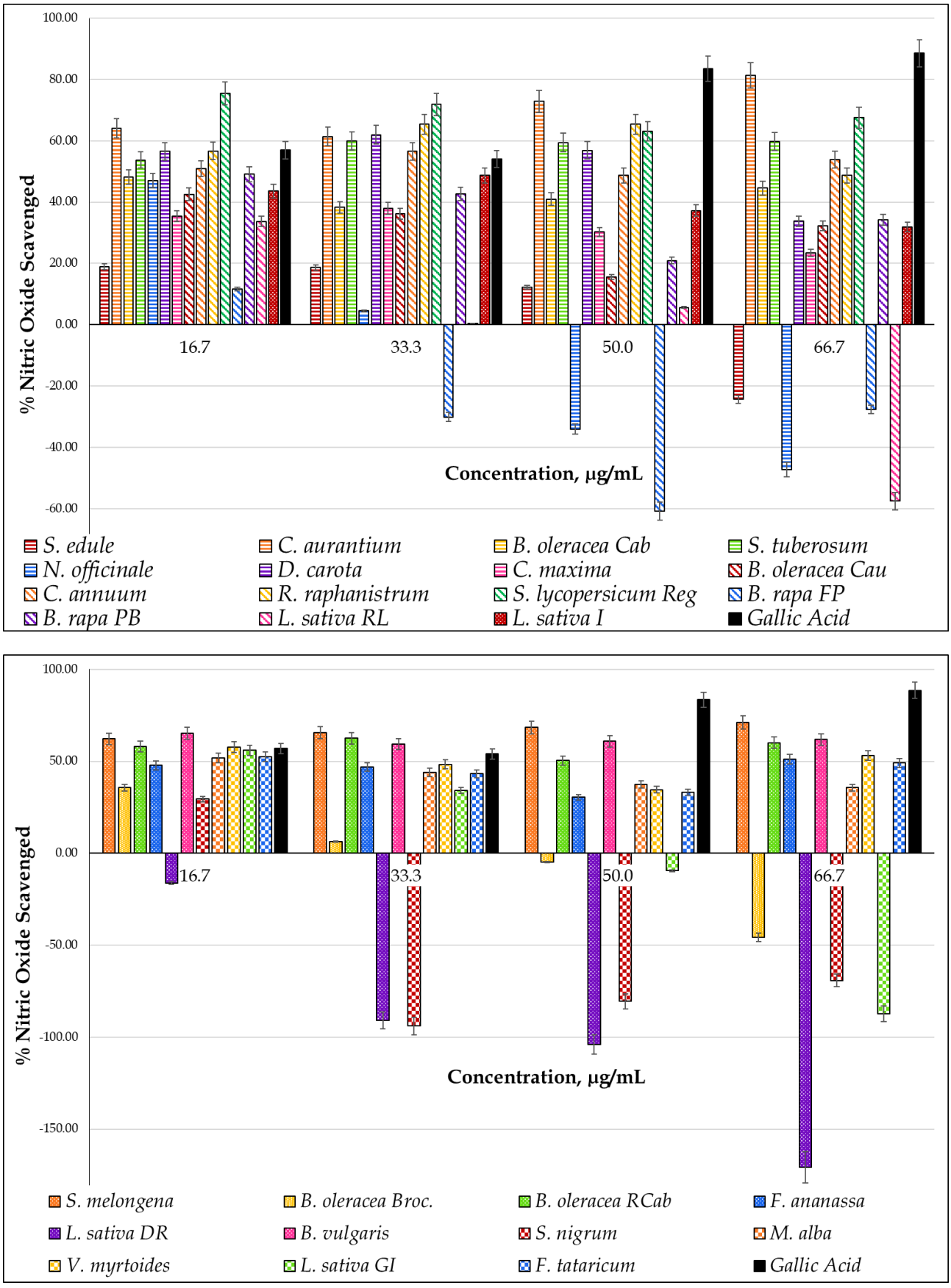

Figure 5. Nitric oxide scavenging activity of Baguio plant foods.

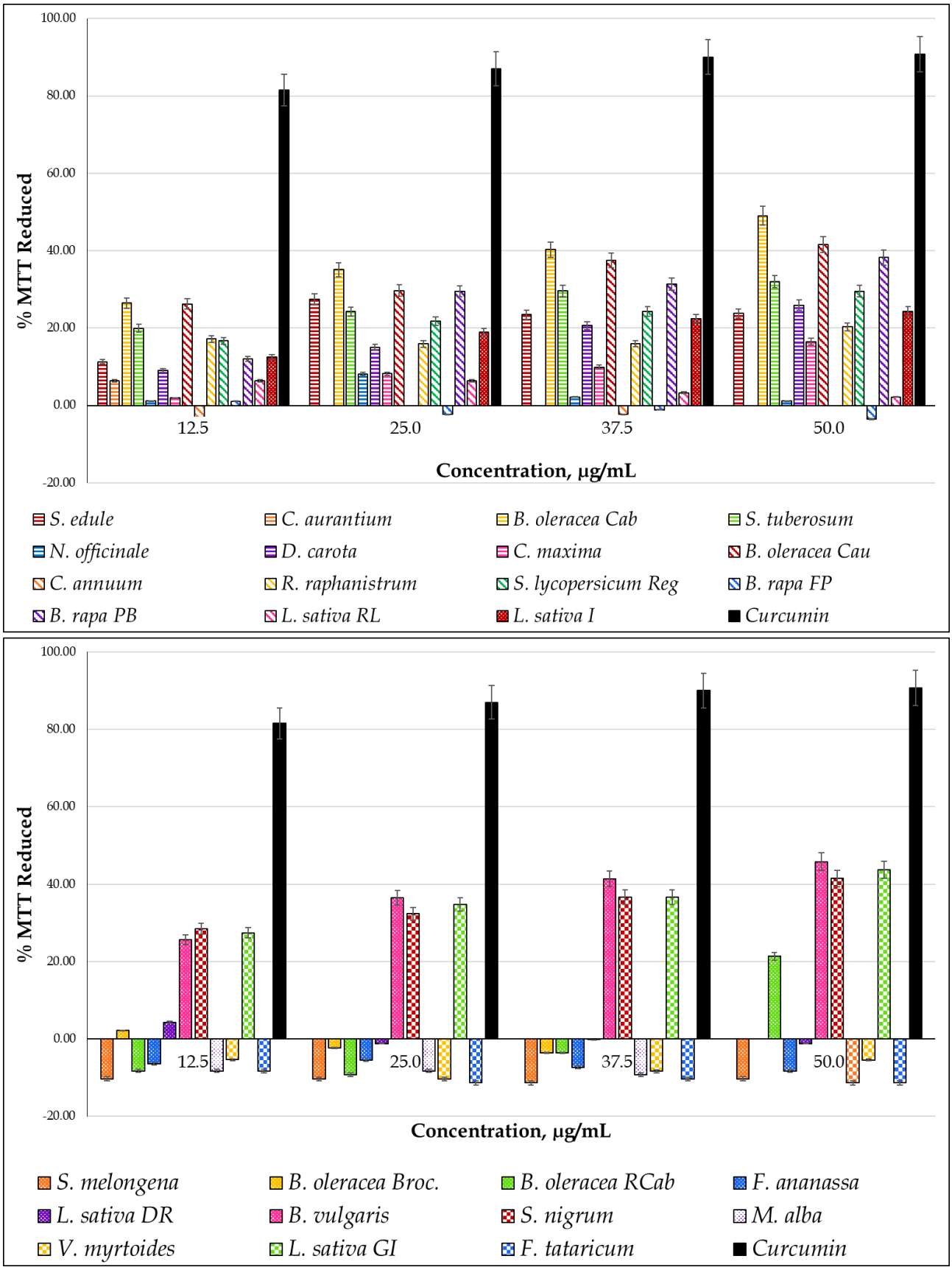

Figure 6. MTT reducing activity of Baguio plant foods.

Hydroxyl radical scavenging assay

Hydroxyl radicals are highly reactive and short-lived molecules. These molecules can react with lipids, polypeptides, proteins, and DNA, especially thiamine and guanosine bases[53]. The high reactivity of hydroxyl radicals may result in reduced disulfide bonds in fibrinogen, resulting in abnormal folding[54]. Therefore, it is vital to remove hydroxyl radicals.

Baguio plant foods showed scavenging ability against hydroxyl radicals. Among these plant foods, *B. oleracea* Cab showed the highest inhibitory activity against hydroxyl radicals at 166.7 µg/mL by 54.36±2.38%. Antioxidant activity and polyphenol content of *B. oleracea* varieties, it was revealed that methanolic extracts of *B. oleracea* varieties exhibited varying hydroxyl radical inhibitions in the order: red cabbage > green cabbage > broccoli > cauliflower[27]. Comparable scavenging to that of *B. oleracea* Cab was also observed in *C. maxima* (53.26±0.82%), *C. annuum* (48.38±2.14%), *L. sativa* RL (48.37±0.819%), and *L. sativa* GI (46.18±1.2%) at the same concentration. In addition, varieties of *L. sativa* exhibited different nitric oxide radical scavenging activities. The four varieties of *L. sativa* exhibited scavenging activity at 166.7 µg/mL in decreasing order: *L. sativa* RL > *L. sativa* GI > *L. sativa* I > *L. sativa* DR. These conformed with results using various Serbian lettuce varieties[55]. Among the three plant varieties, the Neva variety possessed the highest hydroxyl scavenging activity (IC_{50}=87.56±1.05 µg/mL), followed by Emerald, then Vera[54]. Other commonly consumed plant foods in Baguio exhibited ~30-40% scavenging activity at 166.7 µg/mL. The overall results are presented in **Figure 7**.

Phytochemical analysis

All Baguio plant samples contained reducing sugars and quinone compounds. Terpenes and terpenoids were found in all extracts except *B. rapa* FP, *N. officinale*, and *S. nigrum*. Only *L. sativa* Green GI, *L. sativa* DR, *B. oleracea* RCab, *F. tataricum*, and *V. myrtoides* revealed the presence of phenolic and tannin compounds. Alkaloids were found in *D. carota*, *L. sativa* DR, *B. oleracea* Cab, *B. oleracea* RCab, *R. raphanistrum*, *C. aurantium*, and *S. lycopersicum* Reg. A few plant samples contained flavonoid and saponin compounds. Among all samples tested, *N. officinale* possessed the least phytochemicals present. The overall results are presented in **Table II**.

Many phytochemicals support the body's innate antioxidant machinery. Phytochemicals: polyphenols, flavonoids, anthocyanins, and carotenoids are the major contributors to their antioxidant properties[56]. Polyphenols, which include flavonoids, stilbenes, lignans, and phenolic acids, are chemical substances characterized by aromatic rings with one or more hydroxyl groups. These compounds react with free radicals, resulting in the delocalization of the gained electron and stabilization of the aromatic nucleus through resonance. This, in turn, stops the free radical chain reaction[57]. Carotenoids, which exhibit a characteristic, symmetrical tetraterpene skeleton found in colored pigments of plant foods, are effective "radical-trapping antioxidants" and one of the most efficient singlet oxygen quenchers[58]. Other phytochemicals such as terpenes and terpenoids, alkaloids, and saponin compounds were also reported for antioxidant activities[59-61].

However, several studies reported that phenolic antioxidants can also act as prooxidants under certain conditions, like high concentrations of transition metal ions, alkali pH, and the presence of oxygen molecules[62-64]. Large molecular weight phenolics, such as hydrolysable and condensed tannins, have little or no prooxidant properties compared to simple phenol. Polyphenols with low oxidation potentials (Epa) exhibit antioxidant activity, while those with high Epa values act as prooxidants[5]. This characteristic could describe a dual action of phenolic compounds, where high-Epa polyphenols exist in some extracts that simultaneously exhibit antioxidant and prooxidant activities.

In summary, the study revealed that phytochemicals from these plant foods exhibited various degrees and types of antioxidant components and activities. For example, some plant foods, *F. tataricum*, *V. myrtoides*, and *M. alba*, possess higher or equal antioxidant activity than known standards. The various composition of phytochemicals in plant foods exerts different "strength" against oxidants. Reports have shown that exogenous antioxidants may show prooxidant activities, especially when administered at high doses. Despite these drawbacks, food-based secondary metabolites hold promising avenues for health benefits. Further work may be done to elucidate these results *in vivo* and identify compounds responsible for these activities. Moreover, continuous studies on phytochemical mechanisms and interactions may be done to establish the most significant impact of antioxidant systems on alleviating chronic diseases.

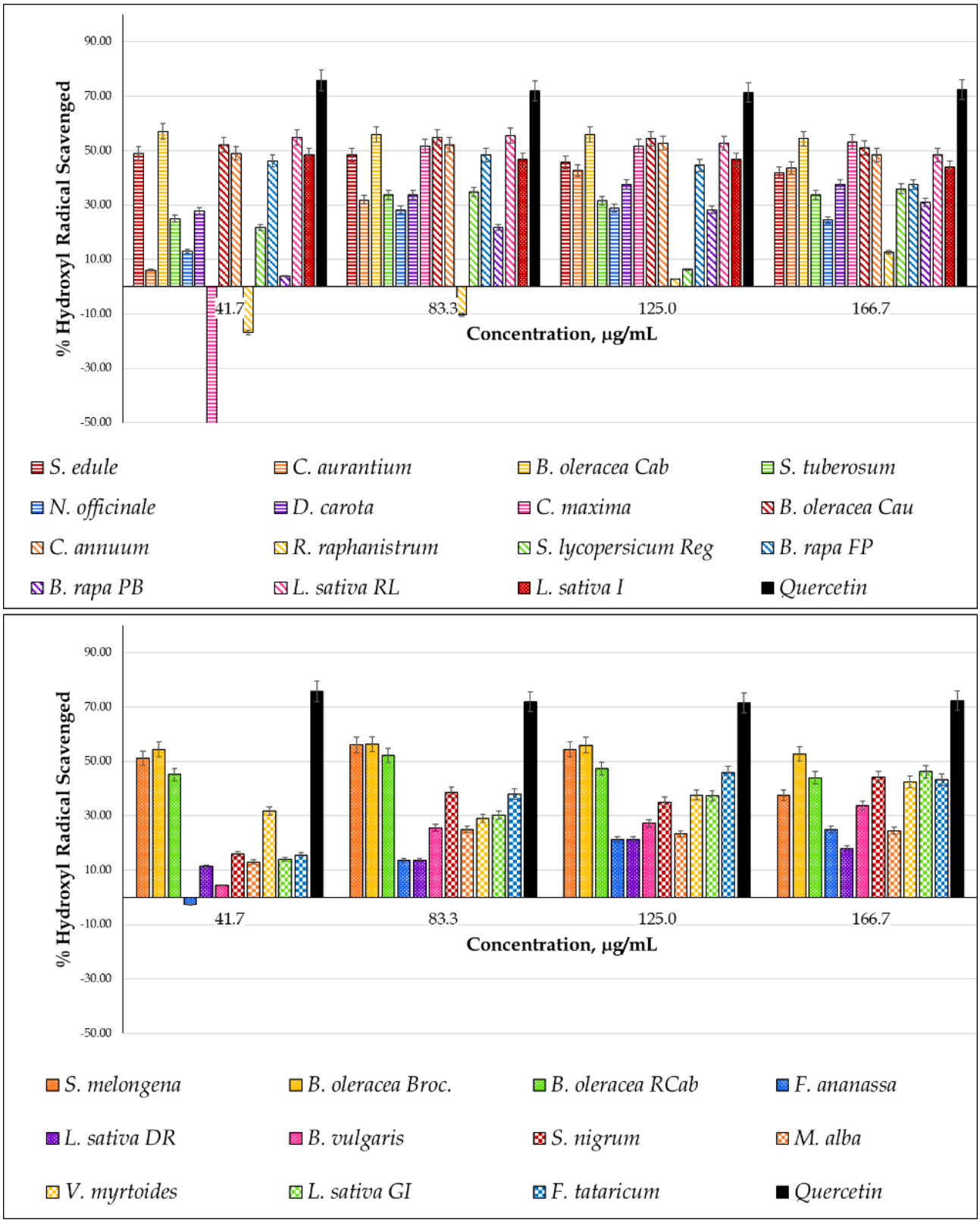

Figure 7. Hydroxyl radical scavenging activity of Baguio plant foods.

Table II. Phytochemical profile of methanolic extracts of Baguio plant foods

Plant Sample	Flavonoids	Coumarins	Reducing sugars	Proteins	Saponins	Glycosides	Alkaloids	Terpenes/ Terpenoids	Tannins	Phenolics	Steroids and phytosterol	Quinones	Anthraquinones
B. vulgaris	-	-	+	+	+	+	-	+	-	-	-	+	-
D. carota	-	+	+	+	+	+	+	+	-	-	+	+	+
L. sativa I	-	+	+	+	-	-	-	+	-	-	-	+	+
L. sativa RL	+	+	+	+	-	-	-	+	-	-	-	+	-
L. sativa GI	-	+	+	+	-	+	-	+	+	+	+	+	-
L. sativa DR	-	+	+	-	-	+	+	+	+	+	+	+	-
B. oleracea Cab	+	+	+	+	-	-	+	+	-	-	-	+	-
B. oleracea RCab	+	+	+	+	-	+	+	+	+	+	+	+	-
B. oleracea Broc	-	+	+	-	-	+	-	+	-	-	+	+	+
B. oleracea Cau	-	+	+	+	-	+	-	+	-	-	+	+	-
B. rapa PB	-	+	+	+	+	+	-	+	-	-	-	+	+
B. rapa FP	-	+	+	+	-	+	-	-	-	-	+	+	-
N. officinale	-	+	+	-	-	-	-	-	-	-	+	+	-
R. raphanistrum	+	+	+	+	-	+	+	+	-	-	+	+	+
S. edule	+	+	+	-	+	+	-	+	-	-	+	+	-
M. alba	+	+	+	-	-	+	-	+	-	-	+	+	+
F. tataricum	-	+	+	-	-	-	-	-	+	+	-	+	-
F. ananassa	+	+	+	+	-	+	-	+	-	-	+	+	+
C. aurantium	+	+	+	+	-	+	+	+	-	-	+	+	-
S. melongena	-	+	+	-	-	-	-	+	-	-	+	+	-
C. annuum	-	+	+	+	-	+	-	+	-	-	-	+	-
S. lycopersicum Reg	-	+	+	+	+	+	+	+	-	-	-	+	-
S. tuberosum	-	+	+	+	+	-	-	+	-	-	-	+	-
C. maxima	-	-	+	+	+	-	-	+	-	-	+	+	+
S. nigrum	-	+	+	+	+	-	-	-	-	-	-	+	-
V. myrtoides	+	+	+	-	-	+	-	+	+	+	+	+	+

CONCLUSION

In conclusion, the study revealed that Baguio plant foods contained various phytochemicals and showed promising antioxidant capacities. The findings of this study may provide information that consuming Baguio-cultivated plant foods are beneficial and may be applied in the management of various free radical-linked diseases.

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AUTHORS' CONTRIBUTION

PRB performed laboratory experiments and wrote the paper. **RCC** performed laboratory experiments; **GFY** supervised the flow, conceptualized, and advised ideas, and edited the manuscript.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Research Article

Analysis of Management Elements in Antibiotic Inventory Control with EOQ and MMSL Methods at Aisyiyah Bojonegoro Hospital

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Abstract

Inventory control is important in managerial activities because it involves investment and is hospitals' most significant expenditure component. The Aisyiyah Bojonegoro Hospital requires a logistics management system that can maintain the safety and effectiveness of the use of drugs for the smooth running of hospital pharmaceutical services in the long term. This analytical observational study analyzes management elements in controlling antibiotic inventory by simulating the Economic Order Quantity (EOQ) and Minimum-Maximum Stock Level (MMSL) methods. Determination of the sample purposively, i.e., 17 types of antibiotic drugs category A from the results of the ABC analysis, with the inclusion criteria being high cost, high volume, clinically important drugs for antibiotic drugs that are included in the 2020 Hospital Formulary and the exclusion criteria are drugs with inadequate supply. The analysis technique used the Mann-Whitney test and the Kruskal-Wallis test. Based on the results of the study, it was concluded that. Applying the EOQ and MMSL methods has been proven to increase the efficiency and effectiveness of the supply of category A antibiotics at Aisyiyah Bojonegoro Hospital.

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INTRODUCTION

Based on The Law of the Republic of Indonesia Number 44 of 2009 concerning Hospitals, it is stated that the hospital is a health service institution that provides complete individual health services that provide inpatient, outpatient, and emergency services. Meanwhile, pharmaceutical services are an integral part of integral part of the health care system within the hospital. Pharmaceutical services are supporting services and, at the same time, the main revenue center[1]. The purpose of pharmaceutical services is to ensure the availability of good quality, smooth distribution and affordability of drugs, and the availability of types and quantities of drugs to meet public health needs[2].

Drug management in hospitals is a vital management aspect because its inefficiency will negatively impact hospitals medically and economically[3]. Inventory management is the core of the system. The pharmaceutical inventory aims to minimize total inventory costs and optimize quality[4], balance stock out and over stock, and avoid financial losses as the ultimate goal[5]. Procurement is a continuous activity starting from the selection, determination of the required amount, adjustment between needs and funds, selection of procurement methods, supplier selection, determination of contract specifications, monitoring of the procurement process, and payment[6]. Effective procurement must ensure availability, quantity, and time at an affordable price and by quality standards[7].

The percentage of spending on antibiotics from the total spending on pharmaceutical supplies at Aisyiyah Bojonegoro Hospital shows that in 2018 it reached 28.25% with an antibiotic spending value of IDR 6,045,618,320. Meanwhile, in 2019 it reached 20.08% with an antibiotic spending value of IDR 5,043,476,179. Considering that the percentage of antibiotic

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spending at Aisyiyah Bojonegoro Hospital is stable at 20-30% per year, the success of management in controlling the value of spending on antibiotics means being able to control most of the spending value at the Pharmacy Installation. Based on the initial survey at the Aisyiyah Bojonegoro Hospital Pharmacy Installation shows that inventory control management implemented using the consumption method has not been able to control drug supplies optimally. The consumption method can provide good accuracy predictions in planning drug needs but only predicts how many drug needs will be planned without knowing when to order drugs again[8].

The management of the Aisyiyah Bojonegoro Hospital Pharmacy Installation has attempted to create budget efficiency without reducing the quality of health services in the post-Covid-19 pandemic, whose impact is still being felt today. The quality service in question is an integrated and comprehensive service system through appropriate drug planning management to guarantee that individual patients may obtain qualified drugs, increase drug use efficiency, and reduce drug costs for patients[9]. The efficiency of antibiotics supplies drugs can be achieved using Economic Order Quantity (EOQ) inventory method based on ABC (Always, Better, Control) analysis which is expected to reduce inventory value, increase Inventory Turn Over Ratio (ITOR), and increase customer service level[10]. Economic Order Quantity is the amount of inventory that can be ordered in a period to minimize inventory cost[11]. This method can answer questions about conditions that often occur in hospital pharmacy installations: determining the number of pharmaceutical supplies and medicines that are by hospital needs, neither too high nor too low, to reduce losses that occur in hospitals due to management inaccuracy in processing inventory[12].

The decline in the quality of services in hospitals, especially pharmacy installations, can also be caused by the stock of drug supplies[13,14]. To anticipate the tendency of hospitals to carry out excessive procurement patterns, but on the other hand, they can also find out what the minimum stock is so that there is no stock out; it is necessary to calculate the maximum and minimum stock values using the Minimum-Maximum Stock Level (MMSL) method. This method is an inventory control by considering the safety stock so that the reorder point for each item of pharmaceutical supplies can be determined[15,16]. Previous research from Dewi *et al*.[12] analyzed cost-efficiency management of drug supply via EOQ and MMSL in hospitals. It shows that EOQ is the most effective and efficient method to manage the stock of drugs at hospital pharmacies. It causes minimum opportunity loss and opportunity cost and thus is an effective and efficient drug inventory method compared to MMSL and usual consumption methods at RA Basoeni Hospital, Mojokerto.

Analysis of antibiotic drug inventory planning by applying the EOQ and MMSL methods related to inventory control at Aisyiyah Bojonegoro Hospital is needed to avoid high inventory values at the end of the year, anticipate death stock and stockouts, and increase the ITOR value for antibiotic drugs. Therefore, this study aimed to determine the method of planning antibiotics supply by the characteristics of pharmaceutical supplies at Aisyiyah Bojonegoro Hospital using the EOQ and MMSL methods. Then, the control of antibiotic drug inventory that had been simulated was associated with the impact of the application of the two methods on the management element analysis, including the elements of man, material, and method.

MATERIALS AND METHODS

Materials

This study was an analytical observational study to analyze management elements in antibiotic inventory control, and observations were carried out sequentially in time based on secondary retrospective research data from logistics management data for category A antibiotics at the Aisyiyah Bojonegoro Hospital Pharmacy Installation for one period at 2022.

Methods

The research method that the author used was a qualitative analysis method as a form of analysis of the simulation of the implementation of logistics management methods that have been carried out by comparing the value of inventory, ITOR, death stock, and stock out, as well as the aftermath of the impact on management elements. Determination of the sample purposively with antibiotic drugs category A from the results of the ABC analysis, with inclusion criteria being high cost,

high volume, clinically important drugs in antibiotic drugs that were included in the Hospital Formulary in 2020 and the exclusion criteria were drugs with unstable supply.

Data analysis

A comparison test between the new methods (EOQ and MMSL) and the existing method (Consumption method) was carried out to find out whether there were differences in the dependent variable resulting from the independent variables with unpaired non-parametric data with the Mann-Whitney test analysis and a comparison test of more than two groups with Kruskal-Wallis.

RESULTS AND DISCUSSION

Results of analysis with ABC method

Before analyzing the application of consumption methods and simulations, ABC analysis or Pareto analysis was carried out on data on the use of antibiotics to obtain drugs in category A according to the inclusion criteria and stability of drug supply during 2020; therefore, more is needed to just once data collection. Based on data on drug grouping at Aisyiyah Bojonegoro Hospital with ABC analysis, it is known that from 1442 items of drugs and medical devices managed by the Aisyiyah Bojonegoro Hospital Pharmacy Installation, 138 types of antibiotic drugs are included in groups A, B, and C.

The results of ABC analysis based on usage value and investment value are shown in **Table I**, which shows that the study sample consisted of 17 types of antibiotic drug category A (high cost, high volume) with a cumulative value of usage between 0-80% and included in the 2020 National Formulary (clinically important drug), and the supply drugs during January to December 2020 was stable. Stable supply means that there is a similarity between the number of orders and the number of receipts or deliveries from suppliers or distributors[13].

Table I. ABC analysis in determining research samples at Aisyiyah Bojonegoro Hospital in 2020 (Source: Aisyiyah Bojonegoro Hospital Pharmacy Installation Logistics Data, 2020)

No	Types of Antibiotics	Cost (IDR)	Cumulative value (IDR)	Classification
1	Cefxon Inj 1 g	1,643,426,475	1,643,426,475	A
2	Terfacef Inj 1 g/28	921,094,980	2,564,521,455	A
3	Cefila Cap 100 mg/30	758,669,309	3,323,190,764	A
4	Cefim Inj 1 g	568,639,060	3,891,829,824	A
5	Fourcef Inj 1 g	402,322,800	4,294,152,624	A
6	Broadced Inj 1 g	363,448,800	4,657,601,424	A
7	Cefobactam Inj	327,172,230	4,984,773,654	A
8	Cefspan Cap 100 mg/30	266,021,141	5,250,794,795	A
9	Baquinor Inf 100 mL	207,974,268	5,458,769,063	A
10	Cravox Inf 500 mg	203,132,754	5,661,901,817	A
11	Cravox Tab 500 mg/10	161,426,567	5,823,328,384	A
12	Starxon Inj 1 g	144,317,580	5,967,645,964	A
13	Sporetik 100 mg/30	140,049,301	6,107,695,265	A
14	Simextam Inj 1 g	138,560,400	6,246,255,665	A
15	Cefila Ds 30 mL	127,400,402	6,373,656,067	A
16	Pelastin Inj	127,053,432	6,500,709,499	A
17	Sporetik 200 mg/10	126,444,197	6,627,153,696	A

ABC analysis in 2020 showed that category A antibiotics amounted to 17 items (12.32%) of the total antibiotic items in the Pharmacy Installation with an investment value of IDR 6,627,153,696 (86.90%) of the total investment value of antibiotics or 20.5% of the total value of pharmaceutical inventory in the Aisyiyah Bojonegoro Hospital Pharmacy Installation. Category B antibiotics were 48 items (34.78%) of the total antibiotic items with an investment value of IDR 876,478,766 (11.49%) of the total antibiotic investment value or 2.7% of the total value of pharmaceutical inventory in the Aisyiyah Bojonegoro Hospital Pharmacy Installation. At the same time, category C antibiotics were 73 items (52.90%) of the total antibiotic items with an investment value of IDR 122,951,269 (1.61%) of the total investment value of antibiotics or 0.38% of the total value of pharmaceutical inventory in the Aisyiyah Bojonegoro Hospital Pharmacy Installation. The results of the ABC analysis based on the investment value of antibiotics are presented in detail in **Table II**. Thus, category A antibiotic drugs occupied the most significant investment value in Aisyiyah Bojonegoro Hospital Pharmacy Installation, with 86.90% of the total

investment value for antibiotics or 20.5% of the total investment in pharmaceutical supplies at the Aisyiyah Bojonegoro Hospital Pharmacy Installation. This becomes a reason that category A antibiotics need to be a priority for inventory control.

Table II. Classification of antibiotic drugs with ABC analysis based on investment value at Aisyiyah Bojonegoro Hospital in 2020 (Source: Aisyiyah Bojonegoro Hospital Pharmacy Installation Logistics Data, 2020)

Categories	Items	% Items	Investment value (IDR)	% Investment cumulative
A	17	12.32	6,627,153,696	86.90
B	48	34.78	876,478,766	11.49
C	73	52.90	122,951,269	1.61
Total	**138**	**100**	**7,626,583,731**	**100**

Recording of category A antibiotic procurement calculation based on consumption method

Table III shows data on inventory value, ITOR, death stock, and stockout of category A antibiotics from January to December 2020 based on the consumption method. **Table I** shows that the highest price of category A antibiotic drug is Pelastin Inj of IDR 433,727 per vial, and the lowest price for category A antibiotic is Sporetik 100 mg of IDR 25,450. The inventory cost of category A antibiotics is calculated by multiplying the purchase price of antibiotics by the amount used. The highest inventory cost was Cefim Inj 1 g, with a value of IDR 1,242,019,334, and the lowest inventory cost was Cefspan Cap 100 mg, with a value of IDR 33,134,640. The most significant number of early inventories was Cefila Cap 100 mg amounting to 916, and the smallest number was Starxon Inj 1 g amounting to 12. Meanwhile, the most significant number of the ending inventory was Cefila Cap 100 mg totaling 485; the smallest number of the ending inventory, empty or depleted, was Broadced Inj 1 g. Inventory value, ITOR, and death stock are efficiency indicators, while stockout values are effectiveness indicators. The following is the calculation of this study's efficiency and effectiveness indicators[17].

1. Inventory Value
   Inventory value is obtained from the calculation of the final stock of the drug multiplied by the price of each drug item[18]. The most considerable inventory value for the consumption method was Cefim Inj 1 g of IDR 55,205,454, while the smallest inventory value for the consumption method was Cefspan Cap 100 mg/30 of IDR 145,200.
2. Inventory Turn Over Ratio
   Inventory Turn Over Ratio is one of the benchmarks in the efficiency of antibiotic inventory control. Inventory Turn Over Ratio shows the inventory turnover ratio between sales and purchases of a particular antibiotic inventory. Inventory Turn Over Ratio comparison of each method is based on calculating the cost of good sold divided by the average inventory value[10]. Inventory Turn Over Ratio data in **Table III** shows the highest ITOR value for the consumption method is Cefim Inj 1 g (36.19), while the lowest ITOR value is Cefspan Cap 100 mg/30 (3.23).
3. Death Stock
   Death stock is the stock of antibiotic drugs at the Pharmacy Installation that does not move for three months or more, resulting in stockpiling of drugs, even expiry[19]. The calculation of the death stock of category A antibiotics in the Aisyiyah Bojonegoro Hospital Pharmacy Installation for 2020 using the consumption method is shown in **Table III**. There are six types of category A antibiotics recorded as death stock in the application of the consumption method (the existing method), including 1 g Broadced Inj, Cefobactam Inj, Cefspan Cap 100 mg, Baquinor Inf 100 mL, Cravox Inf 500 mg, and Simextam Inj 1 g, while the other 11 category A antibiotics did not experience death stock. The highest death stock value was Cefspan Cap 100 mg/30, 210 items or IDR 6,098,400. The lowest death stock value was Baquinor Inf 100 mL for 10 items or IDR 3,000,250.
4. Stock Out
   Measurement of effectiveness indicators uses stockout value data. Stockout is a condition where the amount of stock of antibiotic drugs in the Pharmacy Installation is empty, or the stock is 0 (zero) when there is a demand for that type of drug so that it fails to be served. Stockout occurs if, in inventory management, there is a final stock of drugs that is less than the average amount of use each month so that when there is a demand for the drugs, the stock is empty. Stockout conditions can cause losses for the patients, material losses for hospitals, and affect the quality of hospital services[20].

Table III shows that there are seven antibiotics category A that experienced stockout during the application of the consumption method, including Cefxon Inj 1 g, Cefila Cap 100 mg, Fourcef Inj 1 g, Broadced Inj 1 g, Cefspan Cap 100 mg, Cravox Inf 500 mg, and Pelastin Inj. Thus, 10 other category A antibiotics did not experience stockout. The most immense

stockout value was antibiotic Cefspan Cap 100 mg/30, as many as 691 caps (IDR 20,066.640), while the smallest stockout value was antibiotic Pelastin Inj, as many as 25 vials (IDR 10,843,175).

Table III. Data on inventory value, ITOR, death stock and stockout of category A antibiotics using Consumption method at Aisyiyah Bojonegoro Hospital in 2020 (Source: Aisyiyah Bojonegoro Hospital Pharmacy Installation Logistics Data, 2020)

No.	Drug names	Price (Rp)	Total use	IC (Rp)	Early invent	Ending invent	Inventory value	ITOR	Death stock		Stock out	
									Item	Rp	Item	Rp
1.	Cefxon Inj 1 g	277,200	3,092	857,102,400	153	91	25,225,200	25.34	0	0	45	12,474,000
2.	Terfacef Inj 1 g/ 28	271,656	1,318	358,042,608	76	55	14,941,080	20.12	0	0	0	0
3.	Cefila Cap 100 mg/30	25,740	13,234	340,643,160	916	485	12,483,900	18.89	0	0	394	10,141,560
4.	Cefim Inj 1 g	206,762	6,007	1,242,019,334	65	267	55,205,454	36.19	0	0	0	0
5.	Fourcef Inj 1 g	382,800	529	202,501,200	75	24	9,187,200	10.69	0	0	39	14,929,200
6.	Broadced Inj 1 g	257,400	223	57,400,200	28	0	0	15.93	37	9,523,800	53	13,642,200
7.	Cefobactam Inj	260,297	583	151,753,151	72	73	19,001,681	8.04	115	29,934,155	0	0
8.	Cefspan Cap 100 mg/30	29,040	1,141	33,134,640	701	5	145,200	3.23	210	6,098,400	691	20,066,640
9.	Baquinor Inf 100 mL	300,025	251	75,306,275	46	27	8,100,675	6.88	10	3,000,250	0	0
10.	Cravox Inf 500 mg	345,284	395	136,387,180	23	15	5,179,260	20.79	28	9,667,952	27	9,322,668
11.	Cravox Tab 500 mg/10	48,555	2,882	139,935,510	144	262	12,721,410	14.20	0	0	0	0
12.	Starxon Inj 1 g	296,340	454	134.538.360	12	51	15.113.340	14.41	0	0	0	0
13.	Sporetik 100 mg/30	25,450	3,151	80,192,950	323	257	6,540,650	10.87	0	0	0	0
14.	Simextam Inj 1 g	231,199	210	48,551,790	33	54	12,484,746	4.83	30	6,935,970	0	0
15.	Cefila Ds 30 mL	109,560	691	75,705,960	48	21	2,300,760	20.03	0	0	0	0
16.	Pelastin Inj	433,727	306	132,720,462	66	24	10,409,448	6.80	0	0	25	10,843,175
17.	Sporetik 200 mg/10	36,861	2,871	105,827,931	141	175	6,450,675	18.17	0	0	0	0

Note: ITOR: Inventory Turn Over Ratio; IC: Inventory Cost

Recording of category A antibiotic procurement calculation based on EOQ method

Table IV contains data for category A antibiotics from January to December 2020 based on the simulation of the EOQ method. The inventory cost for category A antibiotics is calculated by multiplying the purchase price of antibiotics by the amount used, similar to that in planning with the consumption method. The data showed that the most extensive ending inventory was Sporetik 200 mg totaling 196 caps, and the smallest ending inventory was Cefxon Inj and Pelastin Inj, each with three vials. Meanwhile, two antibiotics had an ending inventory value of zero, the Broadced Inj and Cefobactam Inj. The average ending inventory for category A antibiotics with the EOQ method was 44 units or lower than that for the consumption method, 110 units. The following is the calculation of the efficiency and effectiveness indicators used in this study for the EOQ method.

1. Inventory Value
   Inventory value is an indicator in assessing the efficiency of antibiotic inventory control using the EOQ method. **Table IV** shows that the largest inventory value for the EOQ method is Cefim Inj 1 g of IDR 25,638,488, while the smallest inventory value for the EOQ method is Cefspan Cap 100 mg of IDR 580,800. In addition, two antibiotics in category A have a zero-inventory value because the stock for the final inventory is also zero: Broadced Inj 1 g and Cefobactam Inj.
2. Inventory Turn Over Ratio
   Inventory Turn Over Ratio is used to measure the efficiency of antibiotic inventory control. **Table IV** shows that the highest ITOR value for the EOQ method is Cefim Inj 1 g (63.57), while the lowest ITOR value is Cefspan Cap 100 mg/30 (3.17).

3. Death Stock

   Similar to inventory value and ITOR, death stock is also used to measure the efficiency of inventory control for category A antibiotics in this study. **Table IV** shows that two antibiotics in category A experienced death stock in the simulation of applying the EOQ method: Cravox Tab 500 mg and Sporetik 200 mg, while 15 other category A antibiotics did not experience death stock. The lowest death stock value was Cravox Tab 500 mg with 1 item (IDR 48,555), while the highest death stock was Sporetik 200 mg with 63 items (IDR 2,322,243).

4. Stockout

   The stockout value is used to measure the effectiveness indicator of the EOQ method[21]. **Table IV** shows that no category A antibiotics experienced stockout during the implementation of the EOQ simulation method.

Table IV. Data on inventory value, ITOR, death stock and stockout of category A antibiotics using a simulation of EOQ method at Aisyiyah Bojonegoro Hospital in 2020 (Source: Aisyiyah Bojonegoro Hospital Pharmacy Installation Logistics Data, 2020)

No.	Drug names	Price (Rp)	Total use	IC (Rp)	Early invent	Ending invent	Inventory value	ITOR	Death stock		Stock out	
									Item	Rp	Item	Rp
1.	Cefxon Inj 1 g	277,200	3,092	857,102,400	153	3	831,600	39.64	0	0	0	0
2.	Terfacef Inj 1 g/ 28	271,656	1,318	358,042,608	76	9	2,444,904	31.01	0	0	0	0
3.	Cefila Cap 100 mg/30	25,740	13,234	340,643,160	916	114	2,934,360	25.70	0	0	0	0
4.	Cefim Inj 1 g	206,762	6,007	1,242,019,334	65	124	25,638,488	63.57	0	0	0	0
5.	Fourcef Inj 1 g	382,800	529	202,501,200	75	8	3,062,400	12.75	0	0	0	0
6.	Broadced Inj 1 g	257,400	223	57,400,200	28	0	0	15.93	0	0	0	0
7.	Cefobactam Inj	260,297	583	151,753,151	72	0	0	16.19	0	0	0	0
8.	Cefspan Cap 100 mg/30	29,040	1,141	33,134,640	701	20	580,800	3.17	0	0	0	0
9.	Baquinor Inf 100 mL	300,025	251	75,306,275	46	9	2,700,225	9.13	0	0	0	0
10.	Cravox Inf 500 mg	345,284	395	136,387,180	23	9	3,107,556	24.69	0	0	0	0
11.	Cravox Tab 500 mg/10	48,555	2,882	139,935,510	144	140	6,797,700	20.30	1	48,555	0	0
12.	Starxon Inj 1 g	296,340	454	134,538,360	12	27	8,001,180	23.28	0	0	0	0
13.	Sporetik 100 mg/30	25,450	3,151	80,192,950	323	31	788,950	17.80	0	0	0	0
14.	Simextam Inj 1 g	231,199	210	48,551,790	33	31	7,167,169	6.56	0	0	0	0
15.	Cefila Ds 30 mL	109,560	691	75,705,960	48	19	2,081,640	20.63	0	0	0	0
16.	Pelastin Inj	433,727	306	132,720,462	66	3	1,301,181	8.87	0	0	0	0
17.	Sporetik 200 mg/10	36,861	2,871	105,827,931	141	196	7,224,756	17.04	63	2,322,243	0	0

Note: ITOR: Inventory Turn Over Ratio; IC: Inventory Cost

Recording of category A antibiotic procurement calculation based on MMSL method

Table V shows that the inventory management of category A antibiotics using the MMSL method simulation showed that the drug with the most considerable ending inventory value was Cefim Inj 1 g totaling 15 vials. Those with the smallest ending inventory were Fourcef Inj, Broadced Inj, Cefobactam Inj, Cefspan Cap 100 mg, Baquinor Inf 100 mL, Simextam Inj, Pelastin Inj, and Sporetik 200 mg each with ending supply of zero unit or empty. Thus, the average ending inventory for category A antibiotics using the MMSL method was two units. This means that the average supply of category A antibiotics using the MMSL method was smaller than the consumption method (110 units) and the EOQ method (44 units). The following is the calculation of the efficiency and effectiveness indicators used in this study for the MMSL method.

1. Inventory Value

   Inventory value is an indicator in assessing the efficiency of antibiotic inventory control using the MMSL method[22]. **Table V** shows that the drug with the largest inventory value for the MMSL method was Cefim Inj 1 g of IDR 3,101,430, while the drug with the smallest inventory value for the MMSL method was Sporetik 100 mg of IDR 76,350. In addition, eight types of category A antibiotics had zero inventory value because the stock for the final inventory was also zero, i.e.,

Fourcef Inj 1 g, Broadced Inj 1 g, Cefobactam Inj, Cefspan Cap 100 mg, Baquinor Inf 100 mL, Simextam Inj, Pelastin Inj, and Sporetik 200 mg.

2. Inventory Turn Over Ratio

   Inventory Turn Over Ratio is used to measure the efficiency of antibiotic inventory control. **Table V** shows drug with the highest ITOR value for the MMSL method is Cefim Inj 1 gr, which is 150.18, and the lowest ITOR value is Cefspan Cap 100 mg, which is 3.26.

3. Death Stock

   Death stock value is used to measure the efficiency of inventory control for category A antibiotics in the MMSL method. **Table V** shows one type of antibiotic category A that experienced death stock in applying the MMSL method: Cefila Ds 30 mL as many as 97 items (IDR 10,627,320), while 16 other category A antibiotics did not experience death stock.

4. Stockout

   The stockout value is used to measure the effectiveness indicator for the MMSL method. **Table V** shows that there were no category A antibiotics that experienced stockout during the application of the MMSL method.

Table V. Data on inventory value, ITOR, death stock and stockout of category A antibiotics using a simulation of MMSL method at Aisyiyah Bojonegoro Hospital in 2020 (Source: Aisyiyah Bojonegoro Hospital Pharmacy Installation Logistics Data, 2020)

No.	Drug names	Price (Rp)	Total use	IC (Rp)	Early invent	Ending invent	Inventory value	ITOR	Death stock		Stock out	
									Item	Rp	Item	Rp
1.	Cefxon Inj 1 g	277,200	3,092	857,102,400	153	4	1,108,800	39.39	0	0	0	0
2.	Terfacef Inj 1 g/ 28	271,656	1,318	358,042,608	76	1	271,656	34.23	0	0	0	0
3.	Cefila Cap 100 mg/30	25,740	13,234	340,643,160	916	9	231,660	28.61	0	0	0	0
4.	Cefim Inj 1 g	206,762	6,007	1,242,019,334	65	15	3,101,430	150.18	0	0	0	0
5.	Fourcef Inj 1 g	382,800	529	202,501,200	75	0	0	14.11	0	0	0	0
6.	Broadced Inj 1 g	257,400	223	57,400,200	28	0	0	15.93	0	0	0	0
7.	Cefobactam Inj	260,297	583	151,753,151	72	0	0	16.19	0	0	0	0
8.	Cefspan Cap 100 mg/30	29,040	1,141	33,134,640	701	0	0	3.26	0	0	0	0
9.	Baquinor Inf 100 mL	300,025	251	75,306,275	46	0	0	10.91	0	0	0	0
10.	Cravox Inf 500 mg	345,284	395	136,387,180	23	1	345,284	32.92	0	0	0	0
11.	Cravox Tab 500 mg/10	48,555	2,882	139,935,510	144	4	194,220	38.95	0	0	0	0
12.	Starxon Inj 1 g	296,340	454	134,538,360	12	4	1,185,360	56.75	0	0	0	0
13.	Sporetik 100 mg/30	25,450	3,151	80,192,950	323	3	76,350	19.33	0	0	0	0
14.	Simextam Inj 1 g	231,199	210	48,551,790	33	0	0	12.73	0	0	0	0
15.	Cefila Ds 30 mL	109,560	691	75,705,960	48	1	109,560	28.20	97	10,627,320	0	0
16.	Pelastin Inj	433,727	306	132,720,462	66	0	0	9.27	0	0	0	0
17.	Sporetik 200 mg/10	36,861	2,871	105,827,931	141	0	0	40.72	0	0	0	0

Note: ITOR: Inventory Turn Over Ratio; IC: Inventory Cost

Supporting factors and inhibiting factors for the application of EOQ and MMSL methods in antibiotic inventory control

Focus group discussion conducted by the informants of this study, consisting of the parties directly involved in drug management, showed that the management of category A antibiotic drugs, as currently carried out using the consumption method, has proven to produce high inventory values and low ITOR, so it was not economically profitable for Aisyiyah Bojonegoro Hospital. The trials of the EOQ and MMSL methods with ABC analysis of category A antibiotics at the Pharmacy Installation of Aisyiyah Bojonegoro Hospital had been proven to reduce the inventory values and increase the ITOR. The research informants in this study consisted of the Head of Pharmacy Installation, Pharmacy Logistics Supervisor, Head of Finance, Pharmacy and Therapy Committee, Deputy Director of Administration and Finance, Pharmacy Staff, and Ward Nurses. The results of the FGD process by the informants are presented in **Table VI**.

Table VI. Qualitative comparison of consumption, EOQ, and MMSL methods (Source: Focus Group Discussion Aisyiyah Bojonegoro Hospital, 2021)

Method	Supporting factors	Impending factors
EOQ	a. Determination of when to order based on reorder point (ROP) to minimize stockout. b. Determination of the number of orders based on Quantity Order (Qo) to minimize excess inventory. c. Proven to have a significant impact in reducing inventory value compared to consumption method. d. Proven to prevent stockout. e. Budgeting for drug needs can be predicted on an annual scale. f. Able to calculate holding costs and ordering costs.	a. It is difficult for operators because they have to research which items of the drugs reaching the ROP point. b. Difficulty calculating storage costs accurately, given the volume of each drug and the need for different storage resources (e.g. electricity) for each drug. c. Changing the habits of pharmaceutical logistics staff from the consumption method pattern to the EOQ method. d. Highly dependent on information technology system for ROP scrutiny to minimize stockouts.
MMSL	a. Determining when to order based on Minimum Stock (Smin) to minimize stockout. b. Determination of the number of orders based on 7-day service requirements or maximum stock (Smax) to minimize excess inventory. c. Proven to have a significant impact in reducing inventory value compared to consumption method. d. Proven to have a significant impact in increasing the ITOR compared to consumption method. e. Proven to have a significant impact on the decrease in the value of death stock compared to consumption method. f. Proven to prevent stockout. g. Very sensitive to fluctuations in the number of patients and the need for drug prescription.	a. Makes it difficult for the operators because they have to examine any drug items that are less or equal to the minimum stock (Smin). b. The drug order process is carried out weekly, it can even be more than once per week according to fluctuations in real drug needs in the service. c. Budgeting for the cost of drug needs cannot be predicted on an annual basis, because it follows the fluctuating demand for real services. d. Changing the habits of pharmacy logistics staff from the pattern of consumption method to MMSL method. e. Highly dependent on IT systems for Minimum Stock (Smin) scrutiny to minimize stockout.

Analysis of management elements as the impact of EOQ and MMSL method application

Every choice made always has an impact or risk that must be anticipated. **Table VII** shows the impact analysis if the EOQ or MMSL method is applied at Aisyiyah Bojonegoro Hospital. The results of the added value analysis, if the EOQ method is applied at Aisyiyah Bojonegoro Hospital, are shown in **Table VIII**.

Table VII. Analysis of management elements as the impact of EOQ method application at Aisyiyah Bojonegoro Hospital

No	Parameters	Pre/consumption method (IDR)	Post/EOQ method (IDR)	Difference (IDR)
A.	**Human resources**			
1	Logistic Coordinator Allowance (Pharmacist)	-	2,200,000 x 12 months = 26,400,000	26,400,000
B.	**Materials**			
1	IT Team Empowerment	-	3 persons x 7 days x 105,000 per hour = 2,205,000	2,205,000
2	Consultation to IT Consultant	-	3 x 1,500,000 = 4,500,000	4,500,000
3	Program finishing	-	5,500,000	5,500,000
C.	**Methods**			
1	Socialization and technical assistance	-	Participation in annual routine education for inhouse training	-
2	Ordering cost	32,500 x 24 times/years x 17 antibiotic items = 13,260,000	32,500 x 48 times/years x 17 antibiotic items = 26,520,000	13,260,000
	Total			**51,865,000**

Table VIII. Analysis of the added value from the EOQ method application at Aisyiyah Bojonegoro Hospital

No	Parameters	Pre/Consumption method (IDR)	Post/EOQ method (IDR)	Difference (IDR)
1	Inventory cost	215,490,674	74,662,912	140,827,762
2	Death stock	65,160,527	2,370,798	62,789,729
3	Stock out	91,419,443	0	91,419,443
	Total			**295,036,934**

There has been a change in the number of human resources (man), infrastructure (material), system (method), and financing (money). The application of the EOQ method had an impact in the form of increasing the workload of the procurement department because the frequency of procurement increased, so it will be necessary to increase the level of the head of logistics, not equivalent to a supervisor but to become a coordinator led by a pharmacist who has the authority to make

more strategic decisions, one of which is ordering drugs[23]. The pharmaceutical logistics leadership position will also be equivalent to the coordinator with an allowance of IDR 2,200,000 per month, increased from the supervisor-level allowance in the previous system of IDR 500,000 per month. So, IDR 1,700,000 per month or IDR 20,400,000 per year will be added.

Changes also occurred in the facilities and infrastructure needed, such as improving the information system program (software). It will be necessary to empower the IT team with extra working hours considering that this is not a routine activity in the IT team, with overtime of IDR 15,000 per hour for seven hours per day for seven days with three IT staff so that the total overtime financing will be IDR 2,205,000. The overtime value of IDR 15,000 per hour was based on the Regency Minimum Wage of IDR 2,400,000 per month divided by 40 working hours per week, then divided by 4 (a month consists of four weeks). The cost of consulting an IT Consultant needs to be done three times periodically for IDR 1,500,000 with a total of IDR 4,500,000. They were finishing a program of IDR 5,500,000. This information system upgrade will be only done once, then what is needed will be software and hardware maintenance supported by routine maintenance costs budgeted for by the hospital so that it will not require separate financing.

The system is also changing with new procedures and requires socialization and training of related staff. However, the financing for this process has been budgeted for in the annual routine exhouse training in education and training programs, so there will be no need for special financing[24]. In the EOQ method, based on the average simulation, the procurement process was carried out once a week or 48 times a year. This changed the average habit of the existing method that the procurement process was carried out twice a month or 24 times a year. This means that there will be an increase of two times more often than before. The cost of each order was IDR 32,500 if the existing method was carried out 24 times a year with 17 items of antibiotics, so it would require an annual ordering fee of IDR 13,260,000 per year. In the EOQ method, the number of orders 48 times a year with 17 items of antibiotics required a cost of = IDR 26,520,000 per year. There will be a more expensive difference in ordering financing of IDR 13,260,000 annually.

From the man, material, and method analysis, the total additional cost obtained was IDR 45,865,000 per year. However, the positive impact of the application of the EOQ method was a decrease in the value of inventories from the previous method of IDR 215,490,674 to IDR 74,662,912 so that the difference in efficiency was IDR 140,827,762. The decline in the value of the death stock also showed a decrease from the previous IDR 65,160,527 (existing method) to IDR 2,370,798 (EOQ method), so the difference in efficiency was IDR 62,789,729. The stockout value also differed between the existing methods of IDR 91,419,443 to IDR 0 in the EOQ method, with the addition of an efficiency value of IDR 91,419,443. The total efficiency of applying this EOQ method will be IDR 295,036,934 per year. If this value is deducted from the costs that must be met in applying the EOQ method, an added value of IDR 249,171,934 per year will be added. The results of comparing pre- and post-unit costs for applying the MMSL method are presented in **Table IX**, while added value analysis of the MMSL method is listed in **Table X**.

There has been a change in the number of human resources (man), infrastructure (material), system (method), and financing (money). The application of the MMSL method increased the workload so that it required additional new human resources as staff in pharmaceutical logistics. In addition, an increase in the position level will also be needed[25]. The head of logistics will be different from a supervisor but become a coordinator led by a pharmacist with the authority to make more strategic decisions, one of which is ordering drugs. With this illustration, the financial impact of adding one staff with the competence of a diploma in pharmacy will be the allocation of a total salary per month of IDR 2,430,000 per month or IDR 29,160,000 per year. The pharmaceutical logistics leadership position will also be equivalent to a coordinator with an allowance of IDR 2,200,000 per month, an increase from the supervisor-level allowance in the previous system of IDR 500,000 per month, so there will be an additional IDR 1,700,000 per month or IDR 20,400,000 per year.

Changes also occurred in the facilities and infrastructure needed, such as improvement of the information system program (software), so extra or overtime IT team empowerment will be needed considering this is not a routine IT team activity, with overtime wages of IDR 15,000 per hour for seven hours per day for seven days with three IT staff so that the total overtime financing will be IDR 2,205,000. The overtime value of IDR 15,000 per hour was based on the Regency Minimum Wage of IDR 2,400,000 per month, divided by 40 working hours per week, then divided by 4 (a month consists of four weeks). Consultation with an IT consultant will need to be done three times periodically with a fee of IDR 1,500,000 with a total of IDR 4,500,000 and a finishing program of IDR 5,500,000. This information system upgrade will only be done once, so after that, what is needed will be software and hardware maintenance which would be financed from routine maintenance costs budgeted for by the hospital so that it will not require separate financing.

Table IX. Analysis of impact of MMSL method application at Aisyiyah Bojonegoro Hospital

No	Parameters	Pre/consumption method (IDR)	Post/EOQ method (IDR)	Difference (IDR)
D.	**Human resources**			
1	Addition of 1 logistic staff	-	2,430,000 x 12 months = 29,160,000	29,160,000
2	Logistic Coordinator Allowance (Pharmacist)	-	2,200,000 x 12 months = 26,400,000	26,400,000
E.	**Materials**			
1	IT Team Empowerment	-	3 persons x 7 days x 105,000 per hour = 2,205,000	2,205,000
2	Consultation to IT Consultant	-	3 x 1,500,000 = 4,500,000	4,500,000
3	Program finishing	-	5,500,000	5,500,000
F.	**Methods**			
1	Socialization and technical assistance	-	Participation in annual routine education for inhouse training	-
2	Ordering cost	32,500 x 24 times/years x 17 antibiotic items = 13,260,000	32,500 x 96 times/years x 17 antibiotic items = 53,040,000	39,780,000
	Total			**107,545,000**

Table X. Analysis of the added value from the MMSL method application at Aisyiyah Bojonegoro Hospital

No	Parameters	Pre/Consumption method (IDR)	Post/EOQ method (IDR)	Difference (IDR)
1	Inventory cost	215,490,674	6,624,322	208,866,352
2	Death stock	65,160,527	10,627,320	54,533,207
3	Stock out	91,419,443	0	91,419,443
	Total			**354,819,002**

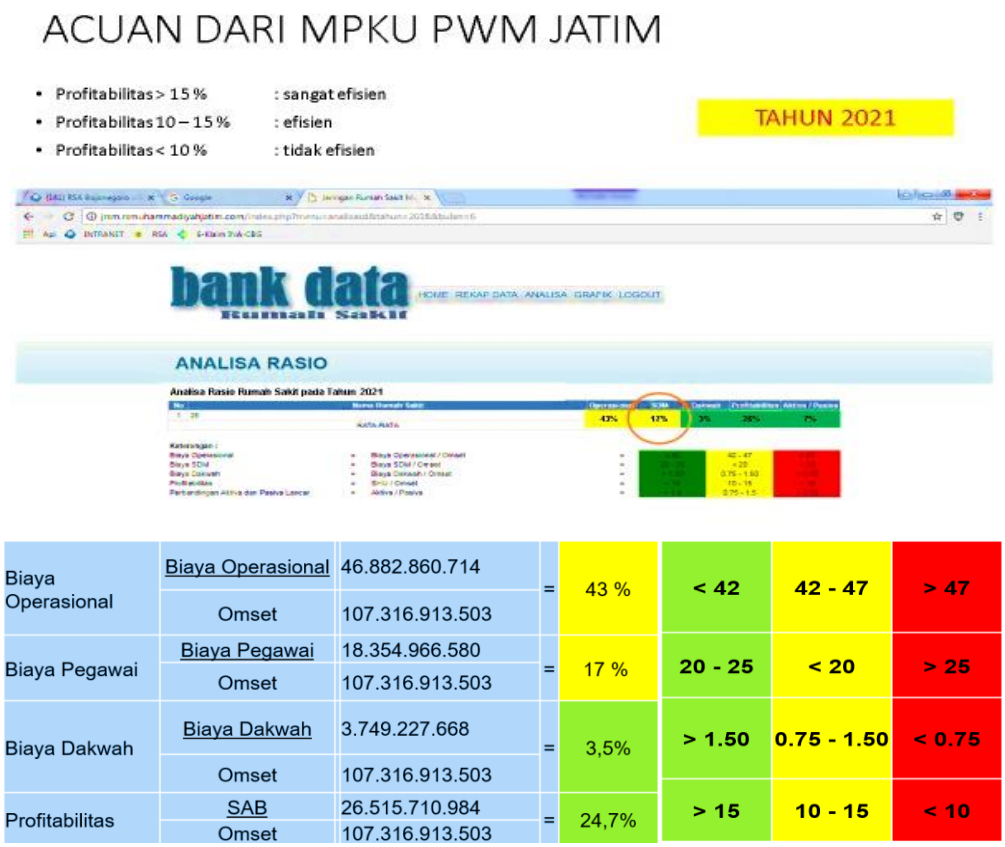
The system was also changing with new procedures that required socialization and training of related staff. However, the financing for this process has been budgeted for in the annual routine exhouse training in education and training programs, so there will be no need for special financing. The MMSL method was based on a simulation that conducted the procurement process on average twice a week or 96 times a year. This changed the average habit of the existing method that carried out the procurement process an average of two times a month or 24 times a year. This means that there will be an increase of four times more often than before. The cost of each order will be IDR 32,500 if the existing method is carried out 24 times a year with 17 items of antibiotics, so that it will require an annual ordering fee of IDR 13,260,000. In the MMSL method, the number of orders was 96 times a year with 17 items of antibiotics, so that it will require a cost of IDR 53,040,000 per year. There will be a more expensive difference in ordering financing of IDR 39,780,000 annually.

Analysis of the man, material, and method approach showed the total additional cost of IDR 101,545,000 per year. However, the positive impact of the MMSL method application was a decrease in inventory value from the previous method of IDR 215,490,674 to IDR 6,624,322, so the difference in efficiency was IDR 208,866,352. The death stock value decreased from the previous IDR 65,160,527 (existing method) to IDR 10,627,320 (MMSL method) with an efficiency difference of IDR 54,533,207. The stockout value was also different from the existing method of IDR 91,419,443 to IDR 0 on the MMSL method, with the addition of the efficiency value of IDR 91,419,443. The total efficiency offered in the MMSL method application was IDR 354,818,802 per year. If this value is deducted from the costs that must be met in the MMSL method application, an added value of IDR 253,273,802 per year will be obtained. In addition, the MMSL method application can prevent drug stockouts and increase service effectiveness and quality.

Tables VIII and **X** show the results of added value analysis of the EOQ and MMSL method application at Aisyiyah Bojonegoro Hospital. The MMSL method is good at decreasing inventory cost than EOQ. The EOQ is better than MMSL in decreasing death stock. Overall, the MMSL method has better different efficiency than EOQ. This finding supported the previous research that EOQ is best in controlling death stock[12]. Optimal ordering planning is obtained based on the proper calculation of the EOQ[26]. The EOQ is best in decreasing death stock. It was observed that the reason for this was the use of ROP (Re-Order Point) that set the minimum limit for reordering in the EOQ method. In the MMSL method, each drug item was determined by its maximum and minimum stock levels, in which when the drug's volume had reached the minimum amount, it would be purchased immediately until the volume reached the optimal stock. This method is appropriate for drugs that are used commonly; as the actively used drug's volume reaches the set minimum level, new purchases will be activated[12].

The analysis also shows the advantages of the EOQ method, in which no additional staff is required, and the frequency of orders is less than the MMSL method[27]. Meanwhile, MMSL requires adding new human resources in the pharmaceutical

logistics department, and the frequency of procurement will be more frequent[28]. However, the added value of efficiency obtained is still superior to the MMSL method. Nevertheless, the priority is referred to the performance assessment of Aisyiyah Bojonegoro Hospital as determined by General Health Supervisory Council (*Majelis Pembina Kesehatan Umum*, MPKU), Muhammadiyah Regional Leadership (*Pimpinan Wilayah Muhammadiyah*, PWM) East Java, Indonesia, as shown in **Figure 1**. According to **Figure 1**, the operational cost ratio score compared to the turnover of Aisyiyah Bojonegoro Hospital is still yellow (sufficient). Better efficiency is needed to go to the color green (good). By comparing the standard of employee or HR costs with turnover, Aisyiyah Bojonegoro Hospital is in yellow (sufficient), which means that adding HR costs by new personnel in pharmaceutical logistics is not a burden but improves the hospital's performance assessment. Thus, the MMSL method, which offers better inventory value efficiency and ITOR by increasing the number of new human resources, is currently the best choice at Aisyiyah Bojonegoro Hospital.



Biaya Operasional	Biaya Operasional	46.882.860.714	=	43 %	< 42	42 - 47	> 47
	Omset	107.316.913.503					
Biaya Pegawai	Biaya Pegawai	18.354.966.580	=	17 %	20 - 25	< 20	> 25
	Omset	107.316.913.503					
Biaya Dakwah	Biaya Dakwah	3.749.227.668	=	3,5%	> 1.50	0.75 - 1.50	< 0.75
	Omset	107.316.913.503					
Profitabilitas	SAB	26.515.710.984	=	24,7%	> 15	10 - 15	< 10
	Omset	107.316.913.503					

Figure 1. Dashboard of performance appraisal reference for Aisyiyah Bojonegoro Hospital (Source: MPKU PWM East Java Website in 2021).

CONCLUSION

As a method of planning and procuring category A antibiotics currently being implemented at the Pharmacy Installation of Aisyiyah Hospital Bojonegoro, the consumption method has resulted in high inventory values, lower ITOR values compared to the new simulated method, and higher death stock and stockout rate. The application of EOQ and MMSL methods has been proven to increase the efficiency of category A antibiotics supply at Aisyiyah Hospital Bojonegoro with the indicators as follows: the decrease in inventory value, the increase in ITOR, and the decrease in the death stock rate. In addition, the application of the EOQ and MMSL methods was also proven to increase the effectiveness of category A antibiotic drug supplies at Aisyiyah Hospital Bojonegoro with an indicator of the decrease in stockout incidence rate. This study showed that applying the MMSL method had a greater chance of reducing the stockout rate than the EOQ and the existing consumption methods. The limitation of this research is that there are several other factors, such as the study location and time.

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AUTHORS' CONTRIBUTION

All authors contributed equally to the research and writing of this article.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The author declares that there is no potential conflict of interest in relation to the authorship and publication of this article.

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