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Combination of *Spirulina platensis* powder and *Stichopus variegatus* powder against Bcl2 expression in the hippocampus of dementia Rats

Lisa Agustina Botutihe, Rizka Safira, Sapto Yuliani, Kintoko*
Faculty of Pharmacy, Universitas Ahmad Dahlan
Jl. Prof. Dr. Soepomo, S.H, Warungboto, Umbulharjo, Yogyakarta, Indonesia

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ABSTRACT

Spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) are known to have antioxidant activity that has the potential to prevent neurodegeneration disease. The aim of this study was to examine the effect of the combination of spirulina and golden sea cucumber on Bcl2 gene expression in pyramidal hippocampus cells of trimethyltin-induced dementia (TMT) rats. The study used *Sprague Dawley* rats which were divided into six groups, namely the normal control group (CMC-Na and NaCl 0.9%), pain control (CMC-Na and TMT), positive control (citicoline dose 200 mg/kg BW and TMT) and and test control injected with TMT and given a combination of spirulina (S) and golden sea cucumber dose (G) with three ratios of SG-3:1, SG-1:1 and SG-1:3 in a single dose of 200 mg/kg BW. Sample and citicoline were given on days 1-28, while TMT injection was given a single dose of 8 mg/kg BW on day 8. On day 36, the rats were sacrificed, brains were removed and the right hemispherium cerebri was fed to 10% formalin in pbs. After 6 days, the hippocampus was separated for immunohistochemical observation. The test result data was statistically analyzed with *a one-way* ANOVA test then followed by post hoc *tukey* to see the differences between groups. Results showed the combination of spirulina and golden sea cucumber can increase the expression of the Bcl2 gene in the hippocampus. The combination of spirulina and golden sea cucumber (SG-1:3) at a dose of 200 mg/kg BW had the ability to increase Bcl2 expression almost the same as citicoline with the number of Bcl2 cell expressions being 27.51 ± 0.70 in the CA1 region and 69.96 ± 1.97 in the CA2-CA3 region. So, it can be concluded that SG-1:3 has the potential to prevent dementia.

Keywords: Bcl2, dementia, *Spirulina platensis*, *Stichopus variegatus*, trimethyltin

Corresponding author:
Kintoko
Faculty of Pharmacy, Universitas Ahmad Dahlan
Jl. Prof. Dr. Supomo, S.H, Warungboto, Umbulharjo,Yogyakarta, Indonesia
Email: Kintoko@pharm.uad.ac.id

INTRODUCTION

Dementia is a clinical syndrome characterized by progressive cognitive decline that appears as memory loss, communication and language disorders, agnosia, apraxia and impaired executive function (reasoning, judgment and planning) (Duong et al., 2017). Dementia symptoms are caused by damage to a part of the brain called the hippocampus, which has a central role in memory (Lavenex & Lavenex, 2013). Oxidative stress participates in the development of dementia. Oxidative stress is an imbalance between free radicals and antioxidants. The most abundant type of free radical in the body is *Reaction Oxygen Specific* (ROS) (Singh, 2022). Excess ROS can cause apoptosis.

Apoptosis is cell death that occurs regularly to ensure a homeostatic balance between the rate of cell formation and cell death. The process of apoptosis can occur when the amount of antiapoptotic and proapoptotic proteins in the cell is out of balance. Apoptosis is regulated by several genes, including Bcl-2 which plays a role in preventing apoptosis (anti-apoptosis) (Yuliani et al., 2021).

*B-Cell lymphoma-2 (*Bcl-2) is a protein that provides key functions in cell health through the mechanism of apoptosis (Callens et al., 2021). Bcl-2 can inhibit the release of cytochrome-c from mitochondria and activation of caspase thereby preventing apoptosis. Caspase is a proapoptotic protein that acts as an executor in the apoptotic cascade (Zhao et al., 2014). For this reason, an antioxidant agent is needed to neutralize free radicals that can cause apoptosis.

Spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) are two marine resources that have high potential as antioxidants. Research by Jadaun et al. (2018), shows spirulina plays a role in the prevention of increased oxidative stress during apoptitosis, increased mitochondrial membarane potential as well as increased Bcl2 expression. Reseacrh by Su et al. (2018), showed that sea cucumbers also showed inhibition of caspases-9 and -3 so as to prevent apoptosis.

The development of herbal-herbal combination therapy or known as polyherbal therapy is widely carried out in the treatment of disease. Drug combinations often produce promising effects in the treatment of diseases (Aslam et al., 2016). Research by Safithri et al. (2021), shows methanol extracts of *S. platensis*, golden sea cucumber, and their mixtures have antioxidant activity.

This study aimed to determine the effect of the combination of *Spirulina platensis* and *Stichopus variegatus* on increasing the expression of the Bcl2 gene in pyramidal hippocampus cells of dementia rats.

MATERIALS AND METHOD

Materials

The tools used in the study were oral needles, 1 mL injection syringes, a set of surgical tools and scales (Ohaus), glass tools (*pyrex*), stirring rods, binocular microscopes (Olympus), optilab (Miconos). The main ingredients used in this study were *Spirulina platensis* powder obtained from PT. Algaepark Indonesia Mandiri, Klaten, Jawa Tengah, Indonesia (Batch No. 11723265) and *Stichopus variegatus* powder (hydrolisat) obtained from CV Rigo Alam Sejahtera, Bogor, Indonesia (Batch No. ST012308). Primary antibody (Bcl2) and secondary antibody (*Biotinylated universal*).

Methods

Sample solution preparation

The sample solution was prepared by suspending spirulina powder and golden sea cucumber powder with a 1% CMC-Na solution. Oral administration of the sample at a dose of 200 mg/kg BW. The volume of the drug solution given to rats weighing 200 grams orally was 2.0 mL (Yuliani et al., 2021).

Test animal preparation

The test animals used were *male Sprague Dawley* rats aged about 2 months (150-200 grams). The use of animals in research has been approved for preclinical research by the Research Ethics Committee of Universitas Ahmad Dahlan, Yogyakarta, Indonesia (approval number 012209148). A total of 48 rats were acclimatized for seven days and divided into six groups.

1) Normal control (CMC-Na and NaCl 0.9%)

2) Pain control (CMC-Na and TMT 8 mg/kg BW)
3) Positive control (citicoline 200 mg/kg BW and TMT 8 mg/kg BW)
4) SG-3:1 (Combination of spirulina and golden sea cucumber 3:1 dose 200 mg/kg BW and TMT 8 mg/kg BW)
5) SG-1:1 (Combination of spirulina and golden sea cucumber 1:1 dose 200 mg/kg BW and TMT 8 mg/kg BW)
6) SG-1:3 (Combination of spirulina and golden sea cucumber 1:3 dose 200 mg/kg BW and TMT 8 mg/kg BW)

CMC-Na, citicoline and sample were administered orally for 28 days, while NaCl and TMT were injected intraperitoneally on day 8.

Hippocampus preparation

Rats are sacrificed by putting them in a container and then flowing with CO_2 gas. The rat was then dissected on its head. After that the brain is taken. Right hemispherium cerebri was introduced in a 10% formalin fixation solution in PBS for 6 days. The hippocampus is then carefully separated from the hemispherium cerebri, then inserted into pots of tissue again (Yuliani et al., 2021).

Paraffin block creation

Paraffin block making is carried out at the Pathology Laboratory, Gadjah Mada University, Yogyakarta. The hippocampus is put in gauze, dehydrated, and immersed in ethanol solutions of 70, 80, 90 and 100%. each for 60 minutes at room temperature. The next process is purification using xylol for 15 minutes. After the *clearing* process, the infiltration process with liquid paraffin is carried out 3 times, each for 60 minutes in an incubator at a temperature of 60^{o}C, then stored at room temperature so that paraffin blocks are formed (Yuliani et al., 2021).

Bcl-2 immunohistochemical procedure

Immunohistochemical staining was carried out at the Pathology Laboratory, Gadjah Mada University, Yogyakarta, using an indirect method. The formed paraffin block is cut horizontally using microtomes with a thickness of 3-4 µm placed on the glass of *the poly-L-lysin* object. Immunohistochemical review uses primary antibodies (Bcl-2) and secondary antibodies (*Biotinylated universal*). The staining results were observed using a binocular microscope connected to a digital camera with a magnification of 400x. Observations were made on hippocampus pyramidal cells in the CA1 and CA2-CA3 areas of 2 tissue slices per hippocampus. In this staining, Bcl2 protein expression is marked with brown color in the cytoplasm and nucleus, while cells that do not express Bcl-2 protein will appear blue in pyramidal hippocampus cells. The Bcl2 cell intensity criterion is based on the average number of cells from 4 CA2-CA3 fields of view (400x magnification) (Yuliani et al., 2021).

Data Analysis

The normality test is performed using the Saphiro-Wilk test and the homogeneity test is performed with the Levene test. Statistical analysis using *one way* ANOVA and followed by Tukey HSD *posthoc* test to see differences between groups.

RESULT AND DISCUSSION

Dementia is a syndrome that can be caused by a number of progressive diseases and affects memory, thinking, behavior and the ability to carry out daily activities (Prince et al., 2014) In this study using trimethyltin (TMT) as a model of dementia in rats. TMT is a toxic organotin compound that selectively induces acute neuron death in the *dentate gyrus* of the hippocampus followed by impaired cognition (Melliou & Chinou, 2014). A collection of evidence suggests that TMT toxicity induces neurodegeneration of the hippocampus and results in cognitive impairment, mental confusion, memory defects, and seizures. Thus, TMT is a useful tool to prevent the most common neurodegenerative disorders such as dementia (Mitrović et al., 2021; Lee et al., 2016; Pompili et al., 2020; Dragić et al., 2021).

Based on the results of immunohistochemical painting, it shows that qualitatively antibodies specific to Bcl2 are selectively painted brown in the cytoplasm and cell nucleus in the CA2-CA3 region and predominantly painted in the cytoplasm only in the CA1 area (Figures 1 and 2). Previous research has said that CA3 and CA2 regions contain relatively higher concentrations of bcl-2 than CA1. Area CA3 holds a strategic position in the hippocampus because it receives sensory information through the main collateral pathway of Schafer (Aboutaleb et al., 2015). In addition, quantitatively (Tables 1 and 2) showed that compared to the normal control group TMT injection can decrease the amount of Bcl-2 gene expression in hippocampus pyramidal cells in the CA1 region and significantly in the CA2-CA3 region. According to Widiyanti et al. (2014), in normal conditions, Bcl2 cells are found in the outer mitochondrial membrane, endoplasmic reticulum and nuclear membrane to determine the response of a cell to the apoptotic stimulus through the intrinsic pathway. In addition, the TMT group rats qualitatively showed less Bcl2 expression compared to the citicoline and extract groups. TMT causes selective neuron death in Cornu Ammonis (CA) pyramidal cells in the hippocampus (Yuliani et al., 2021). So, it can be said that TMT can reduce the amount of expression of the Bcl2 gene in the pyramidal cells of the hippocampus.

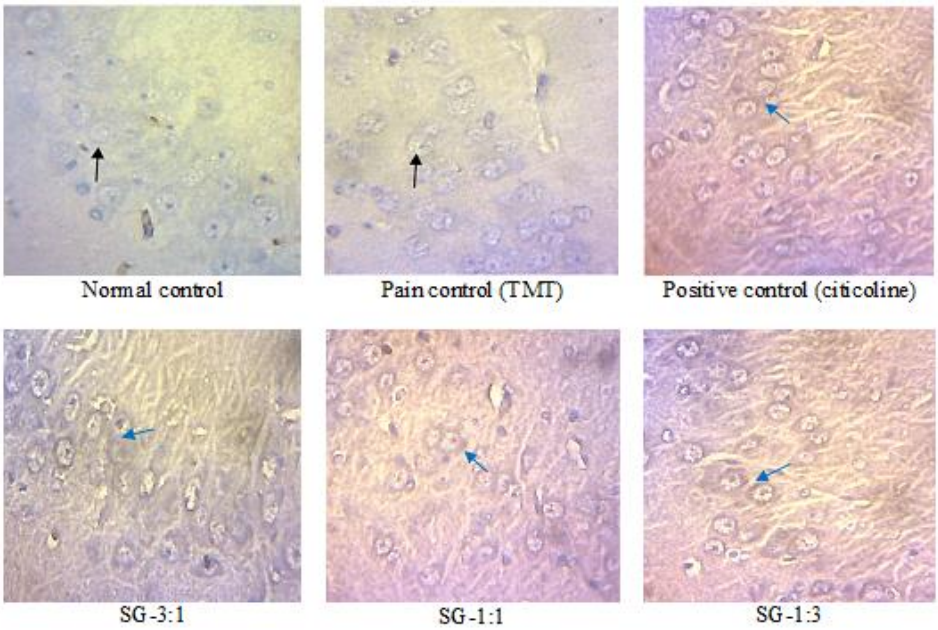

Figure 1. Microscopic image of Bcl2 immunohistochemical painting on hippocampus pyramidal cells in the CA1 region of a mouse model of TMT-induced dementia. Normal cells are expressed in blue (➚), the expression of Bcl2 protein in the cytoplasm is presented in brown (➚). 400x magnification

Table 1. Calculation of the number of cells expressing Bcl2 in the CA1 region

Group	Mean ± SEM
Normal control	12.67 ± 0.85
Pain control (TMT)	10.07 ± 0.47
Positive control (citicoline)	33.32 ± 1.73*
SG-3:1	25.76 ± 1.47*#
SG-1:1	26.24 ± 0.58*
SG-1:3	27.51 ± 0.70*

Description: *significantly different from pain control ($p<0.05$), #significantly different from positive control ($p<0.05$)

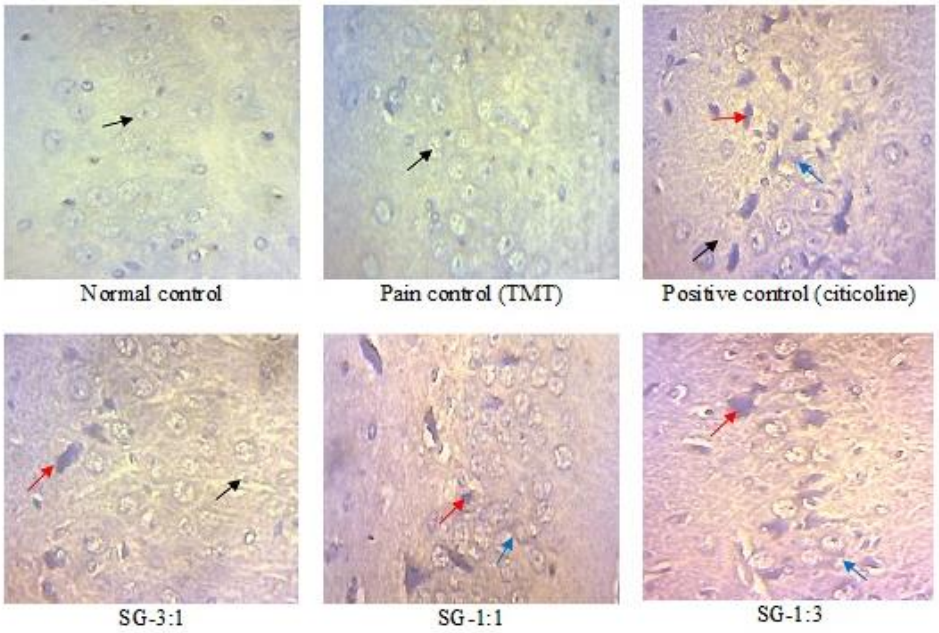

Figure 2. Microscopic image of Bcl2 immunohistochemical painting on hippocampus pyramidal cells in the CA2-CA3 region of a mouse model of TMT-induced dementia. The expression of Bcl2 protein in the cytoplasm (↗) and nucleus (↗) is presented in brown. Normal cells are expressed in blue (↗). 400x magnification

Table 2. Calculation of the number of cells expressing Bcl2 in the CA2-CA3 region

Group	Mean ± SEM
Normal control	23.94 ± 0.33
Pain control (TMT)	13.01 ± 0.53*
Positive control (citicoline)	71.57 ± 1.78#
SG-3:1	57.73 ± 1.44#†
SG-1:1	57.89 ± 1.69#†
SG-1:3	69.96 ± 1.97#

Description: *significantly different from normal control ($p<0.05$), #significantly different from pain control ($p<0.05$), †significantly different from positive control ($p<0.05$)

Intraperitoneal TMT injection increased the rate of ROS production in rats in sensitive areas of the hippocampus and increased the rate of ROS-induced oxidative damage, which contributes to activating the apoptosis signaling pathway (Kang et al., 2016). ROS causes mitochondrial dysfunction by inhibiting the synthesis of *adenosine triphosphate* (ATP). The decrease in ATP causes cytochrome-c which interacts with *apoptic protease-activating factor-1* (Apaf-1) and caspase-9 to form apoptosome. Apoptosome act as activators of caspases 3, 6 and 7 causing apoptosis. Bcl-2 can inhibit the release of cytochrome-c from mitochondria and activation of caspase thereby preventing apoptosis. Caspase 3 is a proapoptotic protein that acts as an executor in the apoptotic cascade (Zhao et al., 2014).

TMT-injected rats differed significantly with citicoline and extract. So, it can be said that the combination of spirulina and golden sea cucumber in this study increased the expression of Bcl2 in pyramidal hippocampus cells. Citicoline is not significantly different from SG-1:3 in both CA1 and CA2-CA3 areas, so it can be said that SG-1:3 has a greater effect in increasing Bcl2 expression than SG-3:1 and SG-1:1. The larger content of golden sea cucumber at SG-1:3 shows a better effect than SG-3:1 and SG-1:1. This may be due to golden sea cucumber samples being used in the form of peptide hydrolysates which can show better antioxidant potential than spirulina powder. In addition, according to research (Windari et al., 2019) golden sea cucumber and the combination of spirulina and golden sea cucumber (81 and 284 mg/kg BW) have antioxidant activity with smaller MDA levels and large SOD and catalase activity compared to spirulina alone. The combination of spirulina and golden

sea cucumber with these three comparisons was chosen because in several previous studies it has been known that both spirulina and golden sea cucumber at doses of 200 mg/kg BW showed good activity in preventing dementia (Ghanbari et al., 2019; Li et al., 2019) In addition, previous studies also showed a combination of spirulina and golden sea cucumber which has antioxidant activity and decreased MDA levels which are important in improving memory function (Windari et al., 2019; Safithri et al., 2021). So that a combination of spirulina and golden sea cucumber was carried out at a dose of 200 mg/kg BW in three concentration ratios. Based on this explanation, it can be said that the combination of spirulina and golden sea cucumber in this study increased Bcl2 expression in pyramidal hippocampus cells.

Spirulina's ability to increase Bcl2 expression according to Jadaun et al. (2018), because spirulina can play a role in the prevention of increased oxidative stress during apoptosis, increased mitochondrial membrane potential as well as increased Bcl2 expression. Several scientific publications have explained its positive effects on various pathologies, one of which is neuroprotective (Trotta et al., 2022). On the certificate of spirulina analysis indicates the content of karatenoids and vitamin E (tocopherol) as described by El-Shall et al. (2023), Spirulina has a large amount of natural antioxidants including polyphenols, carotenoids, and phycocyanin. Spirulina also has a wide variety of antioxidants such as superoxide dismutase (SOD), provitamin-A, vitamins C and E (Wang et al., 2013). Carotenoids act as antioxidants to block triggers of apoptosis and ROS-related mitochondrial dysfunction (Park et al., 2020). In addition, it is known that the content of *Phycocyanin* which is a blue-green pigment in *Spirulina platensis* can increase antioxidant enzyme activity and also suppress the expression of caspase-9 and caspase-3 by providing significant protection from mitochondrial membrane permeability and increasing ATP production and restore Bax/Bcl2 balance and also weaken the release of caspase-3 and caspase-9 (Li et al., 2020).

As for golden sea cucumbers, although there is no supporting data related to the analysis of chemical content, but the material used in this study is peptide hydrolysate obtained from golden sea cucumber and in previous studies it has been explained that protein hydrolysate and peptides obtained from sea cucumbers show antioxidant potential. Sea cucumbers contain various bioactive compounds, namely phenolics, polysaccharides, proteins (collagen and peptides), carotenoids, and saponins, which are abundant in these marine invertebrates and exhibit antioxidant activity (Hossain et al., 2022). According to research by Windari et al. (2019) stated that golden sea cucumber and the combination of spirulina and golden sea cucumber (81 and 284 mg/kg BW) have antioxidant activity with smaller MDA levels compared to spirulina. Research Su et al., (2018) states that sea cucumbers can inhibit caspases-9 and -3 so as to prevent apoptosis. Inhibition of caspases occurs due to the presence of Bcl2 as an antiapoptotic. The peptide effect of sea cucumber can improve memory function (Xu et al., 2020).

Citicoline as a comparison that has a value almost equal to SG-1: 3 can induce a significant reduction of cells undergoing apoptosis, citicoline weakens cell death caused by oxidative stress. Previous studies reported that citicoline may prevent apoptosis through decreased caspase 3 expression in CA2-CA3 regions (González-Pacheco et al., 2014). Research Sugianto et al. (2013), states citicoline can reduce inflammation and apoptosis by decreasing the expression of procaspase -1, -2, -3, -6, -8 and caspase-3 and increasing the excretion of Bcl2 hippocampus Nashine & Kenney (2020). The mechanism of citicoline in repairing neuronal membranes is through increasing phosphatidylcholine synthesis, then repairing damaged cholinergic neuronal membranes through potentiation of acetylcholine production and reducing free fatty acid production at the site of nerve damage (Pathan, 2012), so as to prevent dementia in test animal models.

CONCLUSION

The combination of spirulina (*Spirulina platensis*) and golden sea cucumber (*Stichopus variegatus*) can increase the expression of the Bcl2 gene in trimethyltin-induced dementia rats. The combination of spirulina and golden sea cucumber (SG-1:3) dose of 200 mg/kg BW was able to increase hippocampus Bcl2 expression with the number of Bcl2 cell expression almost the same as citicoline in both CA1 and CA2-CA3 regions, so that it has the potential to prevent dementia.

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Enzymatic virgin coconut oil effect on urea and creatinine levels of hypercholesterolemia-diabetics induced Wistar male rats

Niluh Puspita Dewi[1], Syafika Alaydrus[1*], Nadila[1], Magfirah [2], Joni Tandi[1], Viani Anggi[1], Nani Astria Polontalo[3]

[1]Departments of Pharmacology and Clinical Pharmacy, STIFA Pelita Mas Palu, Jl. Wolter Monginsidi No.106A, Palu, Central Sulawesi, 94111, Indonesia
[2]Departments Pharmaceutics and Pharmaceutical Technology, STIFA Pelita Mas Palu, Jl. Wolter Monginsidi No.106A, Palu, Central Sulawesi, 94111, Indonesia
[3]Department of pharmaceutical biology, Akademi Farmasi Bina Farmasi, Palu, Jl. Wolter Monginsidi No.106A, Palu, Central Sulawesi, 94111, Indonesia

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ABSTRACT

Coconut (*Cocos nucifera* L.) is an Indonesian commodity that has high economic value tall. Virgin Coconut Oil (VCO) is one of the processed coconut products whose selling value is very high, because the composition of VCO consists of medium-chain fatty acids that can maintain a healthy body and prevent various diseases. The process of making VCO used in this research is an enzymatic method using pineapple weevil as a bromelain enzyme. This study aims to evaluate the impact of different doses of enzymatic VCO in reducing urea and creatinine levels in hypercholesterolemic-diabetic male white rats (*Rattus norvegicus*). This study was an experimental laboratory with a modified pretest and posttest randomized controlled group design using 30 test animals which were divided into 6 groups. Each group consisted of 5 test animals, namely normal control, negative control, positive control receiving branded VCO, and test group given 0.2, 0.4, and 0.8 mL/kg BW of enzymatic VCO. The animals were administered enzymatic VCO orally every day for 14 days. Urea and creatinine levels were measured in blood samples taken from the tail at each observation time point, which included day 0, 21, 28, and 35. The results showed that enzymatic VCO at a dose of 0.8 mL/kg BW significantly reduced urea and creatinine blood levels with an average decrease of 17.40 mg/dL and 0.36 mg/dL. Based on the findings, VCO produced through enzymatic methods shows potential for controlling blood urea and creatinine levels in hypercholesterolemic-diabetic conditions. Further research regarding its formulation into a convenient and practical product would be highly advantageous for its use as a supplement.

Keywords: *VCO enzymatically, Streptozotocin, Urea, Creatinine*

Corresponding author:
Syafika Alaydrus
Departments of Pharmacology and Clinical Pharmacy, STIFA Pelita Mas Palu,
Jl. Wolter Monginsidi No.106A, Palu, Central Sulawesi, 94111, Indonesia
Email: syafikaalaydrus39@gmail.com

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by high blood sugar (glucose) levels (hyperglycemia) exceeding normal levels, diabetes mellitus is caused by a lack of insulin in the body (Elmuzghi, 2023; Pai et al., 2023; Pathak et al., 2023). Lack of insulin makes the body unable to use glucose as energy. Hyperglycemia is an early sign of diabetes mellitustable caused by impaired insulin secretion , hyperglycemia is known to increase the formation of free radicals and Reactive Oxygen Species (ROS) which cause lipids peroxidation and cell membrane damage then can cause long-term complications such as kidney damage (Asmat et al., 2016).

Kidneys are the main organs for the excretion of metabolic waste products that are no longer used by the body and control the volume of fluid composition in the body. The smallest functional unit of the kidney is the nephron. The nephron consists of the glomerulus, proximal convoluted tubule, loop of Hanle, distal convoluted tubule, and collagenous duct. Overall kidney function is based on nephron function and impaired kidney function due to decreased nephron work. Laboratory examination method which is used to evaluate the function of the kidneys is to measure the body's metabolic waste substances excreted through the kidneys in the form of urea and creatinine (Pandya, 2016).

Creatinine is the final endogenous product of creatine phosphate metabolism where the levels are relatively more constant and creatinine is also a product of muscle metabolism which is secreted through serum by the body every day, while urea is the main product of protein metabolism in the body. Serum urea levels depend on the catabolism (breakdown) of proteins and amino acids in the liver which are secreted into the kidneys and then excreted through the urine. The body will form antioxidant compounds to control urea and creatinine levels through the release of electrons so that metabolism can take place properly (Sulistyani & Nurkhasanah, 2017).

Diabetes mellitus has a significant scientific correlation with altered urea and creatinine levels, primarily attributed to its proclivity for causing diabetic nephropathy, a progressive kidney disease. This condition, often associated with poorly managed diabetes, leads to a diminished glomerular filtration rate, subsequently impeding the efficient clearance of urea and creatinine from the bloodstream. Consequently, elevated levels of urea and creatinine, termed azotemia and hypercreatininemia, serve as critical clinical markers for renal impairment in diabetics, necessitating vigilant monitoring and diligent glycemic control to mitigate the risk of diabetic kidney disease (Vallon & Komers, 2011). In the other hand, hypercholesterolemia increases the risk of renal failure by promoting conditions such as atherosclerosis, which reduces blood flow to the kidneys (Kon et al., 2011). This compromised blood supply, along with associated factors like inflammation and oxidative stress, impairs renal function and contributes to the progression of chronic kidney disease (CKD).

Virgin Coconut Oil (VCO) is one of the food sources of fat that is in great demand by the wider community because of its potential role in addressing diabetes and hypercholesterolemia, showcasing promising therapeutic effects. The medium-chain fatty acids present in VCO are believed to contribute to improved insulin sensitivity, aiding in the management of diabetes (Narayanankutty et al., 2016). Additionally, VCO has demonstrated the ability to enhance lipid metabolism, leading to a reduction in hypercholesterolemia (Chinwong et al., 2017). Previous studies suggest that the unique composition of VCO, including lauric acid, may positively influence lipid profiles by increasing high-density lipoprotein (HDL) cholesterol while reducing low-density lipoprotein (LDL) cholesterol levels (Marcus, 2013; Savva & Kafatos, 2016). Furthermore, the antioxidant properties of VCO are thought to mitigate oxidative stress, a factor implicated in the progression of diabetes and associated complications (Garkuwa et al., 2023; Iranloye et al., 2013; Narayanankutty et al., 2016).

The role of bromelain in the production of Virgin Coconut Oil (VCO) is pivotal and closely associated with the enzymatic method of VCO production (Rahmalia & Kusumayanti, 2021). Bromelain is a protease enzyme naturally occurring in pineapple plants, and it plays a key role in catalyzing the hydrolysis of peptide bonds within proteins (Agrawal et al., 2022). The enzyme is primarily found in the extract of the pineapple's pulp or stem. In the enzymatic VCO production process, specifically the fermentation method, pineapple stem extract containing bromelain is

introduced into coconut milk. During this enzymatic process, bromelain's proteolytic activity becomes instrumental. It acts upon the protein layer within the coconut milk emulsion, breaking down these proteins. As a result, the oil and water components are effectively separated, allowing for the extraction of pure VCO. The use of bromelain in this process enhances the efficiency of VCO production by aiding in the complete separation of oil from water, ensuring a higher-quality end product (Harimurti et al., 2022).

Previous research on VCO that compared with olive oil and red fruit oil at a dose of 0.2 mL/KgBW, VCO is more effective in reducing blood glucose levels in mice. The antidiabetic effect of virgin coconut at a dose of 0.8 mL/KgBW can reduce blood glucose levels (Sulistyani & Nurkhasanah, 2017). Nonetheless, research investigating the effects of enzymatic Virgin Coconut Oil (VCO) on the regulation of urea and creatinine levels in the context of hypercholesterolemia-diabetics complications has not been previously conducted. Hence, this study was conducted to assess the impact of enzymatic VCO at various dosages on blood urea and creatinine levels in hypercholesterolemia-diabetic-induced Wistar male rats.

MATERIALS AND METHOD

Materials

Preparation of test materials

Coconut (*Cocos nucifera* L.) and young pineapple (*Ananas comosus*) used in this study were obtained from the Dolo area in the Central Sulawesi region. 15 coconuts (*Cocos nucifera* L.) were taken and 5 pineapples (*Ananas comosus*) were then cleaned of skin and other impurities and the required parts were taken. The material is washed with running water and then drained so that it is free from the rest of the washing water and then weighed.

Methods

Cream preparation

Mature coconuts, once peeled, their flesh were extracted, grated using a grating machine, and then combined with water in a 1:1 ratio. This mixture was then kneaded and squeezed until all the coconut milk was extracted. The resulting coconut milk was placed in a jar and sealed tightly for 2 hours, allowing two distinct layers to form. The upper layer is referred to as cream, while the lower layer is known as skim or coconut milk. For the production of VCO, 2000 mL of coconut cream was extracted.

Preparation pineapple juice

The pineapple fruit was cut into small pieces and mashed using a blender then filtered with filter paper to obtain the juice. The pineapple weevil juice was then taken as much as 500 mL.

Preparation of VCO by enzymatic method

The 2000 mL of coconut cream was put into a jar and add 500 mL of pineapple weevil juice. The mixture was stirred well and covered with aluminum foil and then labeled. The mixture was standed for 22 hours until three layers are formed, namely oil, blonde and water. The oil was separated by centrifugation at 3000 rpm for 10 minutes. The yield of VCO obtained is calculated (Palilingan, & Pungus, 2018).

$$\% \text{ Yield} = \text{Oil volume} / \text{Cream volume} \times 100\ \%$$

Bromelain enzyme identification test

The Bromelain Enzyme Identification Test was conducted by first preparing 2 mL of pineapple weevil juice. Subsequently, 5 mL of 10% NaOH was added, and the mixture was heated for 5 minutes. Following this, 2 drops of 5% Pb-acetate solution were introduced. The heating process was sustained until a noticeable change in color took place within the solution. The outcome of this test yielded a

positive result, characterized by the development of a brownish solution and the formation of a black precipitate within the solution.

Fat feed manufacturing

The high-fat diet utilized consisted of a mixture of pig oil (50%) and quail egg yolk (50%). The diet was prepared as follows: The pig oil was heated until it transformed into a liquid state. Meanwhile, the quail eggs were separated into yolks and whites, and the yolks were then combined with the liquified pig oil. The mixture was thoroughly stirred until it achieved a uniform consistency. Each rat had a maximum daily food intake of 20 grams, and a fresh batch of the diet was prepared and administered orally using a probe every day for a period of 14 days (Anggraeni et al., 2021).

Preparation of streptozotocin (STZ) solution

Streptozotocin powder at a dose of 40 mg/kg BW was weighed as much as 0.32 grams and then dissolved using citrate-buffer saline with a pH of 4.5 and then injected into rats intraperitoneally (ip).

In vivo test

All experiments were conducted in strict accordance with the animal welfare guidelines established by the World Organisation for Animal Health (OIE) and were granted approval by the Research Ethics Committee at the Faculty of Medicine, Tadulako University, with approval number 2576/UN.28.1.30./K/2019. Male Wistar rats, weighing between 200 and 250 grams, were introduced to designated local animal cages. These rats underwent a 14-day acclimatization period. The criteria for rat selection included an age of approximately three months, a body weight falling within the 200-250 g range, white fur, male gender, and the demonstration of active behavior.

The experimental subjects were divided into six groups: normal control (group I), negative control (group II), positive control receiving branded VCO (group III), and test groups receiving enzymatic VCO at doses of 0.2 mL/kg BW (group IV), 0.4 mL/kg BW (group V), and 0.8 mL/kg BW (group VI).

Cholesterol levels in each group (group II-VI) were measured on the 14th day after the high-fat diet was administered during the two weeks of acclimatization period (Salim et al., 2018; Nurmasitoh, 2015). The rats with cholesterol levels exceeding 200 mg/dL were subsequently induced with STZ. To induce the blood glucose, 1 mL of STZ solution was injected peritoneally just after the acclimatization period ended. Three days after STZ induction, the blood glucose was measured. According to the literature, Wistar rats are classified as diabetic if their blood sugar levels exceed 135 mg/dL (Hidayaturrahmah et al., 2020), and they are categorized as hypercholesterolemic if their cholesterol levels exceed 200 mg/dL (Supriatna et al., 2018). Rats meeting these two criteria were subsequently selected for the treatment. The treatment was administered daily for 14 days (type of treatment given was based on the group). The urea and creatinine levels were measured in blood samples taken from the tail, starting from the initiation of the treatment and continued until day 35, which included day 0, 21, 28, and 35.

Data Analysis

The data are presented as mean ± standard deviation (SD). All data underwent a normality test and homogeneity test. In cases where the data exhibited a normal and homogeneous distribution, data analysis was performed using a One-Way ANOVA. However, if the data did not follow a normal distribution and were not homogenous, non-parametric statistics were employed, specifically the Kruskal-Wallis test, followed by the Mann-Whitney test to assess the differences between treatments, with the assistance of statistical analysis software, SPSS (SPSS for Windows, Version 16.0. Chicago, SPSS Inc.).

RESULT AND DISCUSSION

Bromelain identification

Bromelain is a proteolytic enzyme found in pineapples, particularly in the stem and flesh of the fruit. It plays a significant role in the production of Virgin Coconut Oil (VCO). In the context of VCO production, bromelain is used as a protein-cleaving agent, aiding in the separation of protein components from the extracted coconut oil (Harimurti et al., 2020, 2022). This is a crucial step in the VCO extraction process because the resulting coconut oil needs to be protein-free to ensure its high quality. Bromelain helps ensure that the extra virgin coconut oil is free from protein contamination, resulting in a clearer and higher-quality coconut oil (Hamzah et al., 2021; Hamdan et al., 2022; Ng et al., 2021; Natalia et al., 2019). In the VCO production process, we conducted a qualitative study to identify the presence of bromelain in both pineapple juice and the VCO obtained through the enzymatic approach (Table 1). The presence of bromelain in both components suggests that bromelain may play a role in expediting the production of VCO.

Table 1. Qualitative identification of bromelain enzyme in pineapple juice

Test	Observation	Results
Pineapple hump juice test	Black precipitate brown color	(+)
Enzymatic VCO test	Brownish color with a little black precipitate	(+)

Description: (+) means the presence of bromelain

Enzymatic VCO effect on serum urea level

All test animals underwent a baseline check for urea and creatinine levels before being subjected to a high-fat diet and streptozotocin induction. With the exception of the normal control group, the five rat groups were fed a high-fat diet for 14 days. The high-fat diet aimed to elevate the free fatty acid content in plasma cells, subsequently leading to a decrease in insulin sensitivity. After 14 days, the average cholesterol level in male white rats exceeded 200 mg/dL. Subsequently, the male white rats developed hypercholesterolemia and were intraperitoneally injected with streptozotocin at a dose of 40 mg/kg BW. Streptozotocin exerts a cytotoxic effect, potentially damaging pancreatic beta cells. It specifically causes DNA damage in pancreatic beta cells through the formation of NO, hydroxyl radicals, and hydrogen peroxide, all of which are potent free radicals that quickly harm cell tissues.

On day 0, which indicates that the urea levels across all groups were within the normal range. This is because on day 0, the test animals had not received any induction aside from standard feeding. This aligns with the literature, which indicates that the normal urea levels in rats fall within the range of 15-21.8 mg/dL (Tandi et al, 2022). The results of the Kruskal-Wallis statistical test on day 0 demonstrated that all treatment groups did not exhibit significant differences, with a p-value of 0.937 (p value > 0.05). This implies that the urea levels at the study's outset were consistent with normal levels.

Urea level measurements on day 21 following the induction of a high-fat diet and streptozotocin showed an increase in urea levels in male white rats. This elevation in urea levels was a consequence of the high-fat diet and streptozotocin induction. The results of the Kruskal-Wallis statistical test indicated significant differences among all treatment groups on day 21, with a p-value of 0.022 ($p < 0.05$), signifying that the high-cholesterol diet and streptozotocin induction had a noticeable effect. Subsequently, Mann-Whitney tests were conducted to discern the differences between all treatment groups. The Mann-Whitney test results revealed significant distinctions between the negative control, positive control, and various enzymatic VCO dosage groups (0.2 mL/kg BW, 0.4 mL/kg BW, and 0.8 mL/kg BW), in comparison to the normal control. This suggests that the animals in the treatment groups exhibited an increase in urea levels due to the induction of a high-fat diet and streptozotocin. In this observation point, animal group with 0.8 mL/kg BW enzymatic VCO has the lowest average serum urea level among the treated groups but the value was not significant (Table 2).

Table 2. Average serum urea levels of male white rats in each observation point during treatment

Day	Normal Control	Negative control	Positive control	Enzymatic VCO 0,2 mL/kg BW	Enzymatic VCO 0,4 mL/kg BW	Enzymatic VCO 0,8 mL/kg BW
0	16.60±2.07	17.20±1.92	17.60±2.88	17.80±1.30	18±2.24	17.40±2.61
21	18.40±1.14[a]	43.40±10.78	45.80±15.07	46.20±10.45	49.20±6.02 [a]	42.50±7.01
28	17.60±1.14[a]	57.40±14.93	31.80±10.96[a]	33.40±5.68 [a]	33.80±3.90 [a]	32.40±5.22 [a]
35	18 ± 1.22[a]	61.40±7.16	16.20±2.68[a]	21.6±0.55 [a]	21±1.22 [a]	17.40±0.89[a]

Note: ([a], $p<0.05$) shows a significant differences to negative control

Measurement of urea levels on the 28th day after administration of enzymatic Virgin Coconut Oil (VCO) for 14 days, the urea levels of male white rats showed a slight decrease. This is because the treatment group has been induced with Enzymatic Virgin Coconut Oil (VCO). The results of the Kruskal Wallis statistical test were significantly different with $p<0.05$, which indicated that there were significant differences in all treatment groups on day 28, so it was continued with the Mann Whitney test to see the differences between all treatment groups. Mann Whitney further test results showed that the doses of Enzymatic Virgin Coconut Oil (VCO) 0.2 mL/kg BW, 0.4 mL/kg BW, and 0.8 mL/kg BW were not significantly different from the positive control but significantly different from negative control. This indicates that these three doses had an impact on reducing urea levels but didn't fully restore urea levels to those observed in the normal control. This suggests that the active components within Virgin Coconut Oil (VCO) were not completely absorbed enzymatically, thus not achieving the maximum effect (Puspita et al., 2023).

The reduction in blood urea levels by Virgin Coconut Oil (VCO) can be attributed to several potential mechanisms related to the active components present in pure coconut oil. VCO, containing medium-chain triglycerides (MCTs), may enhance fat metabolism, thereby reducing the accumulation of fats and triglycerides in the body, alleviating kidney stress and subsequently lowering urea levels (Wang et al., 2018; Zicker et al., 2019). Additionally, VCO exhibits anti-inflammatory and antioxidant properties, which can help mitigate kidney inflammation, maintain renal health, and ultimately reduce blood urea levels (Sinaga et al., 2019). Furthermore, VCO's potential to improve insulin sensitivity, notably through MCTs, may aid in regulating excessive urea production in the liver (Thomas et al., 2019). Moreover, certain VCO components, such as lauric acid, may exert direct effects on metabolic processes associated with urea reduction.

The measurement results on the 35th day revealed that within each group, the dose variations of enzymatic Virgin Coconut Oil (VCO) at 0.2 mL/kg BW, 0.4 mL/kg BW, and 0.8 mL/kg BW had an impact on reducing urea levels. Among these doses, the enzymatic Virgin Coconut Oil (VCO) dose of 0.8 mL/kg BW proved to be the most effective. This dose was chosen because it was the one closest to the normal and positive control values, yielding the most substantial reduction in urea levels.

Although not significantly different between treatment groups, the decrease in serum urea levels on day 35 follows a decreasing pattern with the increase in VCO dosage. This indicates that VCO treatment is dose-dependent. This is consistent with the results reported in previous studies where VCO therapy yielded dose-dependent results, both in reducing serum metabolic levels and enzymatic activity (de Moura e Dias et al., 2018; Rahim et al., 2017).

Enzymatic VCO effect on serum creatinine level

This study was also conducted to determine the initial creatinine levels before treatment. On day 0 the mean creatinine levels were obtained for the normal group, negative group, positive group, Enzymatic Virgin Coconut Oil (VCO) 0.2 mL/kg BW, 0.4 mL/kg BW, and 0.8 mL/kg BW are 0.42±0.12, 0.36±0.06, 0.41±0.05, 0.31±0.05, 0.34±0.06, and 0.32±0.08 mg/dL, respectively, which

indicates that the initial creatinine levels of male white rats are in the normal range. This is in accordance with the literature which states that the normal level of rat creatinine is 0.2-0.8 mg/dL (Huseyin et al, 2022). The results of the Kruskal Wallis statistical test on day 0 showed that all treatment groups were not significantly different with p value = 0.098 (p value> 0.05). This means that creatinine levels at the beginning of the study were homogeneous.

Table 3. Average serum creatinine levels of male white rats in each observation point during treatment

Days to-	Normal Control	Negative Control	Positive Control	Enzymatic VCO 0.2 mL/kg BW	Enzymatic VCO 0.4 mL/kg BW	Enzymatic VCO 0.8 mL/kg BW
0	0.42±0.12	0.36±0.06	0.41±0.05	0.31±0.05	0.34±0.06	0.32±0.08
21	0.41±0.05	1.29 ±0.18 [a]	1.36±0.15 [a]	1.31±0.10 [a]	1.40±0.04 [ab]	1.34±0.14 [a]
28	0.38±0.10	1.60±0.21[a]	1.02±0.04 [ab]	1.11±0.10 [ab]	1.07±0.08 [ab]	1.04±0.19 [ab]
35	0.37±0.07	1.65±0.20[b]	0.34±0.07[b]	0.45±0.10 [ab]	0.40±0.11[ab]	0.36±0.06[b]

Note: ([a], p<0.05) shows a significant differences to normal group. ([b], p<0.05) shows significant differences to negative control

Measurement of creatinine levels on the 21st day shows significant differences in all treatment groups and negative group to normal control (Table 3), which means that there was an effect on feeding high-fat and high-fat diets. Mann Whitney test results further showed that the normal control was significantly different from all treatment groups. This was because the test animals in the treatment group were had increased creatinine levels (Cullaro et al, 2018).

Measurement of creatinine levels on the 28th day shows significant differences in all treatment groups on day 28, so that it was continued with the Mann Whitney test to see the differences between all treatment groups. The results of the Mann Whitney test showed that normal control was significantly different from all treatment groups, and negative control was also significantly different from all treatment groups. Positive control was not significantly different with all test group, but significantly different from normal and negative controls. These findings suggest that the three dosage levels have influenced a reduction in creatinine levels but were unable to fully normalize creatinine levels due to inadequate suspension of enzymatic Virgin Coconut Oil (VCO) at that dose. This resulted in imperfect absorption of the active components, preventing the attainment of maximum effects (Lie et al, 2019).

The measurement results on the 35th day demonstrated that all test group produced effects in decreasing creatinine levels. Among these doses, the enzymatic Virgin Coconut Oil (VCO) at 0.8 mL/kg BW proved to be the most effective, as it led to a reduction in creatinine levels close to those observed in the normal control and positive control groups.

The decline in both urea and creatinine levels can be attributed to the presence of antioxidants in Virgin Coconut Oil (VCO) supplements, leading to an increase in glutathione peroxidase levels and a decrease in lipid peroxidation characterized by reduced MDA levels. Medium Chain Fatty Acids (MCFAs) like lauric acid, palmitic acid, and capric acid in Virgin Coconut Oil (VCO) can enhance insulin secretion by increasing intracellular calcium release within cell plasma membranes. Elevated insulin secretion, in turn, reduces the production of free radicals, thereby mitigating cell damage. Furthermore, the presence of vitamin E in Virgin Coconut Oil (VCO) serves to neutralize accumulated free radicals in diabetes, effectively inhibiting cell damage (Supriatna, 2018).

CONCLUSION

In summary, this study concludes that Virgin Coconut Oil (VCO) has the potential to reduce urea and creatinine levels in hypercholesterolemic-diabetic male white rats. Notably, the administration of

Virgin Coconut Oil (VCO) at a dosage of 0.8 mL/kg BW proved to be effective in reducing urea and creatinine levels, resulting in values of 17.40 mg/dL and 0.36 mg/dL, respectively.

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Antiparkinsonian effect of Nutmeg ethanolic extract (*Myristica fragrans* Houtt.) in haloperidol-induced Mice

Dwi Hadi Setya Palupi*, Isma Galuh Fekhayanti
Pharmacology-Clinical Pharmacy Department, Yayasan Pharmasi College of Pharmacy, Semarang
Jl. Letjen Sarwo Edi Wibowo, Km 1, Plamongansari, Semarang

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ABSTRACT

Parkinson's disease is a persistent neurological disorder that could potentially arise from the neuronal degeneration responsible for dopaminergic signals in the brain. Extrapyramidal syndrome, which is distinguished by motor dysfunctions including tremors, rigidity, and postural instability, is the defining feature of this disease. One of the contributing factors to the development of Parkinson's disease is drug-induced parkinsonism, which is precipitated by the administration of antipsychotic medications. The bioactive compounds myristicin, eugenol, and flavonoids found in nutmeg (*Myristica fragrans* Houtt.) have the potential to be utilized in the treatment of Parkinson's disease. The objective of this research endeavor was to ascertain the antiparkinsonian effect of ethanol extract of nutmeg on rodents with Parkinson's disease induced by haloperidol. A seven-day course of induction with haloperidol 1 mg/kg was administered intraperitoneally. Behavioral evaluations were conducted utilizing the cylinder and rotarod tests. Cylinder score and latency time were utilized to evaluate extrapyramidal symptoms. The therapeutic approach involved the oral administration of ethanol extract of nutmeg in varying concentrations (5, 10, and 20 mg/kg) over a period of seven days. The findings indicated that the administration of nutmeg at a rate of 20 mg/kg resulted in noteworthy enhancements ($P < 0.05$) in the motor function of animal models induced by haloperidol. Furthermore, this effect was comparable to that of the standard drug Pramipexole.

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Keywords: *Myristica fragrans*, antiparkinsonian, cylinder test, rotarod test

Corresponding author:
Dwi Hadi Setya Palupi
Pharmacology-Clinical Pharmacy Department
Yayasan Pharmasi College of Pharmacy, Semarang
Jl. Letjen Sarwo Edi Wibowo, Km 1, Plamongansari, Semarang
Email: dwi.palupi@ymail.com

INTRODUCTION

Parkinson's is a progressive neurodegenerative disease that manifests through both motor and non-motor symptoms (Hayes, 2019; Malaiwong et al., 2019). The prevalence of Parkinson's disease increases with age, affecting around 1–3% of sufferers over 60 globally (Ball et al., 2019). One of the causes of Parkinson's is the use of antipsychotic drugs (Drug-Induced Parkinsonism), which arise due to extrapyramidal side effects through dopamine receptor blockade (Grubor et al., 2020). Clinically, this condition is characterized by akinesia, tremors, bradykinesia, muscle rigidity, and postural instability that appear within days or weeks after the use of antipsychotic drugs (Erjavec et al., 2022).

Drug-induced Parkinson's (DIP) is the second-most basic etiology of parkinsonism in the elderly (Kabra et al., 2020) and is the most common type of Parkinsonism in the productive age group, with the youngest being 39 years old. DIP occurs in 11 of 15 cases of Parkinsonism (Jeong et al., 2021). Haloperidol is a typical antipsychotic that has stronger extrapyramidal effects than atypical antipsychotics. The increase in Parkinsonism due to extrapyramidal effects can trigger an increase in the prevalence of Parkinson's disease in the productive age group in Indonesia.

In psychiatric treatment, antimuscarinic antiparkinsonian medicines are commonly used to alleviate extrapyramidal motor symptoms caused by neuroleptic antipsychotic medications. However, when used in conjunction with antipsychotics, antimuscarinic antiparkinsonian agents have been reported to antagonize the therapeutic effects of neuroleptics; there have also been several reports of antimuscarinic antiparkinsonian agents actually causing various psychotic syndromes, elevated mood and stimulant effects, stereotypy, dyskinesia, and behavioral agitation. Extensive data has demonstrated that antimuscarinic antiparkinsonian medicines have the additional effect of acting as strong, indirect dopamine-agonists Vaiman et al., 2022. Antiparkinsonian alternatives are needed for safe antipsychotic adjunct therapy for patients with psychotic disorders. Many naturally derived medicines are now being researched for parkinsonism.

Indonesia has a biodiversity of plants that have the potential to be used as medicinal plants, one of which is nutmeg. Nutmeg (*Myristica fragrans* Houtt.) contains secondary metabolites of alkaloids, saponins, tannins, flavonoids, terpenoids, and essential oils (Noviyandri et al., 2021). Tannic acid (tannin) and myristicin (essential oil) are secondary metabolites that possess the potential to exhibit antiparkinsonian actions. Kawano et al. (2020) demonstrated the mechanism by which tannic acid functions as a dopamine agonist in mice with experimentally induced colitis. Prior experiments conducted on animals utilizing the Forced Swim Test (FST) demonstrated that the ethanol extract derived from nutmeg seeds effectively diminished anxiety levels. The observed impact is linked to the mechanism of inhibiting the Mono Amine Oxidase (MAO) enzyme, with the chemical believed to be responsible being Myristisin (Hasanusi et al., 2020). The ethanol extract of nutmeg seeds has been found to contain 11.17% of the myristicin chemical, as reported by Ghorbanian et al. in (2019). In addition, nutmeg has been scientifically demonstrated to elevate serotonin (5-HT), norepinephrine, and dopamine levels in the hippocampus (Plaingam et al., 2017) and myristicin (essential oil) through inhibition of the monoamine oxidase enzyme (Hasanusi et al., 2020). This research was to determine the antiparkinsonian effect of an ethanol extract of Myristica fragrans on haloperidol-induced mice using the rotarod and cylinder tests.

MATERIALS AND METHOD

Materials

Drugs and chemical

Haloperidol injection (Haldol®), pramipexol tablets (Sifrol®), and other chemicals used in this study were procured from Sigma-Aldrich.

Methods

Extract preparation

Nutmeg (*Myristica fragrans* Houtt.) was purchased from Ungaran, Semarang Regency, Central Java. A fine powder is obtained by grinding dried nutmeg. The powder was extracted using the maceration method, and the sample was soaked in 96% ethanol solvent for 3 x 24 hours at room temperature. The ethanol extract of nutmeg (EEN) is filtered using Whatman filter paper and then concentrated using a rotary evaporator at a temperature of 40°C.

Phytochemical screening

The prepared nutmeg ethanol extract was put to different qualitative tests according to the color assay procedure to evaluate the presence of phytochemical components such as saponins, flavonoids, alkaloids, tannins, and saponines.

Animals

The animals used were mice of the Balb/C strain obtained from the animal experimental laboratory of the Semarang Yayasan Pharmacy College of Pharmaceutical Sciences, which weighed 20–40 g. All animals were kept in a room heated to 25–30 degrees Celsius and kept on a 12/12 hour light-dark cycle. The animals were acclimatized for 7 days, had free access to water, and were not overfed in any way besides the regular fare. The procedures and methods in this research have been approved by the ethics committee of the Semarang Yayasan Pharmacy College of Pharmacy with number 375/YP-NA/KEPK/STIFAR/EC/ V/2022.

Experimental design

We picked six groups of mice at random. Group 1 was a normal group that got CMC-Na solution instead; Group 2 was a negative control group that got haloperidol intraperitoneally and CMC-Na solution (as a vehicle) orally; Group 3 was given intraperitoneal haloperidol and 0.5 mg/kg pramipexole solution orally as a positive control group; and finally, Groups 4, 5, and 6 were given haloperidol and ethanol extract of nutmeg, respectively 5, 10, and 20 mg/kg orally. Mice were induced by administering 1 mg/kg haloperidol intraperitoneally every day from day 1 to day 7. The induction dose and duration are determined according to the methodology described by Saeed (2017). During induction, extrapyramidal effects were observed 45 minutes after the injection of haloperidol. Nutmeg ethanol extract and pramipexole were intentionally given every day for a week from day 8 to day 15. The changes in motor condition were determined on day 15 with rotarod and cylinder scores.

Motor coordination test

The test animal is placed on a rotarod device with a maximum observation time of 300 seconds and a speed of 10 rpm for the rotarod test. Before beginning therapy, each mouse was trained to adjust to the rotarod equipment. For the three experiments, the average time (fall latency) was obtained.

Locomotor activity test

The cylinder test is carried out in an acrylic tube of a certain size. Observations were carried out for 3 minutes in a dark and quiet environment. The score in the cylinder test analysis is obtained from the ability of the test animal to lift its two front arms, touch the cylinder wall, and land, which is observed via video recording at a speed of 0.5x slower.

Data Analysis

Standardized mean values were reported with a margin of error. The data was analyzed using one-way ANOVA and LSD post hoc test.

RESULT AND DISCUSSION

Bioactive compounds from nutmeg were extracted by maceration in 96% v/v ethanol solvent and produced a yield of 29.98%. The results of phytochemical screening and TLC from ethanol extract of nutmeg (EEN) showed the presence of several bioactive contents such as flavonoids, triterpenoids and essential oils (Table 1).

Table 1. Phytochemical screening of ethanol extract of Nutmeg (*Myristica fragrans* Houtt.)

Phytochemical	Observation
Flavonoids	+
Tannin	-
Saponin	-
Triterpenoid	+
Steroid	-
Essential oil	+

(+) present; (-) absent

Phytochemical tests reveal that nutmeg extract includes flavonoid chemicals, as evidenced by the production of a red colour in the amyl alcohol layer. According to Harborne (1984), the Liebermann-Bucchard method is used to determine the terpenoid or steroid content of plants, with terpenoids appearing orange and steroids appearing blue or purple. The reaction of nutmeg extract with the Liebermann-Bucchard reaction yielded a purple colour, indicating the presence of triterpenoid chemicals. Thin-layer chromatography tests of nutmeg extract to detect essential oils revealed the presence of red stains on silica plates. These findings support the presence of essential oils in nutmeg extract. The result of phythochemichal screening of EEN allign with previous study by Sultan et al. (2023) which also verified the existence of essential oil, flavonoids, and triterpenoids in nutmeg. Geographic region and environmental factors play an imperative role in forming the phytochemical composition (Vignesh et al., 2024).

The rotarod and cylinder tests were used to measure the antiparkinsonian effect by watching how the test animal's motor skills changed. Impaired motor function is the main symptom of Parkinson's disease. The rotarod test is used to measure motor performance, including motor coordination, in animal models of Parkinson's disease (Leem et al., 2022). The assessment of latency time obtained from the lenght of time the animal model persist on the rotarod device in rotating condition until it falls down.

The results of observing the latency time (Figure 1) show that haloperidol induction causes a decrease ($p < 0.05$) in the motor coordination of mice. Muscle stiffness in the limbs is the most common sign in Parkinson-like syndrome, which makes it hard for the animal to move its body. Muscle weakness in the legs, tremors, and muscle stiffness are associated with neurodegeneration and basal ganglia dysfunction (Skinner et al., 2019; Saleem et al., 2021). The selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) as well as oxidative stress due to α-synuclein (Lewy body) aggregation and mitochondrial dysfunction (Harris et al., 2020; Saleem et al., 2021) cause neurodegeneration in Parkinson's disease. Mice with healthy motor conditions were able to survive for a long time beyond the testing time. On the other hand, mice with decreased motor conditions will fall faster than the specified time (Yulianita, 2019).

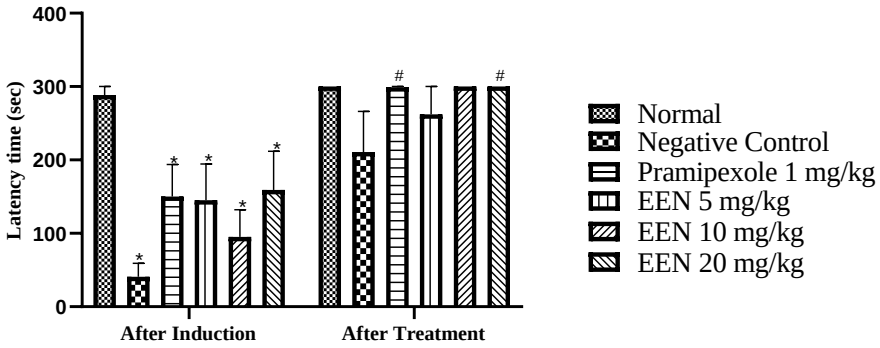

Figure 1. Latency time of haloperidol-induced parkinson's mice after administering ethanol extract of Nutmeg (*Myristica fragrans Houtt.*). *The values are mean ± SEM; n = 5; *p < 0.05 when compared with the normal group; # p < 0.05 when compared with the negative control group*

Haloperidol causes dopamine to be metabolized to 3,4-dihydrophenylacetic acid by the oxidation of the enzyme monoamine oxidase (MAO) and hydrogen peroxide. Increasing the amount of hydrogen peroxide causes oxidative stress and triggers extrapyramidal side effects (Saleem et al., 2021). Extrapyramidal symptoms include decreased motor conditions such as stationary tremor, bradykinesia, and postural instability (Hayes, 2019). Twenty to forty percent of people who take antipsychotics also have Parkinson's signs. These symptoms often start slowly, and most of the time, they start within a few days of starting antipsychotics. The intensity of symptoms can vary. They may get better on their own or get worse over time (D'Souza & Hooten, 2023).

In Figure 1, a slight increase in the latency time on negative control indicates the possibility of improvement, even if the statistics do not support it. After 7 days of therapy, it was revealed that only EEN 20 mg/kg resulted in a substantial increase ($p<0.05$) in latency time when compared to the negative control group.

Although Parkinson's disease is frequently associated with motor symptoms such as stiffness and poor balance, the early signs are often sensory, such as a loss of touch and scent. Sensory impairment can eventually lead to motor abnormalities via the sensorimotor integration process (Ketzef, 2017). The cylinder test is used to assess the antiparkinsonian effect through behavioral changes, specifically the tendency of the rodent's forelimbs to be used (Magno et al., 2019; Jiang et al., 2019) by placing the test animal in a glass cylinder and counting the number of times it uses both forelimbs and touches the cylinder walls. Sensorimotor disorders are linked to these behavioral abnormalities.

Pramipexole is a class of first-line drugs for Parkinson's disease therapy. This drug has mechanism as partial dopamine receptor agonist that acts on D2 receptors with preferential affinity for D3 receptors. As a partial agonist, pramipexole can unblock D2 receptors in the substantia nigra and striatum that have been blocked too much. This is why this drug is also used to treat extrapyramidal side effects (Weng et al., 2019).

The presence of spesific bioactive compound in nutmeg can offer assistance in managing Parkinson’s disease. Flavonoid, triterpenes and essential oil as neuprotective agents, protect the dopaminergic neurons by reducing oxidative stress and neuroinflammation generated by the disease (Devi et al., 2021), against dopaminergic cell death and ameliorating the behavioural impairement in Parkinson’s disease animal model (Spisni et al., 2023). This activity is attributed to the inhibition of monoamine oxidase (MAO) and the regulation of neurotransmitters, including dopamine, norepinephrine (NE), and serotonin (5-HT), inside the substantia nigra (Khazdair et al., 2020).

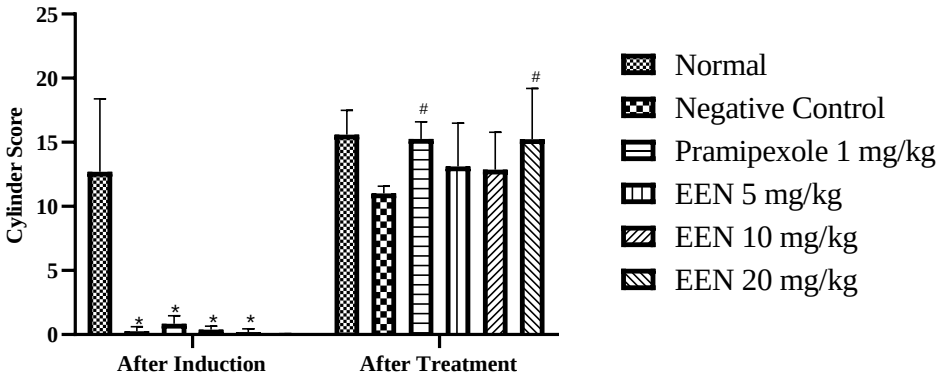

Figure 2. Cylinder score of haloperidol-induced parkinson's mice after administering ethanol extract of Nutmeg *(Myristica fragrans Houtt.)*. *The values are mean ± SEM; n = 5; *p < 0.05 when compared with the normal group; # p < 0.05 when compared with the negative control group*

In this current study, the antiparkinsonian effect of nutmeg was proven, and it is suspected that several of its bioactive compounds play a role in providing the effect through various mechanisms. Further research is needed to determine more specific bioactive compounds that provide antiparkinsonian effects in isolated form, accompanied by brain histopathology and measurements of dopamine neurotransmitter levels in brain samples from test animals.

CONCLUSION

The administration of a 20 mg/kg dose of ethanol extract derived from nutmeg (*Myristica fragrans* Houtt.) has been found to ameliorate the motor condition of mice with Parkinson's disease induced by haloperidol. This improvement is evidenced by an increase in both latency time and cylinder score.

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Antibiotic consumption and resistance: a 3-years ecological study for four critical groups of bacteria in a general regional hospital

Dwi Arymbhi Sanjaya[1], Nyoman Budiartha Siada[1], Rr. Asih Juanita[1], I Putu Yudistira Mahaputra[2], Made Gek Adisti Kamalia[2], Herleeyana Meriyani[1*]

[1]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Mahasaraswati Denpasar
Jl. Kamboja No.11 A, Dangin Puri Kangin, Denpasar, Bali 80236, Indonesia
[2]Program Studi Sarjana Farmasi, Faculty of Pharmacy, Universitas Mahasaraswati Denpasar,
Jl. Kamboja No.11 A, Dangin Puri Kangin, Denpasar, Bali 80236, Indonesia

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ABSTRACT

Pseudomonas aeruginosa, *Acinetobacter baumannii*, *Escherichia coli*, and *Klebsiella pneumoniae* are the most critical groups of multi-drug resistant (MDR) bacteria that cause a threat in hospitals. This study identified the trend of antibiotic consumption, antibiotic resistance pattern, and the relationship between antibiotic consumption and antibiotic resistance in a critical group of bacteria in a general regional hospital. This ecological study was based on retrospective data from inpatient databases in a general regional hospital over three years (2017-2019). The trend for annual antibiotic consumption over 2017-2019 was defined as defined daily doses/100 bed-days. The relationship between total antibiotic consumption and the percentage of antibiotic resistance among four isolated critical bacteria was explored in time series analysis and linear regression. The most frequently used antibiotic was ampicillin (220.33 DDD/100 bed-days), ciprofloxacin (126.86 DDD/100 bed-days), and ampicillin-sulbactam (126.34 DDD/100 bed-days). There was a significant relationship between antibiotic consumption (ampicillin, ampicillin-sulbactam, ceftazidime, gentamicin, amikacin, and ciprofloxacin) in DDD/100 bed-days and antibiotic resistance in *E. coli*, *K. pneumoniae*, and *P. aeruginosa* ($p<0.05$) but not statically significant in *A. baumannii* ($p=0.062$). The annual usage fluctuated or remained stable, with no statistically significant trends change. The relationship between antibiotic consumption and antibiotic resistance was significant in three out of four critical groups of bacteria.

Keywords: *Acinetobacter baumannii*, *antibiotic consumption*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*

***Corresponding author:**
Herleeyana Meriyani
Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Mahasaraswati Denpasar
Jl. Kamboja No.11 A, Dangin Puri Kangin, Denpasar, Bali 80236, Indonesia
Email: herleeyana.m@unmas.ac.id

INTRODUCTION

The increasing level of antibiotic resistance threatens the Sustainable Development Goals (SDGs). This phenomenon significantly influences economic, social, and healthcare changes (Gajdács et al., 2021; Laxminarayan et al., 2013). Antibiotic resistance is one of the main global health concerns that can lead to increased financial burden, length of stay, morbidity, and mortality. Misuse, overuse of antibiotics, and high levels of antibiotic consumption are considered to exert selective pressure, thereby accelerating antibiotic resistance and leading to the emergence of multi-drug resistant (MDR) bacteria (Amaha et al., 2020; Mascarello et al., 2017; Shafiq et al., 2016).

Pseudomonas aeruginosa, *Acinetobacter baumannii*, *Escherichia coli*, and *Klebsiella pneumoniae* can cause healthcare-associated infections and fatal nosocomial infections such as bloodstream infections, surgical site infections, and pneumonia (Kousovista et al., 2021; Veličković–Radovanović et al., 2015). They are the most critical groups of MDR bacteria that cause a threat in hospitals. Several studies showed that *A. baumannii* is significantly resistant to meropenem, cefepime, and ciprofloxacin correlated with meropenem, cefepime, and ciprofloxacin use (Kousovista et al., 2021). *P. aeruginosa* was resistance to ciprofloxacin, meropenem, and cefepime. Gentamycin, ciprofloxacin, and ceftriaxone resistance were found in *E. coli* isolates. *K. pneumoniae* isolates showed resistance to ceftazidime, amikacin, ceftriaxone, and ciprofloxacin. The increasing antibiotic resistance was correlated to antibiotic consumption (JoSeph et al., 2015; Veličković–Radovanović et al., 2015).

A previous study in Indonesia's general regional hospital analyzed the relationships between antibiotic consumption and antibiotic resistance to coagulase-negative staphylococci (Meriyani et al., 2021). In Indonesia, there are no studies on the relationship between antibiotic consumption and antibiotic resistance in a critical group of MDR bacteria. The sensitivity of antibiotics to bacteria differs by region, and the regional reported antibiotic resistance varies widely due to differences in environment and antibiotic consumption (Tao et al., 2017). The local antibiotic resistance pattern is essential to confirm the choice of antibiotics against critical groups of MDR bacteria (Luyt et al., 2014; Rezaie et al., 2016). Therefore, this study identified the relationship between antibiotic consumption and antibiotic resistance in a critical group of bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii, Escherichia coli,* and *Klebsiella pneumoniae*) in a general regional hospital. They are the most bacteria that cause infection in general hospital. Information about the trend of antibiotic consumption, antibiotic resistance pattern, and the relationship between antibiotic consumption and antibiotic resistance in a critical group of bacteria can be used to control antibiotic usage and antibiotic resistance. Knowledge of this relationship is needed to decrease the irrationality of antibiotic consumption.

METHOD

Methods

This was an ecological study based on retrospective data from inpatient databases in Indonesia's general regional hospital, over three years, from January 1st, 2017, until December 31st, 2019. This is a 588-beds tertiary care hospital as a regional referral medical centre. The bed occupancy rate (BOR) was 0.88 in 2017, 0.84 in 2018, and 0.85 in 2019. This study was approved by the general regional hospital's institutional review board (IRB) and the hospital research committee on June 2020 (070/5035/RSD/2020). The committee waived the informed consent requirement because this was a retrospective study without human subjects, and all the secondary data would be used.

Data Analysis

Antibiotic consumption

Antibiotic consumption was collected from electronic pharmacy records in a general regional hospital, from 2017 to 2019. The annual antibiotic consumption was defined as the number of defined daily doses/100 bed-days (DDD/100 bed-days) based on the Anatomical Therapeutic Chemical/Defined Daily Dose (ATC/DDD) classification. DDD/100 bed-days is calculated by

dividing the numbers of DDDs by patients-days and multiplying by 100 Only antibiotics for systemic treatment (oral and injectable) in ATC class J01 were used, and the other antibiotic agents as a topical were excluded in this study.

Microbial resistance data

Data on the susceptibility of four critical bacteria (*P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae*) to antibiotics were collected from the antibiogram. The antibiogram susceptibility results were based on the Clinical and Laboratory Standards Institute (CLSI) with the disk diffusion method. The database included all positive clinical specimens from sputum, blood, and urine. The percentage of antibiotic resistance was defined as the percentage of resistant bacterial isolates compared to the total number of isolates, including isolates from urine, blood, pus, and sputum culture. Results for susceptibility were susceptible to antibiotics (S), intermediately resistant (I), and resistant (R). In this study, intermediately resistant and resistant strains were considered resistant (Center for Disease Control and Management, 2019).

Statistical analysis

The trend for annual antibiotic consumption over 2017-2019 was explored by time series analysis using linear regression, which assesses the changes in trends (i.e., slopes) of the response (i.e., antibiotic consumption) during the study period. Correlation and linear regression analyses were used to analyze the relationship between total antibiotic consumption (ampicillin, ampicillin-sulbactam, ceftazidime, gentamicin, amikacin, and ciprofloxacin), described as DDD/100 bed-days as the independent variable, and the percentage of antibiotic resistance among four isolated critical bacteria as the dependent variable. All statistical tests were considered statistically significant at a p-value < 0.05. The Statistical Program for Social Science (SPSS) 26.0 (IBM Corporation, USA) was used for all statistical analyses (Barton & Peat, 2014; George & Mallery, 2019).

RESULT AND DISCUSSION

Trends in antibiotic consumption

The overall trend of annual antibiotic consumption over 2017-2019 is presented in Table 1. The most frequently used antibiotic in this study was ampicillin (220.33 DDD/100 bed-days) from total antibiotic consumption during 2017-2019, followed by ciprofloxacin (126.86 DDD/100 bed-days) and ampicillin-sulbactam (126.34 DDD/100 bed-days). However, ampicillin, ampicillin-sulbactam, and ceftazidime consumption decreased from 2017 to 2019. Time series analysis demonstrated that the annual usage fluctuated or remained stable, with no statistically significant changes in trends (p>0.05). During the study period, the trend of gentamicin indicated an increasing trend in DDD/100-bed days, although this was not statistically significant. Amikacin and ciprofloxacin are given a stable trend, although slopes showed an increasing trend.

Data on the consumption of antibiotics in this study show that the most frequently used antibiotic with an increasing trend was ciprofloxacin (126.86 DDD/100 bed-days and *slope* (b) = 0.220). Based on data from India, Bangladesh, Sri Lanka, Thailand, and Indonesia, the World Health Organization (WHO) Report on Surveillance of Antibiotic Consumption 2016 - 2018 indicated that South-East Asia had a high consumption of cephalosporins and quinolones (World Health Organization, 2018). A cross-sectional audit of antibiotic prescribing practices at hospitals in 53 countries, including Asia, found that penicillin with a β-lactamase inhibitor and fluoroquinolone (levofloxacin and ciprofloxacin) was the most commonly prescribed in the east and south Asia in 2015. A high level of fluoroquinolone consumption in hospitals is associated with the prevalence of pneumonia (Versporten et al., 2018). Based on surveillance data from the Ministry of Health, Republic of Indonesia, from 2017 until 2019, pneumonia was one of Indonesia's three most common diseases (Kementerian Kesehatan Republik Indonesia, 2020; Ministry of Health, 2018).

In addition, the high prevalence of antibiotic-resistant bacteria in Southeast Asia is associated with increased consumption of broad-spectrum antibiotics, such as ceftriaxone, ceftazidime, cefotaxime,

levofloxacin, ampicillin, gentamicin, and meropenem (Honda et al., 2017; Lee et al., 2013; Versporten et al., 2018). The potential nephrotoxicity caused by aminoglycosides is related to the trend of annual usage of gentamicin and amikacin (Lee et al., 2013). However, in this study, ampicillin, and ceftazidime consumption trend decreased from 2017 to 2019 (Table 1). Moreover, some studies suggest that trend of annual antibiotic consumption fluctuating is most likely related to multifactorial, such as physician's attitudes and knowledge about bacterial infection and antibiotic prescription, lack of well-established infectious disease and treatment, and the lack of infectious diseases experts to manage the program for antimicrobial stewardship (Honda et al., 2017; Kim et al., 2018).

Antibiotic resistance

During the study period, 266 isolates from the four critical bacteria were separated from the clinical sample. The pattern of antibiotic resistance in four critical bacteria is described in Table 2. The percentage of antibiotic resistance was highly resistant (more than 60%) are not recommended for therapy. *K. pneumonia is* only resistant to ampicillin and a combination of sulfamethoxazole and trimethoprim. *E. coli* showed resistance to ampicillin, ampicillin-sulbactam, cefotaxime, ceftriaxone, and ciprofloxacin. *P. aeruginosa* was resistant to ampicillin, ampicillin-sulbactam, piperacillin-tazobactam, aztreonam, and nitrofurantoin. The antibiotic resistance pattern in *A. baumannii* is similar to *P. aeruginosa*, except for piperacillin-tazobactam and nitrofurantoin. In addition, *A. baumannii* was resistant to ceftriaxone.

The relationship between antibiotic consumption (ampicillin, ampicillin-sulbactam, ceftazidime, gentamicin, amikacin, and ciprofloxacin) in DDD/100 bed-days and antibiotic resistance percentages from the four critical bacteria is summarized in Figure 1. There was a significant relationship between DDD and antibiotic resistance in *E. coli* (p-*value*=0.038; r= 0.837) (Figure 1A), *K. pneumoniae* (p-*value*=0.031; r= 0.851) (Figure 1C), and *P. aeruginosa* (p-*value*=0.039; r= 0.833) (Figure 1D). The coefficient correlation (r) between antibiotic consumption (DDD/100 bed-days) and antibiotic resistance percentages in *A. baumannii* has a high correlation (r=0.790) (Figure 1B) but is not statically significant.

Antibiotic consumption may increase selective pressure on certain classes of antibiotics. Although antibiotic consumption in hospitals is much lower than in the community, the intensity of use ensures a high rate of antibiotic resistance in the hospitals (Cižman & Srovin, 2018). Previous studies used aggregated population-level data to investigate the correlation between antibiotic consumption and antibiotic resistance in *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae* (JoSeph et al., 2015; Kousovista et al., 2021; Mladenovic-Antic et al., 2016; Sedláková et al., 2014). Several studies suggested that increased consumption of antibiotics has a negative correlation and is not statically significant with resistance rate (Kim et al., 2018; Mascarello et al., 2017). In our research, the relationship between antibiotic consumption (DDD/100 bed-days) and antibiotic resistance in *P. aeruginosa*, *E. coli*, and *K. pneumoniae* was statically significant but not in *A. baumannii* (Figure 1). This indicates that antibiotic resistance in hospitals varies commonly due to differences in environment and antibiotic consumption and is influenced by multi-factors, such as misuse and overuse of antibiotics, inappropriate prescribing, horizontal gene transfer, and resistance mechanisms that might differ between species (Tao et al., 2017; Ventola, 2015).

Interestingly, in this study, *P. aeruginosa*, *A. baumannii*, and *E. coli* were MDR species, except for *K. pneumoniae* (Table 2). MDR is defined as resistance to at least one antibiotic in three or more antibiotic classes (Center for Disease Control and Management, 2019). WHO reported *Acinetobacter*, *Pseudomonas*, and some Enterobacteriaceae (including *Klebsiella* and *E. coli*) as critical MDR bacteria resistant to various antibiotics, posing a threat in hospitals (World Health Organization, 2017). *P.* aeruginosa, *A. baumannii*, *E. coli*, and *K. pneumoniae* produce antibiotic-inactivating enzymes, such as β-lactamases. This enzyme can break the amide bond of the β-lactam ring, which results in the inactivation of β-lactam antibiotics. This is closely related to the contribution of the *amp*C gene encoding the β-lactamases enzyme (Pang et al., 2019; Sedláková et al., 2014). Through limited outer

membrane permeability and efflux systems that push antibiotics out of the cell, *P.* aeruginosa and *A. baumannii* have a high level of resistance to most antibiotics. In addition, the ability of *P.* aeruginosa and *A. baumannii* to produce biofilm contributes to antibiotic resistance (Gedefie et al., 2021; Pang et al., 2019). Moreover, *E. coli* and *K. pneumoniae* are the main bacteria that produce extended-spectrum β-lactamases (ESBLs). Like β-lactamases, the ESBLs can break down penicillin, cephalosporins, and fluoroquinolones. Through the production of ESBLs, *E. coli* and *Klebsiella pneumoniae* become highly resistant to antibiotics (Brolund, 2014; Dupouy et al., 2019; Mansouri et al., 2019; McDanel et al., 2017).

This study has an ecological design with some potential limitations, such as being based on aggregated data. Although DDD measurements are international tools for quantifying antibiotic use, calculating antibiotic consumption using DDD measurements only measures aggregated-population levels. The analysis of the correlation between antibiotic consumption and antibiotic resistance using an aggregated-population level has the potential for ecological bias because resistance selection pressure arises at the individual level (Guo et al., 2015; Plüss-Suard et al., 2013; Zou et al., 2015). Moreover, this study was based on a single hospital setting in Indonesia. Thus, studies with multicenter designs and longer surveillance periods are needed to explain the correlation between antibiotic consumption and antibiotic resistance.

Table 1. Annual usage trends of inpatient antibiotic consumption at a general regional hospital, 2017-2019

ATC Code	Antibiotics	Antibiotic consumption (DDDs/100 bed-days) per year				Time series analysis		
		2017	2018	2019	Total 2017-2019	Slope (b)	*p-value*	Trend
J01CA01	Ampicillin	80.04	78.05	62.24	220.33	-8.900	0.268	Decreasing
J01CR01	Ampicillin-sulbactam	45.07	40.82	40.45	126.34	-2.310	0.287	Decreasing
J01DD02	Ceftazidime	15.77	14.87	11.67	42.31	-2.050	0.199	Decreasing
J01GB03	Gentamicin	22.45	20.82	27.03	70.30	2.290	0.496	Increasing
J01GB06	Amikacin	15.45	20.53	17.37	53.35	0.960	0.756	Stable
J01MA02	Ciprofloxacin	40.43	45.56	40.87	126.86	0.220	0.951	Stable

Table 2. The pattern of antibiotic resistance in four critical bacteria at a general regional hospital, 2017-2019

ANTIBIOTIC	ANTIBIOTIC RESISTANCE PERCENTAGES (%)			
	E. coli	***A. baumanii***	***K. pneumoniae***	***P.aeruginosa***
	(N=83)	**(N=70)**	**(N=64)**	**(N=49)**
J01A-TETRACYCLINES				
J01AA-Tetracyclines				
J01AA12-Tigecycline	0.00	5.71	0.00	50.00
J01C-BETA-LACTAM, PENICILLIN				
J01CA-Penicillins with extended-spectrum				
J01CA01-Ampicillin	96.39*	100.00*	100.00*	100.00*
J01CR-Combinations of penicillins				
J01CR01-Ampicillin-Sulbactam	81.82*	100.00*	36.40	100.00*
J 01CR05-Piperacillin-Tazobactam	24.00	5.71	-	100.00*
J01D- OTHER BETA-LACTAM				
J01DB-First-generation cephalosporins				
J01DB04-Cefazolin	100.00*	-	-	-
J01DD-Third-generation cephalosporins				
J01DD01-Cefotaxime	63.64*	-	14.00	-
J01DD02-Ceftazidime	45.45	41.43	37.50	32.70
J01DD04-Ceftriaxone	63.63*	89.39*	25.50	-
J01DE-Fourth-generation cephalosporins				
J01DE01-Cefepime	40.91	54.55	25.00	18.40
J01DF-Monobactams				
J01DF01-Aztreonam	59.09	100.00*	27.30	69.40*
J01DH-Carbapenems				
J01DH02-Meropenem	13.64	0.00	-	50.00
J01DH03-Ertapenem	4.55	-	0.00	-
J01E-SULFONAMIDES AND TRIMETHOPRIM				
J01EE-Combinations of sulfonamides and trimethoprim				
J01EE01-Sulfamethoxazole and trimethoprim	43.18	5.71	100.00*	50.00
J01G-Aminoglycoside				
J01GB03-Gentamicin	40.91	21.43	35.94	50.00
J01GB06-Amikacin	2.41	11.43	0.00	6.10
J01M-Quinolone				
J01MA02-Ciprofloxacin	79.55*	40.00	37.50	57.10
J01X-OTHER ANTIBACTERIALS				
J01XE-Nitrofuran derivatives				
J01XE01-Nitrofurantoin	43.18	0.00	-	100.00*

* The percentage of antibiotic resistance was highly resistant (more than 60%)

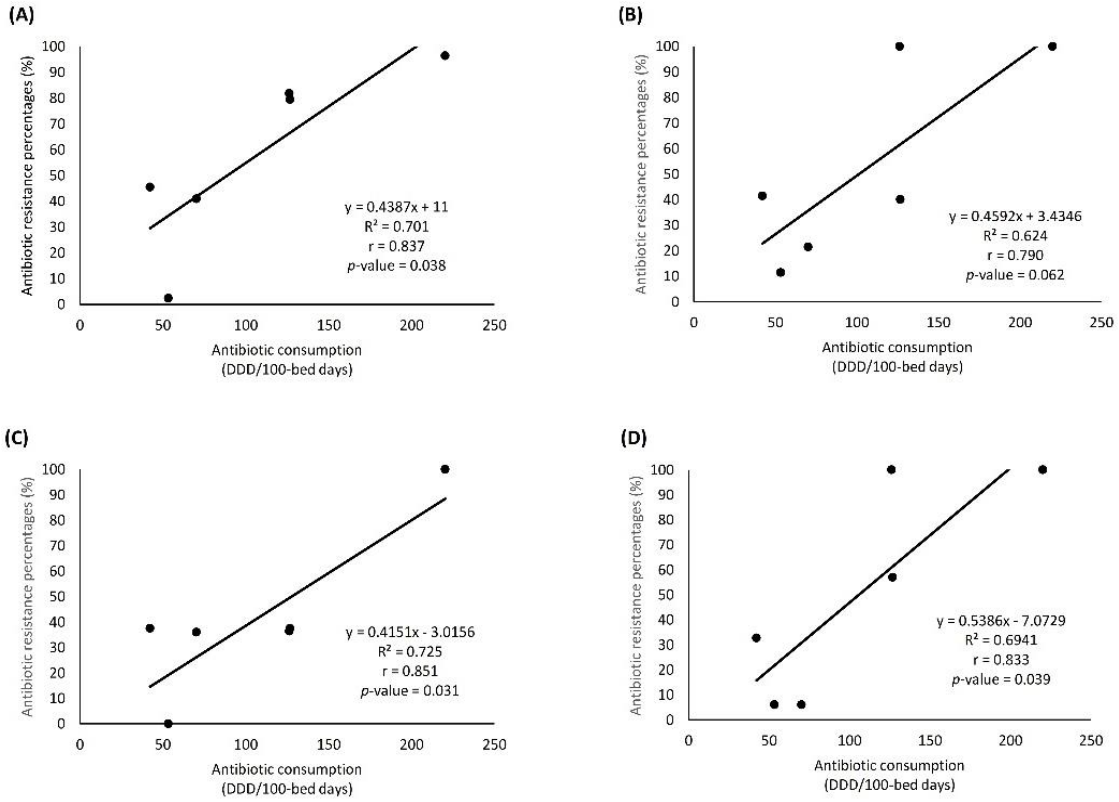

Figure 1. Linear regression analysis of antibiotic consumption and antibiotic resistance percentage in critical group bacteria. (A) *E. coli*; (B) *A. baumanii*; (C) *K. pneumoniae*; (D) *P. aeruginosa*

CONCLUSION

The overall trend of annual antibiotic consumption during the three years fluctuated; the most frequently used antibiotic with an increasing trend was ciprofloxacin. *P. aeruginosa*, *A. baumannii*, and *E. coli* as critical MDR bacteria resistant to various antibiotics. There was a significant relationship between antibiotic consumption (DDD/100 bed-days) and antibiotic resistance in *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, but not statically significant in *A. baumannii*. Antibiotic resistance in hospitals varies commonly due to differences in environment and antibiotic consumption. It is influenced by multi-factors, such as misuse and overuse of antibiotics, inappropriate prescribing, horizontal gene transfer, and resistance mechanisms that might differ between species. This study has an ecological design with some potential limitations, such as being based on aggregated data, although DDD measurements are international tools for quantifying antibiotic use. However, information about the trend of antibiotic consumption, antibiotic resistance pattern, and the relationship between antibiotic consumption and antibiotic resistance in a critical group of bacteria can be used to control antibiotic usage, antibiotic resistance, and decrease the irrationality of antibiotic consumption.

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Medication-related burden of chronic renal failure patients at regional general hospital Sleman Yogyakarta

Woro Supadmi*, Elinda Fitriana, Muhammad Muhlis
Faculty of Pharmacy, Universitas Ahmad Dahlan
Jl. Prof. Dr. Soepomo, S.H, Warungboto, Umbulharjo, Yogyakarta, Indonesia

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ABSTRACT

Patients with chronic renal failure must undergo lifelong treatment. The condition raises treatment-related responsibilities and may affect their treatment adhesion. The aim of this study was to determine the correlation between the burden of medication and the level of medication adherence among chronic kidney failure patients at Sleman Regional Hospital in Yogyakarta. This study took the form of observational study with a cross-sectional design. Data were collected using LMQ (Living with Medicine Questionnaire) and Visual Analog Sacle (VAS) overall burden to determine the burden and MARS (Medication Adherence Rating Scale) to determine medication adherence level. The samples in this study were 60 patients from all patients undergoing hemodialysis who met the inclusion criteria. Sampling was taken using a consecutive sampling technique with inclusion criteria of patients willing to complete the questionnaire and patients diagnosed with chronic renal failure aged ≥18 years. To determine the relationship between medication burden and medication adherence, data was examined using the Spearman test. The results of this study showed that 40 patients (66.7%) had moderate medication burden and 50 patients (83%) had moderate medication adherence. There was a significant correlation between the LMQ score and MARS (correlation-coefficient = 0.581, p=0.000) and a significant correlation between the VAS score and MARS (correlation-coefficient= 0.651, p=0.000). Thus, it can be concluded that there is a positive relationship between treatment burden and the level of treatment compliance, where the higher the burden, the higher the level of compliance in chronic kidney failure patients.

Keywords: adherence, chronic renal failure, medication-related burden

Corresponding author:
Woro Supadmi
Faculty of Pharmacy, Universitas Ahmad Dahlan
Jl. Prof. Dr. Soepomo, S.H, Warungboto, Umbulharjo, Yogyakarta, Indonesia
Email: woro.supadmi@pharm.uad.ac.id

INTRODUCTION

Chronic kidney disease also known as chronic kidney failure is caused by a glomerular filtration rate (GFR) of less than 60 mL/minute/1.73 m^2, and albuminuria does not exceed 30 mg per 24 hours, or signs of kidney damage appear (eg hematuria or structural abnormalities such as kidney polycystic or dysplastic) that lasts more than 3 months. Chronic renal disease is divided into 6 stage based on the Glomerular Filtration Rate (GFR), the last stage of which is considered renal failure. This stage is characterized by GFR <15 mL/minute/1.73 m^2 (NKF KDIGO, 2013). GFR below 15 mL/minute/1.73 m^2 can cause more serious symptoms and complications. In this condition, patients require renal replacement therapy such as dialysis or renal transplantation (Inker et al., 2014). Patients with chronic renal failure must undergo lifelong medication treatment and renal replacement therapy such as dialysis (Sarastika et al., 2019; Wiliyanarti & Muhith, 2019). During treatment, patients can have both good and bad experiences. A good treatment experience includes increased patient control over symptoms or disease conditions and clinical symptoms while a bad experience can be in the form of side effects, poor control of the disease, and discomfort due to side effects. Patients' daily life during medication therapy can be influenced by how the patient responds to the burden experienced (Gallacher et al., 2013).

Medication-related burden can be measured using the Living with Medicine Questionnaire (LMQ) instrument. LMQ covers several domains including relationships with other health workers, technical difficulties, high cost burden, perceived efficacy and side effects, attitudes, impact on daily life, and patient control over the treatment they experience (Krska et al., 2014). Research shows a significant relationship between medication burden and the level of medication adherence in disease treatment (Mohammed et al., 2016; Tesfaye et al., 2020; Tran et al., 2012).

Adherence is defined as a person's behavior in disease treatment (taking medication, following a diet, recommended lifestyle changes) in accordance with the health provider (WHO, 2003). Previous research found that the characteristics of respondents do not influence the level of medication adherence (Naafi et al., 2016). Instead, medication adherence is influenced by several external factors such as the patient's environment and family support (Hannan, 2013). Patient adherence can be measured using the Medication Adherence Rating Scale (MARS) questionnaire. MARS includes several questions related to a person's behavior towards treatment (Fialko et al., 2008). This study aims to determine medication-related burden, medication adherence, and the relationship between medication burden and medication adherence of chronic renal failure patients. The results of this study are expected to be used to solve problems if known factors that affect the burden of medication

METHOD

Methods

This research took the form of observational research and was carried out using a cross-sectional descriptive approach. This research involves observing and measuring variables simultaneously. Data was collected by survey using a questionnaire as a research instrument. The sample for this study was adult chronic kidney failure patients undergoing hemodialysis at Sleman Yogyakarta Regional Hospital who met the inclusion criteria. The inclusion criteria for this study are: (1) Patients who are willing to become research participants; (2) Patients diagnosed with chronic renal failure aged ≥18 years; (3) Patients who routinely undergo hemodialysis. Meanwhile, the exclusion criteria in this study were patients who had limitations in reading and communicating.

The research instrument used in this study consisted of (1) Living with Medicines Questionnaire (LMQ) which consists of 41 items in the form of a Likert scale and open-ended question. Participants were given choices between "strongly agree", "agree", "neutral", "disagree", "strongly disagree". Open-ended question enabled participants to answer freely without any restrictions. This instrument covered 8 domains which include relationship or communication with health workers, technical difficulties, burden related to costs, side effects, medicine effectiveness, concerns about the impact or interference of medicine use in daily life, and control of medicine use. The LMQ score was summed

from the 41 questions to describe the overall score ranged from 41-205. The higher the score, the greater the burden. The scores can be categorized into no-burden = 41-73; low burden = 74-106; medium burden = 107-139; high-burden = 140-172; very-high burden = 173-205 (Katusiime et al., 2018). This questionnaire also contains a Visual Analog Scale (VAS) to determine the overall assessment of the burden experienced by respondents on a scale of 0-10, the higher the VAS score, the greater the burden (Katusiime, 2017; Zidan et al., 2016). (2) Medication Adherence Rating Scale (MARS) questionnaire was also used to assess patient medication adherence. This questionnaire consists of 5 questions with answers in the form of an ordinal scale. Respondents were given choices between "always", "often", "sometimes", "rarely", and "never". The MARS score was obtained from the sum of the 5 questions with the final score ranging from 5-25. The adherence score was categorized into three: high adherence = 25; moderate adherence = 6-24; and low adherence = 5 (Naafi et al., 2016). The questionnaire used has been validated in previous research entitled "Medication-Related Burden in Thalassemia Patients in the Hospital Dr. Hasan Sadikin Bandung" with a p value = <0.05 so that the questionnaire is valid to be used (Kudri, 2019). Ethics approval was obtained from the Regional General Hospital Sleman Yogyakarta Indonesia (No.180/2909: 05 September 2020).

Data Analysis

Descriptive analysis technique was used to obtain the percentage of each variable, namely characteristics, burden, and adherence. We use testing method by Mann Whitney and the method by Kruskall Wallis to determine the connection between respondent characteristics and burden. To determine the relationship between treatment burden and treatment adherence with numerical data analyzed with the Spearman test

RESULT AND DISCUSSION

Patient characteristics

There were 60 respondents who participated in this research. Data on the demographic of participants and medication characteristics is presented in Table 1.

Table 1 shows that the participants in this study consisted of 22 male patients (36.7%) and 38 female patients (63.3%). Similar research also reported that the number of chronic renal failure patients at Dr. Mohammad Hoesin Palembang was dominated by female at 169 (56.3%) compared to male at 131 people (43.7%) (Hervinda et al., 2014). Although there were more female patients involved in this study, gender is not a major risk factor for chronic renal failure since it can occur in both men and women. In terms of age, the respondents were divided into 6 categories. 18 patients chronic renal failure patients (30.0%) in this study were 46-55 years. Clinically, patients aged >60 years are at greater risk of chronic renal failure compared to patients aged <60 years. This is because the renal function decreases as the body gets older (Pranandari & Supadmi, 2015). The participants' employment status was divided into 3 categories: employed, unemployed, and students. A total of 49 chronic renal failure patients (81.7%) were unemployed. This shows that the physical condition of chronic kidney failure patients causes patients to reduce their activity so that it becomes an obstacle in work. The respondents' education level was divided into 2 categories: l ≤12 years of education and >12 years of education. A total of 54 chronic renal failure patients (90.0%) had ≤ 12 years of education which shows that a significant number of respondents have basic education level.

Based on the number of medicines consumed, 56 chronic renal failure patients (93.3%) consumed more than 1 type of medicine. These medicines consist of folic acid supplements, calcium, and other medicines such as antihypertensives. Research showed that the greater the number of medicines consumed, the more the patient's treatment burden increases (Krska et al., 2018). Thus, taking a large number of medications can disrupt patients' daily life. In terms of hemodialysis frequency, 56 chronic renal failure patients (93.3%) had undergone hemodialysis therapy for >6 months and all chronic renal failure patients (100.0%) underwent hemodialysis therapy twice a week. 60 chronic renal failure patients (100.0%) involved in the study were not charged for their treatment because they were National Health Insurance users. A total of 29 chronic renal failure patients (48.3%) needed

companion taking medication in the family for help or supervision in taking medication. The assistance provided is in the form of preparing medication and reminding of the medication schedule.

Table 1. Characteristics of chronic renal failure patients at Sleman regional general hospital, Yogyakarta

	Variable	Frequencies (n = 60)(%)
Gender	Female	22 (36,7)
	Male	38 (63,3)
Age	18 - 25 years	3 (5,0)
	26 - 35 years	9(15,0)
	36 - 45 years	12(20,0)
	46 - 55 years	18(30,0)
	56 - 65 years	15(25,0)
	> 65 years	3(5,0)
Employment Status	Employed	10(16,7)
	Unemployed	50(83,3)
Education Level	Education ≤12 years	54(90,0)
	Education >12 years	6(10,0)
The number of drugs one daily	1 drug (Single therapy)	4(6,7)
	>1 drugs (Combination therapy)	56(93,3)
Dosage Forms	Tablet/Capsule	60(100,0)
frequency of taking the drug in one daily	1 x day	13(21,7)
	2 x day	23(38,3)
	3 x day	24(40,0)
National Health Insurance Participation	Yes	60(100,0)
Companion Taking Medication	Yes	29(48,3)
	No	31(51,7)

Medication-related burden

The analysis results are presented in Table 2. The results of the medication-related burden analysis obtained based on the Living with Medicines Questionnaire (LMQ) scores in chronic renal failure patients revealed that there were more participants with moderate burden (66.70%) than participants with low burden of 20 people (33, 3%). There were no patients with high, very high, and no burden. The lowest LMQ questionnaire score in this study was 87 while the highest LMQ questionnaire score in this study was 135.

Table 2. Medication-related burden of chronic renal failure patients at Sleman regional general hospital, Yogyakarta

Variable	Range	Average	Frequencies (n = 60)(%)
Overall LMQ Score			
No burden	41-73	109.51	0 (0.0)
Low burden	74-106		20 (33,3)
Moderate burden	107-139		40 (66.7)
High burden	140-172		0 (0.0)
Very high burden	173-205		0 (0.0)
VAS: overall burden	0-10	4.14	

Patient adherence

The results by MARS questionnaire, a score was obtained which was used to categorize the level of adherence of chronic renal failure patients as shown in Table 3.

Table 3. The medication adherence of chronic renal failure patients at Sleman regional general hospital, Yogyakarta

Variable	Range	Average	Frequencies (n = 60)(%)
Overall MARS score			
High adherence	25		10 (16.7)
Moderate adherence	6-24	21.78	50 (83.3)
Low adherence	1-5		0 (0.0)

Based on the data in Table 3, it shows that chronic kidney failure patients in this study had an average score of 83.3%. In a previous study of 150 patients, 22% of patients had high adherence to medication use, 55% had low adherence and 23% had moderate adherence (Ahlawat & Tiwari, 2016). Previous research showed that only 61.3% of the study population adhered to their treatment regimen. Forgetfulness in 79.8% was the main reason for non-adherence to treatment (Kefale et al., 2018). Most patients lacking medication adherence forgot to take their medication or took their medication less than the recommended dosage. Based on the results of questionnaire there were patients who stopped taking medication temporarily because they felt bored with the medication routine. Some patients also felt that there was no significant change in their health condition after regularly taking medication so they decided to take less medication than the prescribed dose.

The correlation between the burden of medication and patient adherence

The Spearman rho correlation analysis was performed to analyze the relationship between medication-related burden and the level of adherence in chronic renal failure patients. This analysis was carried out to determine the relationship between the results of the LMQ instrument analysis, LMQ domains, VAS scores, and MARS instruments. Data obtained from this analysis are presented in Table 4.

Based on the results of the analysis, it was found that almost all domains of the LMQ and VAS received a significance value of <0.05, which means there is a relationship between medication-related burden and the level of compliance with taking medication.There were three domains that have a significance value of >0.05, namely domains 1, 3, and 7, so it can be concluded that domains 1, 3, and 7 do not have a significant relationship with MARS (Medication Adherence Rating Scale).

Based on the correlation results of LMQ and VAS scores with MARS, the correlation coefficients were positif 0.581 and 0.651 respectively with a significance of 0.000, indicating that there is a positive correlation between LMQ scores and VAS scores with MARS scores. The higher the burden, the higher the level of adherence in chronic renal failure patients. This is in accordance with research conducted by (Zidan et al., 2016) which showed that the higher the burden felt by the patient during medication treatment, the higher the level of patient adherence (LMQ-MARS correlation coefficient= 0.317; VAS-MARS correlation coefficient= 0.325; $p< 0.05$). Several factors that influence this include family support and attention to the lives of chronic renal failure patients, the family's understanding of chronic renal failure, as well as the attitude and quality of family relationship towards chronic renal failure patients (Saraswati et al., 2019).

Table 4. The relationship between medication-related burden and patient adherence of chronic renal failure patients at Sleman regional general hospital, Yogyakarta

Medication related burden (LMQ Domains)	Adherence	*Coefficient correlation*	*p* value
Domain 1 (Relationship/Communication with Health Professionals Regarding Medicines)	MARS Score	0.212	0.104
Domain 2 (Technical Difficulty)		0.269	0.038*
Domain 3 (Cost-Related Burden)		0.225	0.084
Domain 4 (Side-Effects)		0.463	0.000*
Domain 5 (Medicine Effectiveness)		0.291	0.024*
Domain 6 (Attitudes/Concerns Regarding Medicine Use)		0.347	0.007*
Domain 7 (Impact or Interference on Daily Life_		-0.009	0.994
Domain 8 (Control/Autonomy in Varying Medicine Regimen)		0.385	0.002*
LMQ Score		0.581	0.000*
VAS Score		0.651	0.000*

Note:
*There is a significant relationship < 0.05

CONCLUSION

The results of the LMQ questionnaire showed that 20 patients (33.3%) experienced low burden and 40 patients (66.7%) experienced moderate burden. From the level of compliance, 50 patients (83.3%) had a moderate level of compliance and 10 patients (16.7%) had a high level of compliance. These findings indicate that there is a positive correlation between treatment-related burden (LMQ score and VAS score) and the level of treatment adherence (MARS score) with correlation coefficients of 0.581 and 0.651 respectively, p=0.000 (p<0.05). Thus, it can be concluded that treatment-related burden is positively correlated with treatment adherence.

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The quality of life of hemodialysis patients in Yogyakarta, Indonesia

Siswandi[1], Dyah Aryani Perwitasari[2*] , Lamhot Burju Simanjuntak[1], Gugun Suhendra[1], Dewi Wulandari[1], Haafizah Dania [2], Imaniar Noor Faridah[2], Adnan[2]

[1]dr. Suhardi Hardjolukito Regional Military Hospital,
Jl. Janti Blok O Komplek Lanud Adisutjipto, Yogyakarta, Indonesia
[2]Faculty of Pharmacy, Universitas Ahmad Dahlan,
Jl. Prof. Dr. Soepomo, S.H, Warungboto, Umbulharjo, Yogyakarta, Indonesia

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ABSTRACT

The assessment of quality of life serves as a critical evaluation of the effectiveness of the administered hemodialysis treatment. The measurement of quality of life can be conducted using the Kidney Disease Quality of Life Short Form (KDQoL-SF™) questionnaire. This research aims to gather information concerning hemodialysis patients' quality of life due to chronic kidney failure at the dr. S. Hardjolukito Regional Military Hospital in Yogyakarta. The research employed a cross-sectional design. The respondents consisted of 65 outpatients selected through purposive sampling. The inclusion criteria were patients in the end-stage of hemodialysis, having undergone hemodialysis for at least 3 months, aged 18 years or older, willing to participate in the study, and capable of honestly and voluntarily completing the questionnaire. Subjects experiencing disturbances in consciousness, communication impairments, and those designated as emergency patients (Cito) were excluded. The Independent T-test, Mann-Whitney test, Chi-Square test and Fischer test were conducted to define the predictors of quality of life. Most of the hemodialysis patients was male (50.8%) with average of age was 54.66 years old. The findings revealed that 64.6% of the respondents experienced moderate/poor quality of life, while 35.4% exhibited good quality of life. Statistical analysis established a significant relationship between age and period of hemodialysis and quality of life based ($p<0.05$). Hemoglobin levels, number of prescribed medications, gender, education, occupation and income demonstrated no significant relationship with the quality of life. Among the respondents' characteristics, age and duration of hemodialysis were associated with the quality of life.

Keywords: hemodialysis, KDQoL-SF™, quality of life, Yogyakarta

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Corresponding author:
Dyah Aryani Perwitasari
Faculty of Pharmacy, Universitas Ahmad Dahlan
Jl. Prof. Dr. Soepomo, S.H, Warungboto, Umbulharjo, Yogyakarta, Indonesia
Email: dyah.perwitasari@pharm.uad.ac.id

INTRODUCTION

Chronic kidney failure claims the lives of 850,000 individuals annually, ranking as the 12th leading cause of death worldwide (Ariyanti & Imam, 2021). In Indonesia, there is an increase of the prevalence of chronic kidney failure in 2013 (0.2%), to 2018 (0.38%) (Rohmaniah & Sunarno, 2022). The Special Region of Yogyakarta holds the 12th position with a high prevalence of chronic kidney failure at 4.3% (Hayati et al., 2021). The total number of new patients undergoing hemodialysis in Indonesia witnessed a twofold increase by the end of December 2018 compared to the year 2017, reaching a total of 66,433 individuals, resulting in a rise in the overall number of active patients to 132,142 during the same period (Anonymous, 2018). Notably, dr. S. Hardjolukito Regional Military Hospital Yogyakarta experienced a 17.7% increase in chronic kidney failure patients undergoing hemodialysis between 2017 and 2021. Research findings indicate that individuals with kidney failure undergoing hemodialysis suffer from various effects and conditions that adversely impact their physical, psychological, social, and environmental well-being (Rahman et al., 2013).

The Kidney Disease Quality of Life (KDQoL) and Short Form Transplant Module (SF^{TM}) are two instruments utilized to assess the quality of life in patients with kidney disease. KDQoL incorporates a combination of the Short Form-36 (SF-36) and additional questions specifically developed for kidney disease patients. Similarly, SFTM also employs SF-36 to measure general health status but is supplemented with specific questions. The KDQoL-SF^{TM} version 1.3 differs from version 1.2 by the inclusion of a section on sexual activity (Hays et al., 1995).

This study aims to define patients' quality of life with hemodialysis due to chronic kidney failure at dr. S. Hardjolukito Regional Military Hospital in Yogyakarta using the KDQoL-SF^{TM} questionnaire.

MATERIALS AND METHOD

Materials

This study used the *Kidney Disease Quality of Life Short-Form* (KDQoL-SF^{TM}) as the instrument. This questionnaire employs a Likert scale to assess the health status of the subjects. The instrument comprises 36 questions concerning general health and 43 questions pertaining specifically to kidney-related conditions.

Methods

This study employed a cross-sectional design. Data collection was conducted at dr. S. Hardjolukito Regional Military Hospital from April to September 2022. The subjects included all out patients with chronic kidney disease undergoing hemodialysis at the Hemodialysis Installation. The inclusion criteria were patients in the end-stage of hemodialysis, having undergone hemodialysis for at least 3 months, aged 18 years or older, willing to participate in the study, and capable of honestly and voluntarily completing the questionnaire. Subjects experiencing disturbances in consciousness, communication impairments, and those designated as emergency patients (Cito) were excluded from the study.

The quality of life instrument is the *Kidney Disease Quality of Life Short-Form* (KDQoL-SF^{TM}) as depicted in Figure 1.

Data Analysis

This questionnaire employs a Likert scale to assess the health status of subjects in this study. The instrument comprises 36 questions concerning general health and 43 questions pertaining specifically to kidney-related conditions. General health encompasses role functioning, pain, overall health, emotional well-being, emotional role, social functioning, and fatigue. Function scores range from 0 to 100, where a score of 100 indicates good quality of life. Other items in the questionnaire address the patients' overall health and their feelings regarding their health. Additional patient background information includes gender, ethnicity, education, income, length of hospitalization, and the number of medications received (Joshi et al., 2010; Rokhman et al., 2023). The scoring of the KDQol SF^{TM}

questionnaire follows the procedures outlined in previous research (Hays et al., 1994). The quality of life variables in this study was categorized as follows: good quality of life (76-100), moderate quality of life (60-75), and poor quality of life (<60) (Theofilou, 2013).

To define the predictors of patients' quality of life, the Independent T-test, Mann-Whitney test, Chi-Square test and Fischer test were conducted based on the type of data and the normal distribution.

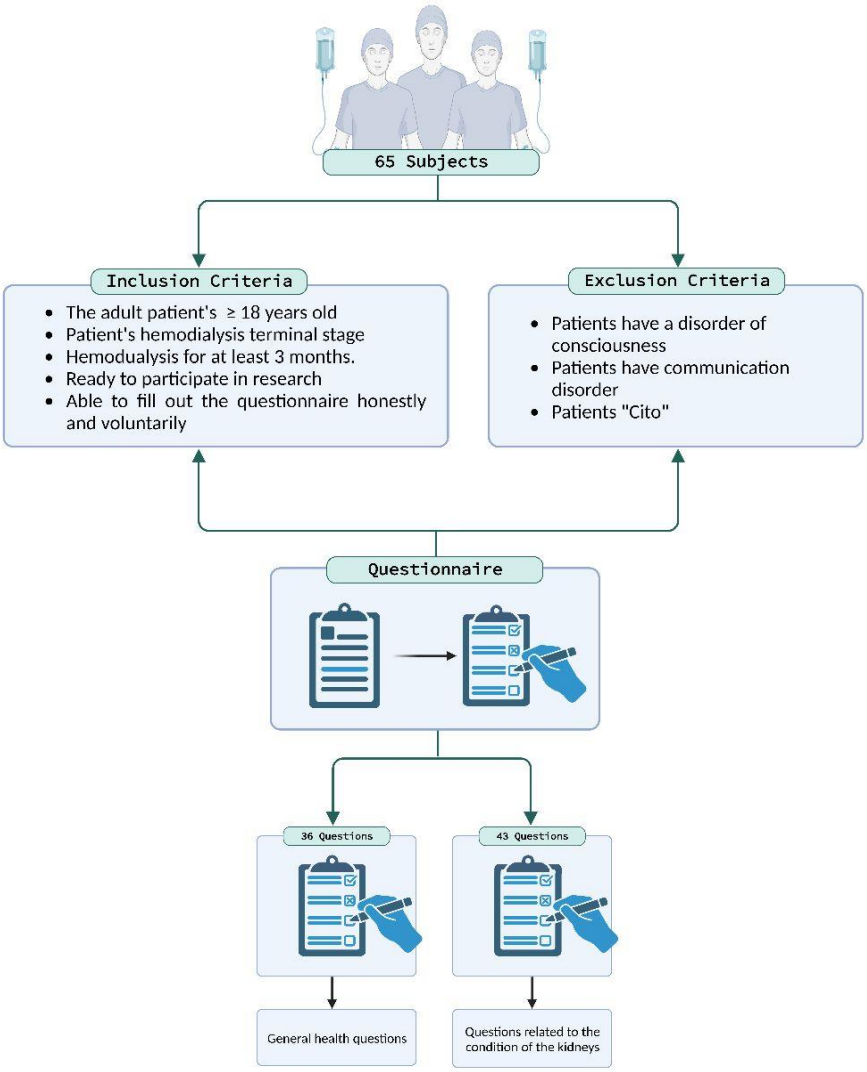

Figure 1. Data Collection using the Kidney Disease Quality of Life Short-Form (KDQoL-SF™) [No copyright: QQ25N06SH0]

RESULT AND DISCUSSION

We recruited 63 hemodialysis patients. The characteristics of the subjects in this study are presented in Table 1.

Most patients are male and their average of age is 54.66 years old. The average hemoglobin level of the respondents is 8.21 ± 1.18 g/dL and the average number of prescribed medications for the respondents is 5.15 ± 1.41 types of medication. The respondents are dominantly within the age group of 45-59 years, which aligns with the data from Riskesdas 2018, where 30% of hemodialysis patients were found in this age range (Anonymous, 2018). The longer the respondents undergo hemodialysis,

the more time they need to adapt to the changes experienced, such as symptoms, complications, and lifelong treatments (Gil Cunqueiro et al., 2003).

Table 1. Characteristics of subjects in this study

Respondents' Characteristics	N	%	$\bar{X}$ ± SD
Age (years old)	65	-	54.66 ± 11.21
Duration of Hemodialysis (months)	65	-	326.98 ± 253.64
Hemoglobin Level (g/dL)	65	-	8.21 ± 1.18
Number of Prescribed Medications (types of medication)	65	-	5.15 ± 1.41
Sex			
Male	33	50.8	-
Female	32	49.2	
Educational Background			
No formal education/Incomplete	3	4.6	
Completed primary education (SD)	8	12.3	
Completed junior high school (SMP)	11	16.9	-
Completed senior high school (SMA)	28	43.1	
Tertiary education (College/University)	15	23.1	
Employment Status			
Unemployed	44	67.7	-
Employed	21	32.3	
Monthly Income (IDR)			
< 6,000,000 per month	63	96.9	-
≥ 6,000,000 per month	2	3.1	

The subjects' quality of life can be observed in Figure 2.

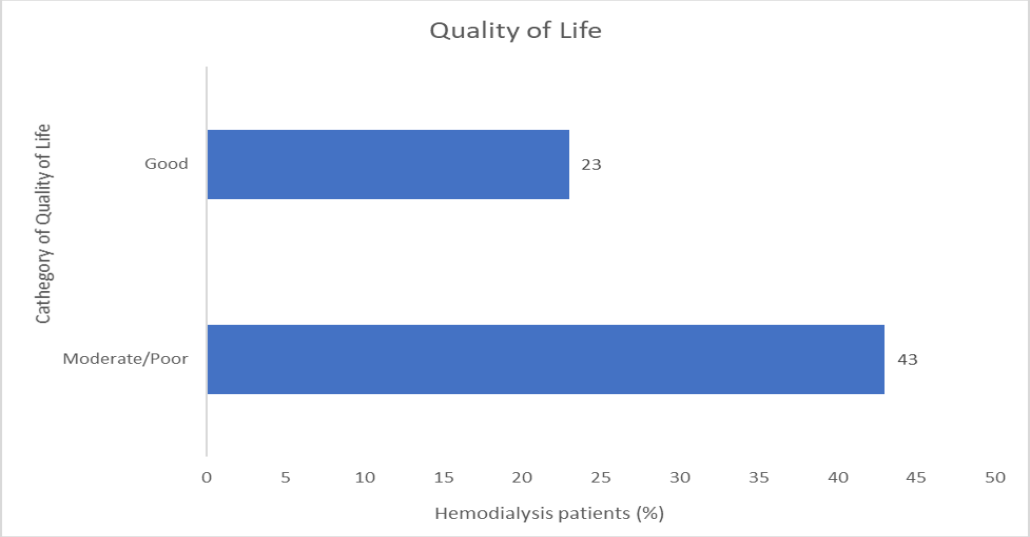

Figure 2. Quality of life of hemodialysis patients with chronic kidney failure at dr. S. Hardjolukito regional Military Hospital, Yogyakarta

Among the subjects, 64.6% experienced moderate/poor quality of life, while 35.4% exhibited good quality of life. These results can be attributed to several factors experienced by the respondents, both upon diagnosis of the disease and during the course of hemodialysis. This finding aligns with previous research indicating that the majority of hemodialysis patients have poor quality of life (Al Salmi et al., 2021; Van Loon et al., 2017).

Parameters for assessing the respondents' quality of life are based on 19 domains grouped into three categories: kidney disease domain, physical health domain, and mental health domain. The mean quality of life score for the respondents in this study is 70.96 ± 10.92, with the highest score found in the mental health domain (78.14 ± 8.95), which is higher than both the kidney disease domain (72.78 ± 20.01) and the physical health domain (58.83 ± 17.47) (Table 2). The physical health domain has lower scores compared to the mental health domain. The lower scores in the physical health domain suggest that over time, respondents can adapt psychologically to the limitations of their health conditions (Adiningrum et al., 2021; Risky, 2019).

Table 2. Quality of life domains of hemodialysis patients with chronic kidney failure at dr. S. Hardjolukito regional Military Hospital, Yogyakarta

Domain	$\bar{X}$ ± SD
Kidney Disease	
Symptoms/Issues	85.63 ± 11.95
Effects of Kidney Disease	85.38 ± 14.98
Burden of Kidney Disease	50.86 ± 19.29
Employment Status	43.07 ± 17.40
Cognitive Function	89.84 ± 20.75
Quality of Social Interactions	96.72 ± 9.14
Sexual Function	46.75 ± 53.44
Quality of Sleep	61.73 ± 22.07
Social Support	95.89 ± 10.22
Quality of Dialysis Staff Services	64.61 ± 14.92
Patient Satisfaction	80 ± 16.97
Physical Health	
Physical Function	61.54 ± 27.08
Physical Role	34.61 ± 26.40
Perception of Pain	76.31 ± 29.13
General Health	62.84 ± 16.86
Mental Health	
Emotional Well-being	87.63 ± 18.02
Emotional Role	83.07 ± 30.68
Social Function	70.96 ± 10.92
Energy/Fatigue	67.61 ± 19.16
Quality of Life Scores	70.96 ± 10.92

Good quality of life is observed in individuals who can carry out their functions and roles in daily life appropriately and in line with their developmental level (Sabaan & Perwitasari, 2016). The SF-36 has been used worldwide to assess quality of life. In China, the SF-36 has been employed in several studies to assess the quality of life in the general population and specific chronic diseases. A study evaluating the quality of life in the general population conducted in Shanghai, China concluded that the SF-36 is acceptable and applicable for evaluating the quality of life in the general population. Chronic diseases significantly disrupt all dimensions of the SF-36, leading to a decrease in the quality

of life among individuals with chronic conditions (Wang et al., 2008). The assessment of quality of life in the general population has also been conducted in India (Sinha et al., 2013) and Iran (Montazeri et al., 2005) where the SF-36 was found suitable for measuring the quality of life in populations and could be used as a basis for comparison with specific populations. Furthermore, the SF-36 has been utilized to evaluate the quality of life in the general population in Indonesia, with the highest physical domain at 91.21 (SD = 12.09) and the lowest general health status at 71.69 (SD = 12.18), indicating that societal characteristics can influence the quality of life in the normal population (Sabaan & Perwitasari, 2016).

Table 3. Predictors of quality of life of hemodialysis patients (age and hemoglobin)

Variable	Mean ± SD		t	*p*	95% CI
	Moderate/Poor (n=42)	Good (n=23)			
Age (year)	57.76 ± 11.26	49.00 ± 8.79	3.227	0.002	3.336 - 14.188
Hemoglobin Level (g/dL)	8.15 ± 1.02	8.322 ± 1.44	- 0.513	0.611	-0.8640 - 0.5158

t = T-test; p = significance/probability; CI = Confidence Interval

As Table 3 indicates, the average age of respondents in the group with moderate/poor quality of life is 57.76 ± 11.26 years, is higher than the average age of respondents in the group with good quality of life, which is 49.00 ± 8.79 years. This is statistically significant ($p<0.05$), indicating a difference between age and quality of life, signifficantly. Our study findings differ from a previous study (Risky, 2019) which found no association between age and quality of life. Age has a significant and negative correlation, meaning that the quality of life of respondents decreases as their age increases (Barzegar et al., 2017). Advanced age can lead to deteriorating organ functions, making the body susceptible to diseases and complications. For instance, kidney function tends to decline with increasing age, affecting physical activity due to impaired organ function. On the other hand, respondents in their productive age can perform activities well, feel motivated for recovery, and have high life expectancy (Istanti, 2014).

The average hemoglobin level of respondents in the group with moderate/poor quality of life is 8.15 ± 1.02 g/dL, which is lower than the average hemoglobin level of respondents in the group with good quality of life, which is 8.32 ± 1.44 g/dL. However, the difference is not statistically significant ($p>0.05$). A decrease in hemoglobin levels is often associated with poor quality of life in chronic kidney disease patients undergoing hemodialysis. However, the relationship between quality of life and changes in hemoglobin levels does not always mean that an increase in hemoglobin levels can improve the quality of life and anemia in chronic kidney disease patients (Lefebvre et al., 2006). Anemia is one of the common complications in patients with chronic kidney failure. The previous research mentioned that hemodialysis patients experienced mild anemia in 45% of cases, moderate anemia in 25%, and severe anemia in 8% (Senduk et al., 2016). Anemia can also impact the quality of life of hemodialysis patients; therefore, proper management of anemia can improve the quality-of-life scores of hemodialysis patients (Gong et al., 2022).

Table 4 shows that the percentage of male respondents with moderate/poor quality of life is 69.7%, which is not significantly different from the percentage of female respondents at 59.4%. The odds ratio (OR) value is 1.57, indicating that male respondents are 1.57 times more likely to have moderate/poor quality of life compared to female respondents. However, this difference is not statistically significant (95% CI; 0.56-4.38), meaning that there is no significant difference between gender and quality of life. Our study results align with previous research (Anggraeni, 2016; Risky, 2019) indicating that gender is not associated with quality of life. In general, any disease can affect anyone, but certain diseases may have different effects on males and females due to differences in employment status, lifestyle, genetic conditions, and physiology (Budiarto & Anggraeni, 2002). Males are considered for predictor of

chronic kidney disease, because of lifestyle factors such as smoking and alcohol consumption, which play a significant role in the development of chronic kidney disease (Astrini, 2013). Researchers assume that both males and females have the same desire for recovery and to feel comfortable with their condition. They have their own ways to adapt to changes and cope with some problems, like symptoms, complications, and lifelong treatment. The methods they employ become the most significant factors in determining their quality of life.

Table 4. Predictors of quality of life in hemodialysis patients (gender, education level, employment status and income)

Variabel	Quality of Life		Total	95% Confidence Interval	OR
	Moderate/Poor	Good			
Sex					
Male	23 (69.7%)	10 (30.3%)	33	0.56-4.38	1.57
Female	19 (59.4%)	13 (40.6%)	32		
Education Level					
Low	13 (59.1%)	9 (40.9%)	22	0.24-2.01	0.69
High	29 (67.4%)	14 (32.6%)	43		
Employment Status					
Unemployed	31 (72.1%)	12 (27.9%)	43	0.88-7.52	2.58
Employed	11 (50.0%)	11 (50.0%)	22		
Income (IDR)/month					
< 6,000,000	41 (65.1%)	22 (34.9%)	63	0.11-31.25	1.86
≥ 6,000,000	1 (50.0%)	1 (50.0%)	2		

X^2 = *Chi-Square* ; *p* = Significance/Probability ; OR = *Odds Ratio*

The percentage of respondents with low education level and moderate/poor quality of life is 59.1%, which is almost twice as high as the percentage of respondents with high education level, which is 67.4%. The odds ratio (OR) value is 0.69, indicating that respondents with low education level are 0.69 times less likely to have moderate/poor quality of life compared to respondents with high education level. However, this difference is not statistically significant (95% CI, 0.24-2.01), meaning that there is no significant difference between education level and quality of life. Our results are consistent with previous research (Anggraeni, 2016; Bosniawan, 2018; Risky, 2019), indicating that education is not associated with quality of life. Education level plays an important role in determining the health status and quality of life of chronic kidney disease patients undergoing hemodialysis, as individuals with higher education are considered to be more knowledgeable, more receptive to information recommended by healthcare professionals for their treatment efforts, and more aware of their medical issues. The quality of life improves with higher education levels (Fadlilah, 2019). The researchers assume that both respondents with high and low education levels are capable of taking care of their health and are receptive to input from medical professionals and their families. They have their own ways of seeking information about their condition and treatment.

The percentage of respondents who are unemployed and have moderate/poor quality of life is 72.1%, which is higher than the percentage of employed respondents, which is 50.0%. The odds ratio

(OR) value is 2.58, indicating that unemployed respondents are 2.58 times more likely to have moderate/poor quality of life compared to employed respondents. However, this difference is not statistically significant (95% CI, 0.88-7.52), meaning that there is no significant difference between employment status and quality of life. Our study results are consistent with previous research, indicating that employment status is not associated with quality of life (Risky, 2019). In the study by previous study, individuals who are employed have stronger social bonds and support. Having a job can help individuals cope with life difficulties and reduce life stress (Primastuti, 2017). The economic status of employed respondents is more stable, which positively impacts their quality of life. The researchers assume that each individual has a different quality of life, and thus, employment status is not always related to quality of life, as many factors can influence respondents' quality of life. Although employment can provide higher income and social status, unemployed respondents can have a good quality of life if they are in good health and have adequate social support.

The percentage of respondents with an income of less than 74 million IDR per year has a moderate/poor quality of life is 65.1%, which is 40 times higher than the percentage of respondents with an income of 74 million IDR or more per year, which is 50.0%. The odds ratio (OR) value is 1.86, indicating that respondents with an income of less than 74 million IDR per year are 1.864 times more likely to have a moderate/poor quality of life compared to respondents with an income of 74 million IDR or more per year. However, the result is not statistically significant ($p>0.05$), meaning that there is no significant difference between income and quality of life. The previous studies mentioned contradictive results that the income had significant association with quality of life (Rustandi et al., 2018; Simorangkir et al., 2021).

Table 5. Predictors of the quality of life in HD patients (Duration of hemodialysis and number of prescribed medications)

Variable	Mean		U	*p*
	Moderate/Poor QOL (n=42)	Good QOL (n=23)		
Duration of hemodialysis (months)	36.55	26.52	334.000	0.041
Number of Prescribed Medications (types of medication)	35.27	28.85	387.500	0.178

U = Mann-Whitney; p = significance/probability; QOL = *Quality of Life*

As Table 5 indicates, the average duration of hemodialysis for respondents in the moderate/poor quality of life group is 36.55 months, which is higher than the average duration of hemodialysis for respondents in the good quality of life group, which is 26.52 months. There is a significant difference in the average duration of hemodialysis between the group of patients with moderate/poor quality of life and the group with good quality of life. This finding is consistent with previous research (Barbosa et al., 2017; Naseef et al., 2023), that indicates the duration of hemodialysis is associated with quality of life.

The average number of prescribed medications for respondents in the moderate/poor quality of life group is 35.27 types of drugs, which is higher than the average number of prescribed medications for respondents in the good quality of life group, which is 28.85 types of drugs. However, there is no significant difference between the number of prescribed medications and quality of life. The number of medications prescribed is associated with the presence of comorbidities or coexisting diseases experienced by the respondents. This finding is different from a previous study (Simorangkir et al., 2021), which found a relationship between the number of prescribed medications and quality of life.

The use of medications in patients with chronic kidney disease undergoing hemodialysis is associated with the respondents' comorbidities and additional symptoms in some cases (Sekti, 2020).

Respondents with multimorbidity tend to have a negative perception of their health, due to the increasing number of prescribed medications (Pratiwi et al.,2019). The researchers assume that respondents view the prescribed medications as a means to help them overcome their illness, so the quantity of medications given does not affect their quality of life. Moreover, they already understand that if their condition is not treated, it could worsen over time.

The limitation of this study is related to the small sample size. This small sample size may impact the statistical power to detect predictors of quality of life in hemodialysis patients. Further research should use a larger sample size and include other factors that can influence the quality of life of patients.

CONCLUSION

The quality of life of hemodialysis patients at dr. S. Hardjolukito regional Military Hospital is mostly moderate/poor (64.6%). In this study, the factors of age and duration of hemodialysis were identified as predictors of quality of life.

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Development of standardized kaffir lime (*Citrus hystrix*) fruit peel extract as a gel for antioxidant and anti-acne

Farida Lanawati Darsono, Lisa Soegianto*, Maria Anabella Jessica
Faculty of Pharmacy, Widya Mandala Catholic University Surabaya,
Jl.Raya Kalisari Selatan No 1, Kampus Pakuwon City Laguna Surabaya, East Java, Indonesia

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ABSTRACT

Topical acne treatment using antibiotics causes an increase in the resistance of acne-causing bacteria. Using natural ingredients is an effort to avoid resistance, such as kaffir lime fruit peel which contains antibacterial substances, namely alkaloids, flavonoids, and tannins. This study aims to determine the effect of increasing the concentration of condensed extract of kaffir lime fruit peel in gel dosage form on physical quality (pH value, viscosity, dispersion) and effectiveness as an antioxidant and anti-acne. The condensed extract was obtained by maceration with 95% ethanol and then standardization of specific and non-specific extracts was carried out. The dosage form chosen is hydrophilic gel. The concentrations of the condensed kaffir lime fruit peel extract used in the gel are F1 (10%), F2 (15%), and F3 (20%). The gel preparation was tested for physical quality and effectiveness, consisting of antioxidant activity (IC_{50}) using the DPPH method and antibacterial activity against *Cutibacterium acnes* using the diffusion method. Experimental data between batches and between formulas were analyzed using the One-Way ANOVA statistical method. If there is a significant difference in statistical analysis between formulas, then the test is continued using the Tukey post-hoc test method. The experimental results showed that increasing the concentration of kaffir lime fruit peel extract (*Citrus hystrix*) caused a decrease in pH and viscosity values as well as an increase in the ability to spread the gel preparation. Increasing the extract concentration also causes an increase in the anti-acne effect with the largest inhibition zone (18.27 ± 0.306 mm), and effectiveness as an antioxidant with the smallest IC_{50} value (15.51 ± 0.15 mg/mL) in formula 3. It was concluded that the best antioxidant and anti-acne gel is the F3 formula with an extract concentration of 20%.

Keywords: *anti-acne, antioxidant, Citrus hystrix, kaffir lime fruit peel extract,* gel

**Corresponding author:*
Lisa Soegianto
Faculty of Pharmacy, Widya Mandala Catholic University Surabaya
Jl.Raya Kalisari Selatan No 1, Kampus Pakuwon City Laguna Surabaya
Email: lisa-s@ukwms.ac.id

INTRODUCTION

Acne is a facial condition with clogged skin pores due to excess oil (sebum) production (Krysandrika, 2023). Acne treatment can be oral or topical. The advantages of using oral and topical antibiotics are more effective in dealing with acne. At the same time, the drawback is easy to develop bacterial resistance to antibiotics, such as irritation (Dessinioti et al., 2022; Kayiran et al., 2020) which states that an increase in the prevalence of resistant *Cutibacterium acnes* was found in clindamycin antibiotics from 45% to 91% and tetracycline antibiotics from 5% to 26.4%.

Based on this, it is necessary to develop anti-acne preparations made from natural ingredients that are expected to be effective and more friendly to facial skin, with the risk of antibiotic resistance being avoided. Many natural ingredients have compounds that have medicinal properties so they have the potential to become an alternative in treatment. kaffir lime (*Citrus hystrix*) is a natural ingredient that is widely used by the community and kaffir lime contains alkaloids, flavonoids (naringenin and hesperidin, and tannins as antibacterial) (Rusmiat et al., 2023). Meanwhile, the most common flavones in kaffir lime fruit peel are naringenin and hesperidin (Guan et al., 2020). The potential effect of kaffir lime fruit peel which has been widely studied, namely as an antioxidant using the DPPH method at a concentration of 100 mg/mL, gives a result of 92.78% of inhibition higher than the percentage of inhibition for other extracts (Ramli et al., 2020). In another study, the IC_{50} value of kaffir lime leaves was 279.03 mg/mL tested using the DPPH method (Othman et al., 2023).

This research was conducted to increase the effectiveness of using kaffir lime peel in the form of a condensed extract as an anti-acne by developing a formulation in hydrophilic gel preparations. The gel form has a better penetration speed than the cream or ointment form, considering that the gel base has a high-water content, which causes an increase in the hydration, making it easier for the gel to penetrate, easy to wash off, and provides a cooling feeling. Application on the skin can reduce the risk of inflammation due to oil production in the skin pores, making it suitable for use as an anti-acne preparation. In addition, the gel form spreads quickly on the skin, the appearance of the color is clear, and it causes a cold sensation so that the acceptability by consumers is greater (Safitri et al., 2020).
This study aimed to determine the effect of increasing the concentration of kaffir lime fruit peel extract gel preparations on physical quality and effectiveness and the correlation between increased antioxidant activity and its anti-acne (antibacterial) properties. The utilization of kaffir lime fruit peel (*Citrus hystrix*), a native plant of Indonesia, as a cosmetic product is expected to contribute to the discovery of complementary and alternative therapies for anti-acne.

MATERIALS AND METHOD

Materials

The kaffir lime fruit peel was used as an active ingredient obtained from the local market around Surabaya. Gel base excipients consisting of carbopol 941 (Corel Pharma, Meshana, Gujarat- India), TEA (Petronas Chemicals, Labuan, Malaysia), Tween 80 (PT Brataco, Surabaya-Indonesia) were selected. Glycerin (PT Suma Asih, Indonesia) and IPM (Oleon Port Klang, Selangor, Malaysia.) as a solubilizer and penetrant enhancer was added to increase the penetration speed of the gel, and distilled water was used as a solvent. All excipients used were of analytical grade. Other ingredients with a pro analytical grade are DPPH reagent (Sigma Aldrich, Germany) as a reagent used in the antioxidant activity test, Ethanol 96% as a solvent (Merck, Darmstadt, Germany), Tryptic Soy Agar as a growth medium for *Propionibacterium acnes* (Merck, Darmstadt, Germany), bacteria *Cutibacterium acnes* obtained from the Microbiology Laboratory of the Faculty of Pharmacy – WMCSU.

Methods

Preparation and Standardization of dried simplicia powder of kaffir lime fruit peel

The kaffir lime fruit used is immature, characterized by green skin with a rough texture. The peel of the kaffir lime fruit dried in an oven at a temperature of around 50℃. The dried powder simplicial of kaffir lime peel is standardized to be specific and non-specific. The Specific standardization includes: water content, total ash content, acid soluble ash content. Water soluble ash content. Specific

standardization includes: organoleptic, pH, solubility in water, solubility in ethanol (Departemen Kesehatan RI, 2000; Darsono et al., 2020).

Preparation and standardization of kaffir lime fruit peel condensed extract (*Citrus hystrix*)

Preparation of condensed extract of kaffir lime fruit peel by weighing 500 grams, then put into a macerator and 96% ethanol solvent (1:2) at room temperature 25℃ for 24 hours. Yield presentation was calculated against the volume of the liquid extract before being evaporated. The condensed extract of kaffir lime peel is standardized to be specific and non-specific (Departemen Kesehatan RI, 2000); Darsono et al., 2022b).

Determination of the profile of active compounds: naringenin and hesperidin in the condensed extract of kaffir lime fruit peel (*Citrus hystrix*)

Analysis of active compounds of naringenin and hesperidin refer to Dianingati et al. (2017) using the TLC method modification. As a standard solution, naringenin and hesperidin were used with a concentration of 0.5% w/v. Each 8 µl of extract and standard solution was individually spotted onto silica gel 60 GF_{254} TLC plates (250 µm thickness). The plates were eluted in ethyl acetate: methanol: formic acid (95:5:0,5 %v/v). After drying, the TLC plates were examined under UV light at 366 and 254 nm (Camag UV cabinet, USA). The Rf values of the interesting spots were calculated.

Preparation of antioxidant and anti-acne gel containing condensed extract of the kaffir lime fruit Peel (Citrus hystrix)

The gel formula based on antioxidant and anti-acne containing a condensed extract of kaffir lime fruit peel in this study refers to the research of Forestryana & Rahman, 2020 and Wulaningsih, 2010, which has been modified as shown in Table 1. Modification of the formula was carried out at the concentration of condensed extract kaffir lime fruit peel starting with a concentration of 10% (F1), 15% (F2), 20% (F3) and gel bases (FB), and adding isopropyl myristate (IPM) as a penetrant enhancer to increase penetration ability can reach the intended target site and glycerin for moisturizing.

Table 1. Reference formula and modified formula for antioxidant and anti-acne gel containing condensed extract of kaffir lime fruit peel (*Citrus hystrix)*

Composition Reference formula	Composition Formula	Modified Formula (%)			
		F1	F2	F3	FB
	Kaffir lime fruit peel condensed extract	**10**	**15**	**20**	-
Carbopol 940	Carbopol 940	0.5	0.5	0.5	0.5
TEA	TEA	0.5	0.5	0.5	0.5
Tween 80	Tween 80	0.1	0.1	0.1	0.1
	IPM	2	2	2	2
	Glycerin	5	5	5	5
Aquadest	Aquadest (until)	100	100	100	100

Physical quality test of antioxidant and anti-acne gel containing of kaffir lime fruit peel extract (*Citrus hystrix*)

Appearance

The appearance of the prepared formulations including constitution, color, smell, and homogeneity was observed by visual observation.

pH test

Thoroughly weigh 100 grams of antioxidant and anti-acne gel in a glass beaker and determine the pH using a Methron 744 pH meter. The pH of antioxidant and anti-acne gel tests were replicated three

times for each batch. The general requirement for the pH of the gel preparation is the skin pH, which is 4.5-6.5. The expected specifications are pH 5.0 ± 0.5.

Viscosity test

The viscosity test refers to the study of Flieger et al., 2021 with the following modifications. A viscosity test was conducted using a Brookfield viscometer LVDV-I+ (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) with spindle S63 and speed 0.6 rpm for 250 mL of antioxidant and anti-acne gel. The general requirements for the viscosity of a gel preparation containing the gelling agent carbopol 940 NF Polymer will provide a thickness between 40,000-60,000 cPs at a pH value of 7.5-7.7 (Power and Ecu, 2013). The expected viscosity specification is 40,000 s / d > 60,000 cPs (Safitri et al., 2020).

Spreadability test

The spreadability test aims to determine the ability of the preparation to spread on the skin's surface. The spreadability test refers to the research of Shriwas et al. 2019 with the following modifications. The spreadability of the anti-acne gel was measured by spreading 0.5 g of the gel on a 2 cm diameter circle marked on a glass plate, then a second glass plate was used and pressed using two types of weight (50 grams and 100 grams). The diameter of the circle after spreading the gel was determined. The general requirements for dispersion for gel are 5-7 cm. The specifications expected in this study are 5.0±0.5 cm, with criteria easy to spread.

Antioxidant activity of antioxidant and anti-acne gel containing condensed extract of kaffir lime fruit peel (*Citrus hystrix*) using the DPPH method with a spectrophotometer microplate reader

Preparation of 0.5 mM of DPPH solution

The DPPH 0.5 mM solution (concentration 200 ppm) was pipetted as much as 5 mL, put into a measuring flask, and diluted with methanol ad 25 mL as a blank solution (40 µg/mL). The DPPH solution with a concentration of 40 µg/mL was homogenized and the absorption was measured at a wavelength of 400-800 nm (Molyneux, 2004).

Preparation of test solution

The gel preparation of each formula (F1, F2, and F3) was weighed as much as 1 gram and dissolved in 10 mL methanol pro analysis and then homogenized using vortex. The preparation of the control solution consisted of 100 µl of DPPH solution mixed with 100 µL of methanol pa. The preparation of the sample solution consisted of a test solution mixed with a 0.5 mM DPPH solution at a ratio of 1:1. The preparation of a blank sample solution consisted of 100 µL of the test solution and 100 µL of methanol pro analysis. The preparation of the control solution, sample solution, and blank solution were pipetted into a 96-well plate well. After incubation for 45 min at room temperature in the dark, the decrease in absorbance of each solution was read at 517 nm using a microplate reader (Multiscan GO *Microplate Reader* (Thermoscientific, Finlandia) against a blank (methanol). A solution of l-ascorbic acid (100 µl), at concentrations between 5 and 50 µg/mL in methanol was used as a positive control. All experiments were determined in triplicate. Furthermore, the absorbance value obtained is used to determine the IC_{50} value. Based on the data obtained, the correlation between extract concentration and increase in the IC_{50} value was determined with the parameter in the form of calculated R values at = 0.05 and df = 1 (Darsono et al., 2020).

Antibacterial activity of anti-acne gel containing condensed extract of kaffir lime fruit peel (*Citrus hystrix*) by well diffusion method

Preparation of Bacteria Suspension

Cutibacterium acnes in TSA slanted taken one ose, suspended into the TSB, and then measured for turbidity with standard Mc Farland I (1.5 x 108 CFU/mL).

Preparation of test solution

Test solutions 10%, 15%, and 20% w/v were made by weighing 0.1 g; 0.15 g; and 0.2 g of kaffir lime fruit peel extract (*Citrus hystrix*), then each was dissolved in 1 mL of 1% DMSO.

Diffusion method antibacterial activity test

The bacterial suspension was first equalized to Mc Farland I (1.5 x 108 CFU/ml), then 0.1 mL was inoculated into 10 mL TSA and poured into sterile Petri dishes. Then pre-curing for 1.5-2 hours at 37°C, then perforating with a 6 mm diameter perforator. Kaffir lime peel extract (*Citrus hystrix*) with a concentration of 10%, 15%, and 20% was put into the well as much as 20 µL. Added positive controls using the antibiotic clindamycin 2 µG/20µL into the wellbore, and negative control was introduced using 1% DMSO 20 µL. The media was incubated for 24 hours at 37°C (Binder, Germany). After incubation, a clear area around the hole was observed, indicating microbial growth inhibition. The antimicrobial activities were measured as the diameter (mm) of the inhibition zone (n = 3). Gel preparations have antibacterial activity if the DHP value is more significant than 6.0 mm (Soegianto et al., 2016).

RESULT AND DISCUSSION

Results of standardization of dried powder of simplicia of kaffir lime fruit peel (*Citrus hystrix*)

Standardization carried out includes non-specific and specific. The results of the non- specific standardization of dried simplicia powder of kaffir lime fruit peel (*Citrus Hystrix*.) can be seen in Table 2**.** Non-specific standardization of Simplicia powder of kaffir lime fruit peel (*Citrus hystrix*.) with parameters of water content aims to provide a minimum limit or range regarding the amount of water content in a material. If the water content value is relatively high, microbes and bacteria will easily contaminate the material. Enzymatic reactions quickly occur, which causes the active ingredients to be hydrolyzed. The results obtained for determining the water content are 8.89± 0.89%, where these results meet the general requirements for water content that have been set, which is less than 10% (Departemen Kesehatan RI, 2000). The total ash content was determined the mineral content in the simplicial powder of kaffir lime fruit peel (*Citrus hystrix*). The test results were obtained at 5.52±0.23%, which means that the mineral content in the simplicial powder of kaffir lime peel (*Citrus hystrix*) met the requirements which are less than 7%.

Result of standardization of condensed extract of kaffir lime peel extract (Citrus hystrix)

The results of making a condensed extract of kaffir lime fruit peel (*Citrus hystrix*) obtained an average yield calculated based on the weight of the dry simplicia powder, which was 17.59±1.70%. The standardization includes non-specific and specific. The results of standardization of the condensed extract of kaffir lime fruit peel extract can be seen in Table 2. The results of an organoleptic test of the condensed extract of kaffir lime fruit peel *(Citrus hystrix*) had a thick greenish-yellow color and a characteristic odor of kaffir lime fruit. The pH value of the condensed extract of kaffir lime fruit peel was 5.59 ± 0.02, which indicated that the viscous extract was acidic. Carried out the determination of water-soluble extract content and ethanol soluble extract content to determine the content of compounds in certain solvents according to their polarity level, kaffir lime peel has a higher solubility in ethanol than water solvents. The results of the determination of the water-soluble extract content of the kaffir lime peel (*Citrus hystrix*) were 58.11 ± 0.78% and the results of the determination of the ethanol-soluble extract content of 65.93±0.35%. The full results of the standardization of condensed extracts can be seen in Table 2**.**

The active compound profile of condensed extract of kaffir lime fruit peel: hesperidin, or naringenin, was determined by thin layer chromatography with UV light 254 and 366 nm VIS. The results of the active substance profiles in the extract with the mobile phase of ethyl acetate: methanol: formic acid (95: 5: 0.5) %v/v and the stationary phase of silica gel 60 GF_{254} showed that in the extract containing active compounds, namely hesperidin, naringenin, and routine.

The stain detected under 366 nm has a value of Rf 0.70 for a fluorescent blue colour is hesperidin, for a bluish-green fluorescent stain with a value of Rf 0.14 is naringenin. It is mostly for a green stain with an Rf of 0.07. The theoretical Rf values for the active substances rutin, naringenin, and hesperidin were 0.167, 0.88, and 0.07, respectively. The complete result of the TLC profile can be seen in Figure 1**.** The difference in Rf value is due to several factors, including extraction and solvent used during the TLC analysis process. Still, in general, it can say that the extract contains active substances that have anti-acne properties.

Table 2. Standardization of dried powder simplicia and condensed extract of kaffir lime fruit peel (*Citrus hystrix*)

Test Type	Observation result	
	dried powder simplicia	condensed extract
Specific Standardization		
Form	Powder	Condesed
Colour	Yellow green	Yellow green
Smell	Kaffir lime	Kaffir Lime
pH (1% solution)	-	5.59 ±0.02
Water Soluble Extract Level ($\bar{x}$ ± SD) (%)	27.82 ± 3.77	58.11 ± 0.78
Ethanol Soluble Extract Level ($\bar{x}$ ± SD) (%)	20.81 ± 0.58	65.93 ± 0.35
Non Specific Standardization		
Water content ($\bar{x}$ ± SD) (%)	8.89 ± 0.89	17.82 ± 0.86
Total Ash Content ($\bar{x}$ ± SD) (%)	5.52 ± 0.23	2.17 ± 0.22
Acid Insoluble Ash Content ($\bar{x}$ ± SD) (%)	0.61 ± 0.001	0.04 ± 0.001
Water Soluble Ash Content ($\bar{x}$ ± SD) (%)	5.17 ± 0.13	2.17 ± 0.42

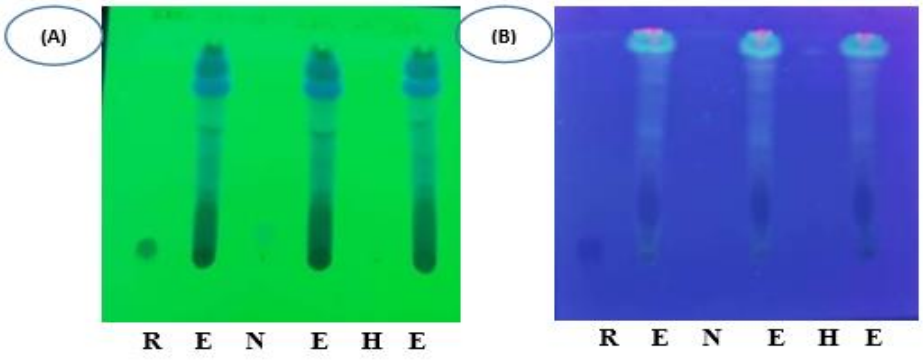

Figure 1. The comparative stain profile of hesperidin, naringin, rutin, and condensed extract of kaffir lime peel (*Citrus hystrix*) by Thin Layer Chromatography (TLC) under UV light 254 nm (A), and UV light 366 nm (B) with R = rutin, N = naringin, H = hesperidin, E = condensed extract of kaffir lime fruit peel

Results of antioxidant activity test of condensed extract of kaffir lime fruit peel (*Citrus hystrix*)

The results of testing the antioxidant activity of the condensed extract of the kaffir lime fruit peel (*Citrus hystrix*) can be seen in Figure 2. Based on the results of the antioxidant power test of the condensed extract of kaffir lime fruit peel, it is known that it has antioxidant power according to the expected specifications. The lower the IC_{50} value of a compound, the greater the antioxidant activity. The test results found that the IC_{50} value of the condensed extract of kaffir lime fruit peel was 2.49 mg/mL (249.43±0.51 µg/mL) and vitamin C was 3.70±0.01 µg/mL.

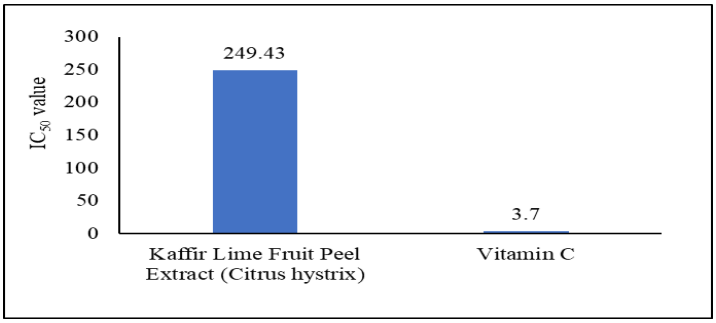

Figure 2. Antioxidant activity of kaffir lime fruit peel extract (*Citrus hystrix*) and vitamin c

Physical quality test results of anti-acne gel containing condensed extract of Kaffir Lime Fruit Peel (*Citrus hystrix*)

Organoleptic test results

Kaffir lime fruit peel extract has an organoleptic appearance such as yellow-green color, opaque gel form, and a characteristic kaffir lime odor. The blank formula has a white appearance, an opaque gel form, and no odor.

pH test results

The purpose of the pH observation was to determine the pH value of the anti-acne gel preparation of kaffir lime peel extract, following the pH of the skin to prevent skin irritation. The pH test results obtained can be seen in Figure 3**.** Formulas F1, F2, and F3 gave pH values of 5.37 ± 0.06, 5.10 ± 0.01, and 4.78 ± 0.00, respectively, which met the dosage specifications of 5.00±0.05. The pH value of the blank formula (FB) is 6.97 ± 0.02. Based on the results of statistical analysis using the one-way ANOVA method, the calculated Fvalue (0.000) < Ftable (α = 0.05) was obtained with a significance value (sig.) or p-value obtained of 0.000 <0.05 which shows a significant difference between the formulas. The blank formula with the formula with condensed extract of kaffir lime peel has a different pH due to the influence of the acidic pH of the condensed extract, which is around 5.59 ±0.02. The formula with a condensed extract of kaffir lime peel has a different pH value for each formula, and this is because the effect of the concentration of the extract used is increasing, increasing the acidity of the preparation.

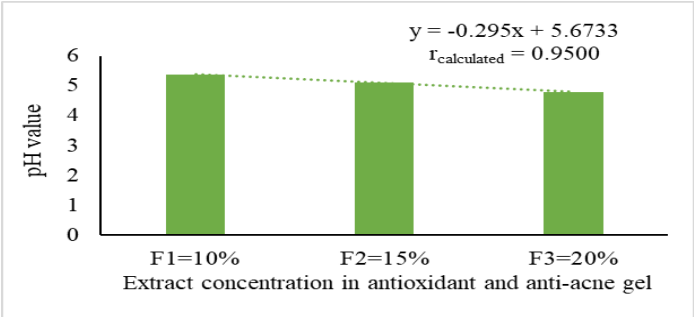

Figure 3. The relationship between pH values and gel formula containing condensed extracts of kaffir lime fruit peel (*Citrus hystrix*) at various concentrations

Homogeneity test results

A homogeneity test was conducted to determine the level of admixture of additives and active ingredients, in this case, the condensed extract of kaffir lime fruit peel. Homogeneous antioxidant and

anti-acne gel preparations can be seen whether there are lumps or coarse particles. The observations showed that the anti-acne gel preparations of condensed extract of kaffir lime fruit peel in each formula, namely F1, F2, F3, and FB, had a good level of homogeneity. Each formula provides an even texture, and no coarse particulates are visible, so the gel preparation can be said to be homogeny.

Viscosity test results

The viscosity test results obtained can be seen in Figure 4**.** Viscosity testing determines the viscosity level of the anti-acne gel preparation of kaffir lime peel extract, which affects the dispersion parameters when applied to the skin surface. Viscosity with a low value will affect a large spread area. If the viscosity value is high, it will reduce the spread area, considering that increasingly viscous preparations will make it difficult to apply on skin reinforcement because the dispersion power is not good. The viscosity value of the anti-acne gel preparation of kaffir lime fruit peel extract is 40,000 to > 60,000 cPs (Power and Ecu, 2013; Safitri et al., 2020). Viscosity observations of each formula were F1, F2, F3 and Fb, respectively: 367,000 ± 21,000, 273,833 ± 1,833, 161,999 ± 2,334 and 36,367 ± 5,067. Based on the results of statistical analysis, they are using the one-way ANOVA method, and the calculated F_{value} (0.000) < F_{table} (α = 0.05) shows that the data between formulas is significantly different. The ANOVA analysis shows that the significance value (sig.) or p-value obtained is 0.000, and this indicates that there are significant differences between the three tested formulas (sig. < 0.05 means that there is a significant difference between the tested groups). The formula with a condensed extract of kaffir lime peel has a different pH value for each formula. The increasing concentration of kaffir lime peel extract causes the viscosity value of the gel preparation to be smaller where the consistency of the preparation becomes thinner. Considering that the optimum pH of carbopol® as a gelling agent is in the range of pH values that are not too acidic, which is around neutral at pH 7.5 - 7.7 because carbopol® is stable at that pH and carbopol® is incompatible with strong acids (Shah et al., 2021). In its neutral form, carbopol is soluble in water, alcohol, and glycerin and will form a clear and stable gel. In an acidic solution (pH 3.5-4.0), carbopol® dispersion shows a low to medium viscosity, and at a pH of 5.0-10.0 and temperatures above 75^0C will show optimal viscosity. In addition, the content of active substances that were successfully extracted in the kaffir lime peel extract also influenced the viscosity of the preparation, wherein the presence of the kaffir lime peel extract. The gel preparations formed a thicker mass with a consistency that could still pour because of the presence of astringent and ballast substances.

Spreadability test results

The spreadability test was carried out on each formula with the aim of knowing the ease with which the preparation spread when used. Determination of spreadability using two types of weights, namely 50 and 100 grams, aims to determine the amount of hand pressure when applied to the surface of facial skin, which provides good spreadability. The results of the dispersion test obtained can be seen in Figure 5**.** The results of observations of dispersion with a load of 50 grams from each formula F1, F2, F3, and FB are as follows: 3.98 ± 0.00, 4.28 ± 0.11, 4.73 ± 0.12, 5.53 ± 0.20. As for the load of 100 grams, the results were as follows: 4.23 ± 0.00 (F1), 4.65 ± 0.20 (F2), 5.01 ± 0.18 (F3) and 6.09 ± 0.02 (FB). Based on the results of statistical analysis using the one-way ANOVA method, the calculated F_{value} (0.000) > F_{table} (α = 0.05) shows that the data between formulas is significantly different. The ANOVA analysis shows that the significance value (sig.) or p-value obtained is 0.000, and this indicates that there are significant differences between the three tested formulas (sig. < 0.05 means that there is a significant difference between the tested groups). The ability of the preparation dispersion to the viscosity value has an inversely proportional value. Each formula has a high viscosity, but low spreadability but is still within the required range. Conversely, if the formula has a low viscosity and high dispersion value. The higher the concentration of the kaffir lime peel extract used in the formula, the lower the viscosity value, so it can say that the preparation is getting dilute, which impacts the higher dispersing ability (Chellathurai et al., 2023).

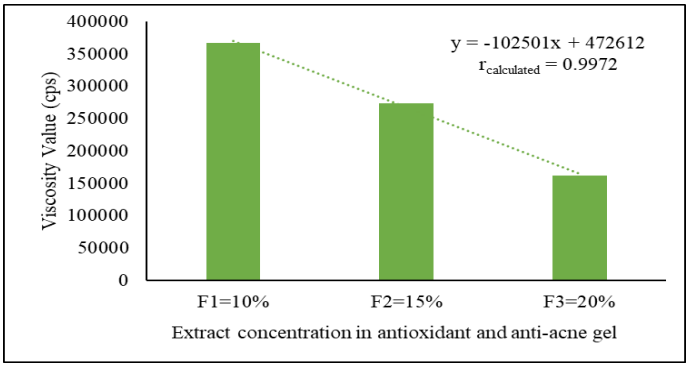

Figure 4. Relationship between viscosity value and gel formula containing condensed extracts of kaffir lime fruit peel (*Citrus hystrix*) at various concentrations

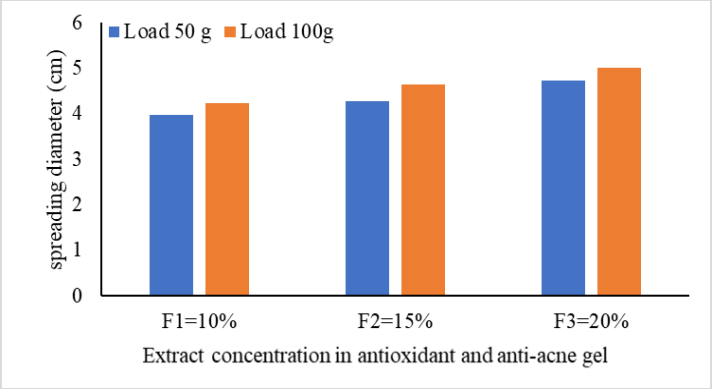

Figure 5. Relationship between spreadability and gel formula containing condensed extracts of kaffir lime fruit peel (*Citrus hystrix*) at various concentrations

Test results of the effectiveness of antioxidant and anti-acne gel containing condensed extract of kaffir lime fruit peel (*Citrus hystrix*)

Antioxidant test results

The results of the antioxidant test preparations obtained can be seen in Figure 6. From the previous research data, the IC_{50} value of the condensed extract of kaffir lime fruit peel was found to be 2.49 mg/mL and the Vitamin C as a comparison was found to be 37 µg/mL. In this research, three gel preparation formulations were made with 3 different concentration of condensed extract of kaffir lime fruit peel (F1, F2, and F3) and the antioxidant activity was tested for each formulation. It was found that the IC_{50} value for F1 was 50.04 ± 11.28 mg/mL, for F2 was 18.45 ± 2.89 mg/mL, and for F3 was 15.51 ± 0.15 mg/mL. The difference in antioxidant power that occurs is due to an increase in the concentration of kaffir lime peel extract from a concentration of 10% (F1), 15% (F2), and 20% (F3), increasing the number of nutritious active compounds contained in the extract so that the impact on increasing their antioxidant power.

The antioxidant activity of kaffir lime peel is due to the content of flavonoid compounds, namely naringenin and hesperidin, which have antioxidant activity. Judging from the basic structure of flavonoids, the presence of hydroxyl group (-OH) attached to the aromatic ring can break down free radicals, or also called ROS (Reactive Oxygen Species) because they have high reactivity as hydrogen donors which will stabilize free radicals. Increasing the concentration in formula 3 causes the amount of flavonoids contained in it to be greater. Therefore, the IC_{50} value in formula 3 shows the lowest

value, or it could be said that the antioxidant activity of formula 3 is the highest compared to the other two formulas. Furthermore, statistical tests were conducted to determine whether there was a significant difference between the formula and the antioxidant activity obtained. The calculation result shows a p-value of 0.02, which is smaller than 0.05 and indicates a significant difference between the formula and the antioxidant activity.

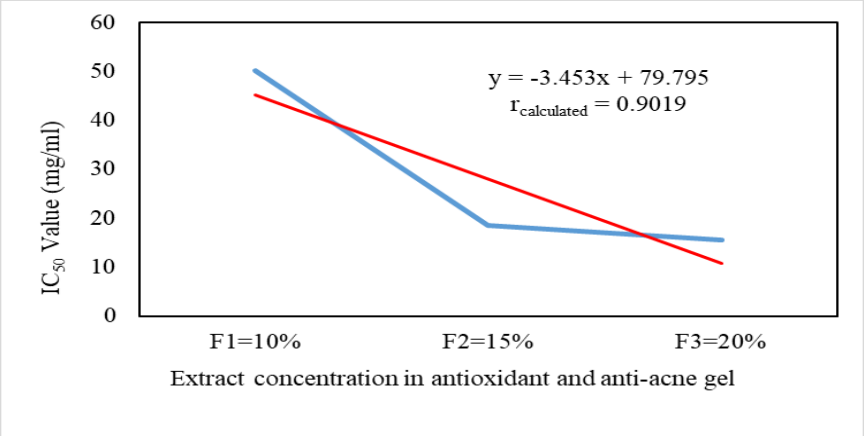

Figure 6. Relationship between increasing concentration (%) of the condensed extract of kaffir lime fruit peel (*Citrus hystrix*) in anti-acne gel preparations on the IC_{50} value

Results of antibacterial effectiveness test against *Cutibacterium acnes* with the well diffusion method of anti-acne gel containing condensed extract of kaffir lime fruit peel (*Citrus hystrix*)

The results of antibacterial activity test of kaffir lime fruit peel extract (Citrus hystix) and anti-acne gel containing kaffir lime fruit peel extract against Cutibacterium acnes

The activity test was carried out using the diffusion method. The results of the antibacterial activity test of kaffir lime fruit peel extract (*Citrus hystix*) and kaffir lime peel extract gel preparations against *Cutibacterium acnes* can be seen in Figures 7. The test results showed the presence of antibacterial activity of antioxidant and anti-acne gel containing kaffir lime fruit peel extract (*Citrus hystix*) at concentrations of 10%, 15%, and 20% with zone inhibition (ZI) areas respectively 12.78 ± 0.370 mm, 13.63 ± 0.154 mm, 13.87 ± 0.118 mm. The results of the ZI data were analyzed using ANOVA statistics, and the significance value (sig.) or p-value was 0.000, and this indicates a significant difference between the three tested formulas (sig. < 0.05 means a significant difference between the groups tested). The test results of ZI with 1% DMSO and gel base had no activity, and this indicates 1% DMSO solution used to dissolve the kaffir lime fruit peel extract and the gel base used to make the kaffir lime peel extract gel did not affect the results of the antibacterial activity against *Cutibacterium acnes*. The difference in antimicrobial activity obtained with the ZI parameters was due to differences in the concentration of the kaffir lime fruit peel extract used. The higher the concentration, the greater the content of the active ingredients diffuse through the agar medium, thereby providing greater ZI. Besides that, it is also supported by the higher concentration of kaffir lime fruit peel extract. The more acidic the gel preparation causes the preparation to be more dilute, making it easier for the diffusion process to the agar medium containing the bacteria *Cutibacterium acnes*. The antibacterial activity of kaffir lime fruit peel extract is due to the presence of phenolic and flavonoid compounds that inhibit bacterial growth by inhibiting cell membrane function, interfering with cell membrane permeability, and inhibiting essential enzymes such as ATPase and phospholipase (Hasan et al., 2022). The antibacterial activity of gel preparations containing kaffir lime fruit peel extract was lower than that of unformulated kaffir lime fruit peel extract. The inhibition zone produced from kaffir lime fruit peel condensed extract differ from the gel form. It is due to the acidic nature of the condensed extract of

kaffir lime fruit peel, which affects the viscosity of the gel, and the ability of the gel to diffuse into media is limited. The effectiveness of carbopol as a gelling agent depends on the pH value of the gel. Based on the study's results, it was concluded that the increase in the concentration of kaffir lime peel extracts affected the physical qualities: pH, viscosity, and dispersion, as well as effectiveness as antibacterial and antioxidant. Increasing the concentration of kaffir lime peel extract led to a significant increase in effectiveness as an antioxidant and anti-acne.

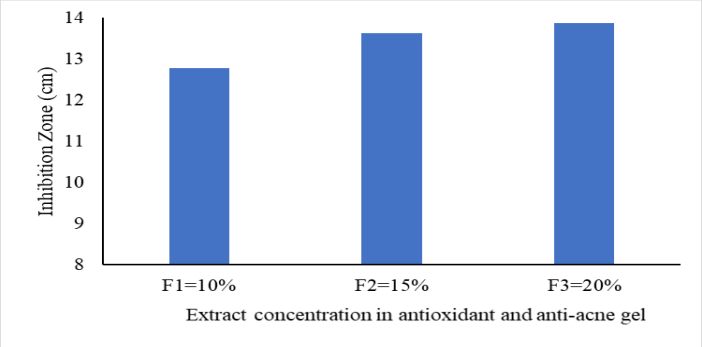

Figure 7. Antibacterial activity of Antioxidant and Anti-acne Gel containing kaffir lime fruit peel extract

The formula was determined as the best formula that fulfilled all the specifications of the physical quality test that referred to the general requirements of the gel preparation and had the best anti-acne effectiveness and antioxidant activity. Based on the overall physical quality evaluation results, formula 3 (20% kaffir lime peel extract concentration) is a formula that better meets all test specifications.

CONCLUSION

Increasing the concentration of kaffir lime peel extract (*Citrus hystrix*) (10%, 15%, and 20%) affects the results of physical quality (pH, viscosity, spreadability) and the effectiveness of the preparation as an anti-acne and antioxidant. Formula 3 (20% concentration of kaffir lime peel extract) is the best anti-acne gel formula that meets the specifications for physical quality and activity antibacterial against *Cutibacterium acnes.*

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Sunscreen effectivity and physical characterization of avocado oil in nanoemulsion using isopropyl myristate variations

Ayu Shabrina[1*], Zuhriya Muna Firdausi[2], Agnes Theresya Poerba[2], Diana Anisa Setyani[2], Junvidya Heroweti[3]

[1]Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmcy, Universitas Wahid Hasyim
Jl. Raya Manyaran-Gunungpati, Nongkosawit, Semarang, Central Java
[2]Bachelor Program Study, Faculty of Pharmacy, Universitas Wahid Hasyim
Jl. Raya Manyaran-Gunungpati, Nongkosawit, Semarang, Central Java
[3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Wahid Hasyim
Jl. Raya Manyaran-Gunungpati, Nongkosawit, Semarang, Central Java

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ABSTRACT

Unsaturated fatty acids in avocado oil can help reduce erythema brought on by prolonged UV-B exposure. The effectivity of sunscreen absorption into the skin will be enhanced by the use of isopropyl myristate (IPM) in nanoemulsion. The purpose of this study was to determine the physical characteristics and sunscreen effectiveness of avocado oil nanoemulsion (AVN) modified with IPM. 1% (FI), 3% (FII), and 5% (FIII) IPM variation were used to make AVN with 5% oil. The AVN were tested for physical characteristics such as organoleptic, pH, viscosity, rheology, particle size and polydispersity index (PI). The products were also tested for sunscreen effectivity by in vitro and Minimum Erythemal Dose (MED) method. The data obtained were analyzed statistically. The results showed that the AVN was pale yellow and clear with transmittance percentage were 96%. The rheogram showed that the products were newtonian. The pH values range were from 6.62 to 6.66; viscosity 1.65-1.84 dPa.s; particle size < 17 nm, zeta potential was in range of -30,54±1,72 to -37,85±3,11 and PI < 0.5 for all formula. In vitro SPF values were 16.43 ± 4.50 (FI), 16.27 ± 4.20 (FII) and 17.88 ± 3.20 (FIII) ($p > 0.05$), and categorized as ultra protection. MED value were 12.28 ± 1.34 (FI); 12.51 ± 1.68 (FII); and 13.22 ± 1.84 (FIII) ($p < 0.05$) and categorized as maximum protection. Isopropyl myristate increased the sunscreen product's MED value without changing its physical characteristics.

Keywords: avocado oil, enhancer, erythema, nanoemulsion

Corresponding author:
Ayu Shabrina
Department of Pharmaceutics and Pharmaceutical Technology, Faculty of Pharmacy, Universitas Wahid Hasyim
Jl. Raya Manyaran-Gunungpati, Nongkosawit, Semarang, Central Java
Email: shabrina@unwahas.ac.id

INTRODUCTION

Chemical sunscreen absorbs sun rays and converts it into heat energy. This sunscreen can work after being absorbed into the skin, so it has the potential to irritate (Minerva, 2019). Sunscreen functions by either scattering or absorbing solar energy to prevent it from striking the skin directly (Riascos, 2012). The effectivity of sunscreen is expressed as SPF value of more than 10, thus describing its ability to prevent erythema due to sun exposure (Araújo et al., 2016). FDA has prohibited sunscreen that contains Para Amino Benzoic Acid (PABA), avobenzone and its derivate due to its side effect (Pirotta, 2020). PABA has the potential to cause many hormone disorders and breast cancer through skin absorption (Barel et al., 2009). Active compound such as Z-3, octyl-methoxycinnamate will increase luteinizing hormone. Due to this side effect, it is necessary to develop natural sunscreen.

One natural component that may be utilized to prepare sunscreen and absorb UV-B radiation is avocado oil (Flores et al., 2019). Avocado oil has bioactive components that benefit the skin such as palmitic acid and linoleic acid, which can increase skin moisture and prevent skin erythema (Li et al., 2019). Components of avocado oil are also used to heal inflammation and skin erythema through skin barrier repair mechanisms (De Oliveira et al., 2013). Previous study had been shown that avocado oil has sunscreen activity with SPF value of 6 to 16 (Fares et al., 2023).

Nanoemulsion system is one of a promising delivery system for cosmetics. For topical usage, this system has good stability and skin penetration capabilities, particularly for active ingredients derived from essential or plant oils (Mohite et al., 2019). Nanoemulsion system can reduce the globule size and increase the efficacy of oil in cosmetic preparations (Hashim et al., 2019). Preparations that are applied topically, such as sunscreen, must be absorbed quickly by the skin to provide a good therapeutic effect (Argenta et al., 2014). Enhancers like isopropyl myristate (IPM) can be added to improve the absorption of active ingredients (Eichner et al., 2017).

Due to the low surface tension and viscosity, isopropyl myristate (IPM) can increase the physical characteristics of nanoemulsion. IPM fits the properties of nanoemulsions, which have a globule size of less than 1000 nm, and is useful in the production of nanoemulsions because of its low molecular weight (Souto et al., 2022). To stabilize the nanoemulsion during storage, intergranular agglomeration can be avoided by adding IPM to the nanoemulsion formula. When IPM and Tween 80 surfactant are used together, the penetration of the active ingredients can be increased, improving the efficacy of the product (Sondari & Tursiloadi, 2018). Based on the background above, it is necessary to characterize and study the effectivity of avocado oil in nanoemulsion using IPM as an enhancer.

MATERIALS AND METHOD

Materials and tools

A magnetic stirrer (Scilogex®), a pH meter (Electro Lab®), a vortex, a nonirritant bandage (Hypafic®), a cone and plate viscometer (Rheosys Merlin VR II®), a particle size analyzer (SZ-100®), a pH spectrophotometer (Shimadzu®), and sterile gauze (Onemed®) were the instruments used in this study.

The primary component of this study was cosmetic-grade avocado oil, which was acquired with an analytical certificate from PT. Daarjeling, Bandung. The carrier materials utilized were methanol with analytical grade acquired from CV. Multi Kimia Raya, Semarang, tween 80, PEG 400, isopropyl myristate, and benzyl alcohol with cosmetic grade. The Parasol SPF 25++ Spray Sunscreen is the comparison utilized.

The test animals used in the research were 3 rabbits. Inclusion criteria were male, 3 months old, with weight at 1,5-2 kg, in good health condition, with strain of New Zealand White. This test had obtained ethical clearance from the Faculty of Medicine and Health Sciences, Muhammadyah University of Yogyakarta with number: 014/EC-KEPK FKIK UMY/III/2023.

Methods

Avocado oil nanoemulsion formula with variations of isopropyl myristate

Table 1 displays the formula for the nanoemulsion of avocado oil. The temperature of the purified water was raised to 30°C. Benzyl alcohol was dissolved in water and agitated until it was homogenous using a magnetic stirrer running at 700 rpm. In order to create a homogenous mixture, Tween 80 and PEG 400 were added to a solution of benzyl alcohol and filtered water during the first mixing stage. Avocado oil and isopropyl myristate were mixed in the second stage until a clear and transparent nanoemulsion was formed. The second stage of mixing was carried out at a speed of 1000 rpm for 30 minutes.

Table 1. Avocado oil nanoemulsion formula (Shabrina & Khansa, 2022)

Components	Concentration (%)		
	F1	F2	F3
Avocado oil	5	5	5
Isopropyl Myristate	1	3	5
Tween 80	40	40	40
PEG 400	30	30	30
Benzyl alcohol	1	1	1
Purified Water		up to 100	

Evaluation of physical properties of avocado oil nanoemulsion

The color, odor, and phase separation of the avocado oil nanoemulsion were visually observed in order to conduct an organoleptic examination. The pH of the nanoemulsion was measured using 10 milliliters of sample and a pH meter that had been calibrated in a buffer solution with pH values of 4 and 7. 5 mL of the sample was put into a cone and plate type viscometer and rotated six times at a speed of 12 rpm for 30 seconds each to measure the viscosity and rheology. Three milliliters of the sample were put in a cuvette to test the sample's clarity, and a spectrophotometer was used to determine the transmittance %. Using a Particle Size Analyzer (PSA) and zeta sizer with 5 mL samples, tests for particle size, polydispersity index (PI), and zeta potential were conducted. Every test was conducted three times (Shabrina et al., 2022).

Effectivity test of avocado oil nanoemulsion

The first step in determining SPF is to compute the Correction Factor (CF) value by comparing it to Parasol SPF 25++. Each of the following: FI, FII, FIII, and Parasol SPF 25++ were measured out to a maximum volume of 0.5 mL, placed in a 10 mL flask, methanol PA was added to the mark, and the mixture was homogenized with a vortex. A UV spectrophotometer was used to measure the absorbance between 290 and 320 nm, and the absorbance values were noted at every 5 nm interval. The CF value was computed by using the Mansur formula to the received results.

The Minimum Erythema Dose (MED) was used to determine the effectiveness of sunscreen. The test rabbits' hair was shaved off with a shaver 24 hours before to treatment. Five treatment locations were marked on the backs of the shaved animals: FI, FII, FIII, comparison (Parasol SPF 25++), and no treatment. Every test point was identified using a 4x4 cm marker. 311 nm UV light was given to the test animals. We counted the quantity of erythema (red spots) that developed in every test animal group. Minimum Erythema Dose (MED) is the amount of UV light exposure time that elapsed before the erythema appeared. The efficiency of sunscreen was calculated by comparing the MED of exposed skin with skin shielded by preparation and Parasol SPF 25++ (Costa et al., 2015; Heckman et al., 2013).

Data Analysis

One way ANOVA was used to statistically assess the data on pH, viscosity, transmittance percentage, particle size, polydispersity index, and zeta potential and SPF value for each formula. The sunscreen effectivity was calculated by independent t-test analysis of in vitro SPF and MED value data.

RESULT AND DISCUSSION

Figure 1 depicts the physical characteristics of the avocado oil nanoemulsion. Table 2 displays the physical properties of the avocado oil nanoemulsion. The findings demonstrated that the pH range of all avocado oil nanoemulsion formulas was 4.5–8.0, which is appropriate for topical applications (Badan Standarisasi Nasional, 1996). The pH of avocado oil before formulation was 6.70 ± 1.50. Additional ingredients such as surfactants, cosurfactants and enhancers had a pH range of 6.0-7.5. The system experienced an increase in pH after avocado oil was incorporated in nanoemulsion yet did not differ significantly. The preparation will cause a skin irritation if the pH is below 4,2 (BPOM, 2014). The pH results of the preparation met the SNI standards for sunscreen preparations. The results showed that increasing the IPM concentration did not affect the pH of the preparation ($p > 0.05$). The results of the viscosity values can be seen in Table 2.

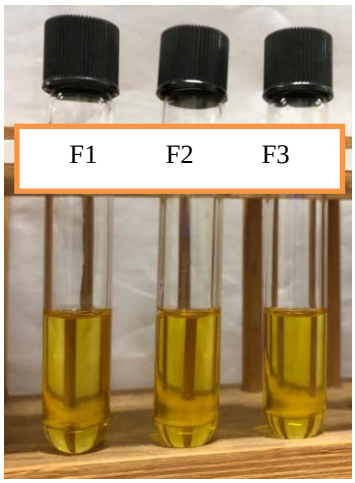

Figure 1. The nanoemulsion of avocado oil

Table 2. Avocado oil nanoemulsion's physical characteristics with IPM variation

Formula	Physical characteristics					
	pH	Viscosity (d.Pas)	Transmittance Percentage (%)	Particle Size (nm)	Polydispersity Index	Zeta Potential (Mv)
FI	6.66±0.03	1.75 ± 0.87	96.50 ± 1.51	15.50 ± 2.13	0.476 ± 0.215	-32.33±2.30
FII	6.65±0.04	1.84 ± 0.83	96.70 ± 1.53	14.75 ± 1.41	0.331 ± 0.227	-30.54±1.72
FIII	6.62±0.03	1.65 ± 0.84	96.90 ± 1.28	13.25 ± 1.35	0.213 ± 0.234	-37.85±3.11

Data displayed were 3 replications with standard deviation

A low viscosity will improve the product's spreadability and user compliance when applied topically. The preparation's viscosity will rise when IPM and Tween 80 are used together as a surfactant (Goyani et al., 2018). This condition can improve the stability of the product due to the inability of globules entrapment and aggregation (Thomas et al., 2014). Compared to other mineral oils, IPM has lower viscosity and interfacial tension, which makes it easier to create small globule

sizes for creating nanoemulsions (Muthi et al., 2016). IPM has the ability to attach to hydrophilic ester groups in the lipid bilayer and integrate with it, making the stratum corneum bilayer membrane's structure more brittle.

IPM belongs to the fatty acid ester group which meets safety requirements and is widely used as an enhancer (Abdullah et al., 2022). Figure 2 showed the outcomes of the rheology analysis of the avocado oil nanoemulsion. Every formula for avocado oil nanoemulsion was Newtonian. According to Marques et al. (2018), a fluid with this rheology has a velocity gradient that is perpendicular to the shear stress and a linearly proportional shear stress. These findings demonstrated that the preparation resembles a solution or liquid that flows readily since the droplets generated were smaller than emulsion (Hasrawati et al., 2016).

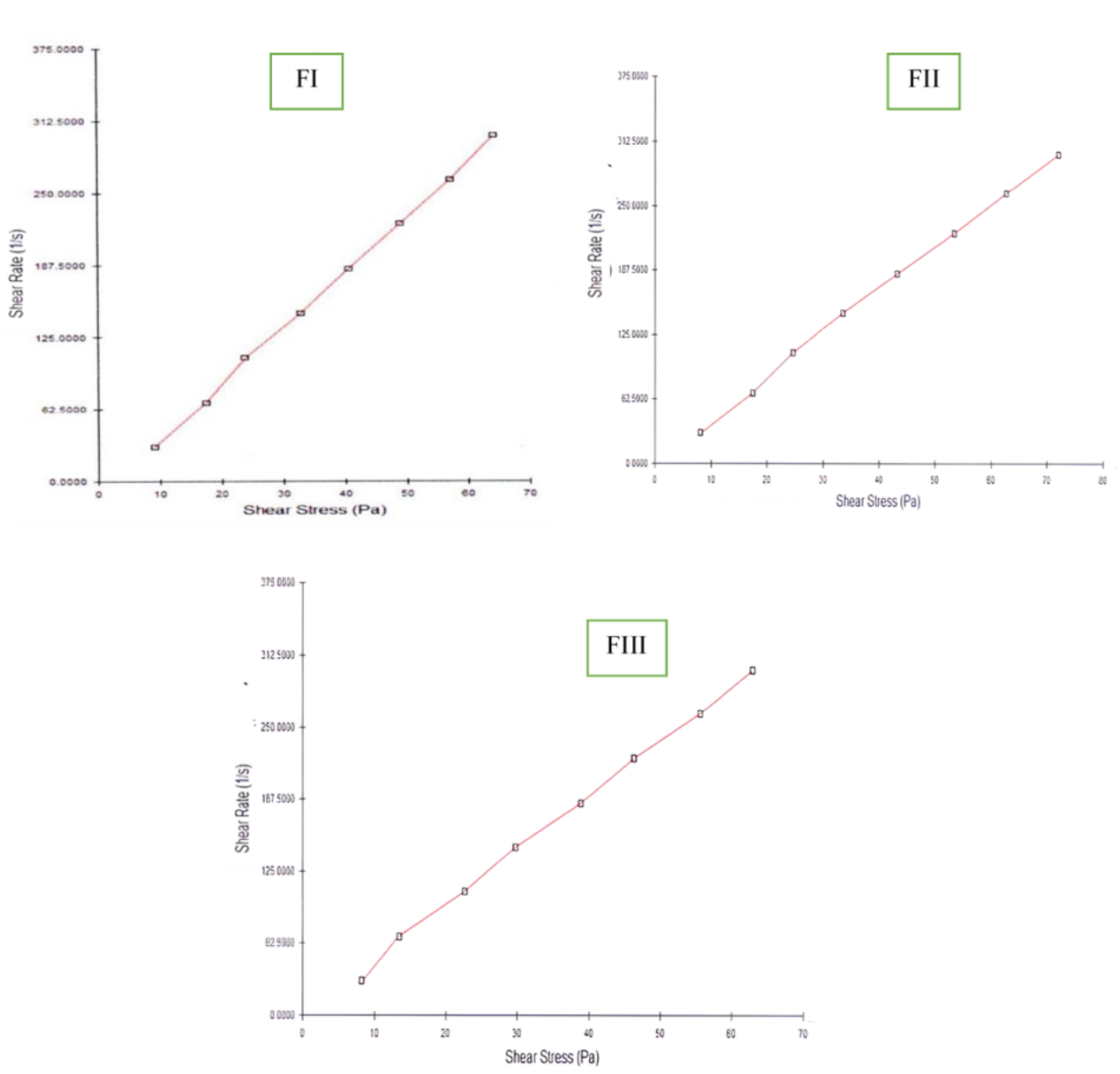

Figure 2. The newtonian rheogram result of avocado oil nanoemulsion with IPM variations

In mixed micelle nanoemulsion formulations, medium chain fatty acids, including IPM, have been employed as permeability enhancers and to promote good absorption of active ingredients (Zhao et al., 2016). Isopropyl myristate when combined with Tween as surfactant will increase the viscosity of the preparation (Sun et al., 2012; Zhao et al., 2016).

Nanoemulsion had a clear and transparent visual appearance with a high transmittance percentage. Nanoemulsions system with smaller dispersed droplet sizes can reduce globule agglomerations to produce a transparrent product. This parameter was determined as transmittance value (%T) nanoemulsion preparations so that transparency can be formed and the percent transmittance value (%T) (Lv et al., 2018). For nanoemulsion, 90–100% percent transmittance is the optimal range (Gurpreet & Singh, 2018). Table 2 showed that the transmittance percentage satisfies the specifications needed for the perfect nanoemulsion. The results of the statistical test indicate that each formula's transmittance % was similar. The preparation of the avocado oil nanoemulsion remained transparent and clear despite variations in the IPM content.

Particle sizes in a suitable nanoemulsion range from 10 to 100 nm (Chen et al., 2017). All avocado oil nanoemulsion formula had particles smaller than 100 nm, according to Table 2's data. This occurs as a result of IPM and surfactant-cosurfactant interaction. In order to facilitate the creation of small particle sizes, Tween 80 and PEG 400 combined with IPM work by the surfactant adsorping the oil and water contact (Cho, 2016; Dalibera et al., 2021). The smaller the globule size will give a slow aggregation of the particles so that creaming in the nanoemulsion can be prevented (Mariadi et al., 2019). The small particle size can extend the shelf life of nanoemulsion preparations, besides that the preparations are not easily damaged and are more easily absorbed by the body (Dwipayana et al., 2022). The globule size results showed that there was a decrease in each formula but it was not significant. IPM is a triglyceride oil that is capable of producing low globule sizes in nanoemulsion systems (De Azevedo Ribeiro et al., 2015).

The PI value < 1 indicates that the nanoemulsion is monodisperse (Eid et al., 2013). The PI value has a correlation with particle size and zeta potential (Gaber et al., 2023). The results of this PI value are in line with previous research that a low PI value indicates a monodispersion system (Caya et al., 2020). Based on Table 2, it is known that variations in IPM concentration did not affect the PI value.

Based on the Table 2, the zeta potential showed that all formula had negative potential. The good zeta potential was close to 30 mv or -30 mv (Shakeel et al., 2021). The statistical analysis showed that there were no significant different of the zeta potential between each formula. The zeta potential of avocado oil nanoemulsion was still fulfill the requirement of nanoemulsion characteristics (Rachman et al., 2023). IPM at 3-5% in formula will show negative zeta potential from -22.5 until -34.6 mV (Abdullah et al., 2022). The zeta potential result that close to zero (0) will intend the short term stability of nanoemulsion and increase the particle aggregation during the storage (Maha & Sinaga, 2018).

The results of the effectiveness of avocado oil nanoemulsion sunscreen using in vitro and MED methods

Their chemical structure, which consists of an aromatic molecule coupled with a carbonyl group, determines the sunscreen's efficacy. This structure facilitates the absorption of high-energy UV radiation, leading to an excited state. The lower energy connected to longer wavelengths is released as the molecule returns to its ground state (Aguilera et al., 2023). A sunscreen's specific wavelength range of absorption varies. In order for topical sunscreen application to effectively prevent sunburn, nourish the skin, and have a photoprotective impact, it must be absorbed into the skin layers (Reza et al., 2023). The use of nanoemulsion systems is one technique for improving sunscreen penetration into the skin. When compared to sunscreen in cream or emulgel, sunscreen in nanoemulsion system has a faster transdermal absorption rate (Chavda et al., 2023).

The results of sunscreen effectivity can be seen in Table 3. A sunscreen product with a known SPF value was used to calculate the correction factor. In order to provide accurate findings, this correction

factor served as a tolerance limit for the spectrophotometer and solvent usage. The Parasol cooling mist sun SPF 25+ PA++® product yielded a correction factor result of 6.68.

All formulations had significant differences, according to the findings of the independent t-test conducted by comparing the SPF values obtained from MED and in vitro experiments. The lack of suitable techniques for evaluating sunscreen products is one factor that may affect the measurement of SPF values in vitro (Zarkogianni & Nikolaidis, 2016). The determination of the SPF value of sunscreen in vitro and MED can differ depending on the combination and concentration of the sunscreen, the type of emulsion, the solvent used to dissolve the sunscreen, and the interaction of other additional components like emulsifiers in a formulation (Heckman et al., 2013; Kausar et al., 2017).

Table 3. Results of SPF values using the MED and in vitro methods

Formula	MED (minutes)	SPF based on MED	Category based on MED Data	SPF in vitro	Kategori SPF in vitro	P Value of In vitro SPF and MED
Unprotected Skin	13.5 ± 7.41	-	-	-	-	-
FI	221.5 ± 6.24$^{c.d}$	16.43 ± 4.50 $^{c.d}$	Ulta Protection	12.28 ± 1.34	Maximum Protection	0.032
FII	228.4 ± 5.31 $^{c.d}$	16.27 ± 4.20 $^{c.d}$		12.51 ± 1.68		
FIII	248.2 ± 3.45$^{a.b.d}$	17.88 ± 3.20 $^{a.b.d}$		13.22 ± 1.84		
Parasol®	326.2 ± 4.60$^{a.b.c}$	24.51 ± 4.60 $^{a.b.c}$		6.68 ± 3.41$^{a.b.c}$		

Notes
n = 3 ± standard deviation
a: Significantly different with F1
b: Significantly different with F2
c: Significantly different with F3
d: Significantly different with Parasol®

According to the findings of the one-way ANOVA test on SPF values based on MED, FI and FII differed significantly from FIII in terms of SPF values. This demonstrates how changes in IPM concentration can lengthen the MED period and result in a higher SPF value. When IPM is present, product absorption into the skin can be enhanced. Sunscreen made of natural substances can work well as long as the preparation is absorbed by the skin (Barradas & de Holanda e Silva, 2021). Elevated concentrations of isopropyl myristate have the ability to break down the inflexible lipid structure and lessen the tension in the stratum corneum, which increases penetration and makes it easier for the active ingredient to enter the skin layers (Jiang et al., 2017). In addition, isopropyl myristate serves as a co-surfactant in the nanoemulsion formulation, giving the final nanoemulsion formula good stability (Dalibera et al., 2021).

The active ingredient components of the avocado oil nanoemulsion can be absorbed by using IPM in conjunction with surfactants and cosurfactants, such as tween 80 and PEG 400, because the nanoemulsion's smaller droplet size can improve the active ingredient's ability to permeate the membrane (Iliopoulos et al., 2022; Pakki et al., 2019). Tween and PEG 400 play an important role in the ability of nanoemulsions to release active ingredients by influencing the surface layer of the nanoemulsion (Akhtar et al., 2011; Nastiti et al., 2017).

CONCLUSION

Avocado oil nanoemulsion has a clear appearance and meets the criteria for a nanoemulsion delivery system. Isopropyl myristate does not affect the physical characteristics of the preparation and at a concentration of 5% shows the highest sunscreen effectiveness.

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Immunomodulator effect of *Cnidoscolus aconitifolius* leaves extract on $CD4^+$ and $CD8^+$ expression in *Salmonella typhimurium*-infected mice

Sholihatil Hidayati[1*], Dhina Ayu Susanti[1], Rian Anggia Destiawan[2], Ahdiah Imroatul Muflihah[2], Rizka Handayani[1], Wima Anggitasari[1]
[1]Pharmacy Study Program, Faculty of Health Sciences, Universitas dr. Soebandi, Jl. Dr. Soebandi No.99, Jember, Indonesia
[2]Medical Laboratory Technology Study Program, Faculty of Health Sciences, Universitas dr. Soebandi, Jl. Dr. Soebandi No.99, Jember, Indonesia

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ABSTRACT

Typhoid fever is a common health problem in the community caused by *Salmonella* bacteria. The incidence rate of this infection will increase if a person's immune system is weakened. Plant extracts have been widely studied for their role in various pharmacological effects, including immunomodulatory effects. Among the plants with the potential to be used as an immunomodulatory substance is *Cnidoscolus aconitifolius*. *Cnidoscolus aconitifolius* leaves extract (CAE) contains flavonoids related to immunomodulatory activity. This study intends to ascertain how administering CAE affects the expression of $CD4^+$ and $CD8^+$ in Babl/c mice that have been infected with *Salmonella typhimurium* bacteria. The study was started by preparing 70% ethanol extract from *Cnidoscolus aconitifolius* leaves. Immunomodulatory activity testing was carried out preparing 30 Babl/C mice as experimental animals. Six mouse groups (the treatment group, the negative control, the positive control, and the healthy control) were allocated at random by giving CAE doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW, and CAE doses of 400 mg/kgBW. Induction was carried out by oral infection with *Salmonella thypimurium* bacteria. After 3 days the infected mice were treated orally once a day for 7 days. Evaluation of $CD4^+$ and $CD8^+$ expression was carried out using the flow cytometry method of the lymph organs. The data was analyzed using the anova test and then the SPSS for Windows tool was used to do the post hoc test (Tukey). The results showed that giving CAE at doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW and CAE doses of 400 mg/kgBW could increase the expression ratio of $CD4^+$ and $CD8^+$. Conversely, administering 400 mg/kgBW of CAE produced noticeably different outcomes ($p<0.05$) from the negative control. This shows that the CAE has potential as an immunomodulatory agent that can improve immune function.

Keywords: *Cnidoscolus aconitifolius*, immunomodulator, *Salmonella typhimurium*, $CD4^+$, $CD8^+$

Corresponding author:
Sholihatil Hidayati
Pharmacy Study Program, Faculty of Health Sciences, Universitas dr. Soebandi
Jl. dr. Soebandi No. 99 Jember, Indonesia
Email: sholihatilhidayati@yahoo.co.id

INTRODUCTION

Infectious diseases are currently the second leading cause of death worldwide in the past ten years, after cardiovascular sickness. The sickness is usually caused by a variety of pathogens, including bacteria, worms, viruses, protozoa, and parasitic fungi that develop on the surface of the body or infect it. Typoid dementia is the infection with the highest incidence in some Asian countries (Fitrya et al., 2020). *Salmonella enterica* infection is the main cause of acute fever, where *Salmonella typhimurium* derivatives are the most abundant (Cordero-Alba et al., 2016). Someone who has a low immune system will be very at risk for contracting this condition (Kalia et al., 2016) . Manusia memiliki sistem kekebalan di bawah sel fagositik yang menjadi pertahanan penting terhadap organisme dan sel ganas (Venkatalakshmi & Brindha, 2016). Activation of the immune system needs to be activated in order to play a role in fighting antigenic substances that enter the body. This activation can be stimulated by the administration of immunomodulatory agents.

Immunomodulatory drugs of synthetic origin have shown benefits, but they have also shown adverse side effects so that the use of such drugs has become less effective. Therefore, the development of research to find safer and more effective immunomodulators is very important (Venkatalakshmi & Brindha, 2016). Potential immunomodulatory agents to be developed from plant extracts, where these plant extracts generally have smaller side effects (Alamgir & Uddin, 2010). Immunomodulators derived from plants work by enhancing the capacity and efficacy of natural killer cells, complement, macrophages, granules, and granules, as well as by generating effector molecules (Jayathirtha & Mishra, 2004). Plants have a bladder of metabolite compounds such as flavonoids, alkaloids, sterols, polysaccharides, glycoproteins, and lectins show activity in increasing endurance (Harun et al., 2015).

Plant extracts have been widely studied for their role in various pharmacological effects, including immunomodulatory effects. Quite a potential plant to be developed as a medicinal plant as an immunomodulatory agent is *Cnidoscolus aconitifolius* leaves. Studies have assessed the therapeutic pharmacological properties of *Cnidoscolus aconitifolius* leaves, indicating potential hepatoprotective, antidiabetic, and anticardiovascular effects (Somade et al., 2021). The findings demonstrated that the flavonoid content of CAE with quercetin standard was 418.46±3.28 mgQE/g extract (Hidayati et al., 2023).

T and B cells produce a variety of unique antigen receptors that control the regulated immune system. Through their interaction, surface T cell receptors (TCRs) and MHC molecules enable T lymphocytes to identify certain antigens, transforming them into $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells. After activation, $CD4^+$ T cells develop into Th helper cells, which produce Th follicular helper cells (Tfh), T regulatory T cells (Treg), and Th1, Th2, Th9, Th17, and Th22 cytokines. In order to destroy infected cells, the activation of $CD8^+$ cytotoxic T cells can control the production of proinflammatory cytokines and cytotoxic mediators (Poon & Farber, 2020). $CD4^+$ cells, a part of humoral immunity, promote the growth and multiplication of B cells. The function of $CD8^+$ cells within the cell is to cause compatible, infected, or malignant (transplant-rejecting) cells to die. In humoral immunity, $CD4^+$ cells serve to stimulate the growth and multiplication of B lymphocytes. The $CD8^+$ cells work intracellularly by triggering apoptose of infected cells, malignant cells, or compatible histoin cells, which are cells that cause transplant rejection (Baratawidjaja & Rengganis, 2014). As a result, $CD4^+$ and $CD8^+$ are crucial metrics for immune system control and the assessment of immunomodulatory effects.

Flavonoids act as costimulatory agents, effectively reducing the release of chemokines and pro-inflammatory cytokines while concurrently upregulating MHC class II expression (Hosseinzade et al., 2019). In mice fed a Western diet, quercetin treatment can increase plasma leptin and TNF-α, decrease the ratio of $CD4^+/CD8^+$ T cells in the adipose tissue of the epididymis, and limit the increase in macrophages (Kobori et al., 2016). This investigation was carried out in Babl/c mice infected with *Salmonella typhimurium* to ascertain the potential of CAE as an immunomodulator to $CD4^+$ and $CD8^+$ T cells.

MATERIALS AND METHOD

Materials

Salmonella typhimurium bacteria were taken from Brawijaya University, Malang. The ingredients used include Ethanol, $CD4^+$ and $CD8^+$ antibodies, phosphate buffer solutions, and aquadest. Tools used include mouse sonde, surgical instruments, propylene tubes, flowcytometry instruments.

Preparation of *Cnidoscolus aconitifolius* leaves extract

Cnidoscolus aconitifolius Leaves were taken from the Patrang area, Jember. The Biology Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University provided the certificate of determination (number 489/Lab. Bio/B/XII/2022). The leaves obtained are wet sorted so that only leaves with a green color are taken, then cleaned and washed using aquadest. After that it is dried in the oven until dry and made powder with a blender. The extraction process begins with sorting the *Cnidoscolus aconitifolius* leaves that have been collected, then the drying process is carried out by aerating for 3 days, then continuing the drying process by ovening at a temperature of 40^{o}C during the formation of dry simplisia and mashed into powder. The finished powder is as much as 500 g, Furthermore, the extraction used 70% ethanol with a total volume of 600 mL for 1 h by ultrasonic maceration, then remaceration is carried out as much as 2 cycle with a volume of 200 mL. The liquid extract obtained is concentrated with a rotary evaporator.

In vivo immunomodulatory activity test in mice

Animal preparation

Thirty male Babl/c strain mice weighing between twenty and thirty grams were housed in the experimental animal laboratory of the Universitas dr. Soebandi Faculty of Health Sciences for a period of fourteen days. This research protocol has been authorized by the Universitas dr. Soebandi Jember's Health Research Ethics Committee under the reference number 096/KEPK/UDS/III/2023. The treatment began by dividing experimental animals into 6 groups, namely the negative control group which were healthy mice without infection, the negative control group which were mice infected with *Salmonella thyparium* bacteria and received placebo CMC Na solution, the positive control group was mice infected with *Salmonella typhimurium* bacteria and received Stimuno® immunomodulator standards, treatment groups 1, 2 and 3 as mice groups infected with *Salmonella typhimurium* bacteria and get CAE doses of 100 mg/kgBW, CAE doses of 200 mg/kgBW and CAE doses of 400 mg/kgBW, respectively. Induction of infection is carried out using *Salmonella typhimurium* bacteria amountingof $1x10^8$ cfu concentration by orally. Three days after induction, mice are examined for bacteremia, including examination of fecal texture and tail blood smear with Giemsa staining. Therapy was started after the mice showed infection. Therapy is administered orally once a day for 7 days (Destiawan et al., 2023).

$CD4^+$ and $CD8^+$ analysis using flow cytometer

$CD4^+$ and $CD8^+$ analysis using the spleen. The spleen organ is washed and crushed in 5 mL of phosphate buffer. After that, it is filtered through the wire and transferred in a 1:3 ratio into a 15 mL propylene tube. The sample was then centrifuged for five minutes at 10°C and 2500 rpm. Take off the supernatant and add 1 mL of phosphate buffer (Kusnul et al., 2017).

After obtaining a 50 µL suspension and centrifuged for 5 minutes at 10°C and 2500 rpm, with the supernatant being disposed of. Incorporate 50 µL of the $CD4^+$ and $CD8^+$ antibody solution, and let it sit at 4°C in the dark for 20 minutes. To prepare a cuvette for flow cytometry analysis, add 400 µL of PBS (Djati et al., 2017).

Analysis of results

Using the SPSS for Windows program, the anova test with significant value ($p<0.05$) was used to analyze the expression of $CD4^+$ and $CD8^+$. Tukey post hoc analysis was used in additional tests to ascertain group differences.

RESULT AND DISCUSSION

The immune response due to *Salmonella typhimurium* bacterial infection is induced by intestinal damage and increased bacterial replication in the intestine. APCs, especially in dendritic cells are the main actors in the immune system's identification mechanism against pathogens. Dendritic cells recognize the lipopolysaccharide structure (LPS) of bacteria via toll-like receptors (TLRs) (Piccioli et al., 2022). Representation of $CD4^+$ T cells via MHC II thereby stimulating increased expression of $CD4^+$ T cells (Leone et al., 2018). However, in *Salmonella typhimurium* bacterial infection, *Salmonella typhimurium* bacteria can inhibit MHC II presentation to $CD4^+$ T cells (Alix et al., 2022) thus, $CD4^+$ T lymphocyte cells are inactive and decrease $CD4^+$ T cell expression when compared to normal (Figure 1 dan Figure 2). This event will lead to excessive systemic infection played by *Salmonella typhimurium* bacteria (Destiawan et al., 2023).

According to the results of flow cytometry analysis, $CD4^+$ T lymphocyte cells express more in lymph tissue cells than $CD8^+$ T lymphocyte cells do. This indicates that $CD4^+$ cells proliferate more quickly than $CD8^+$ cells. This is thought to be because $CD4^+$ plays an important role in secreting various types of cytokines after differentiating into Th1 and Th2. The quantity of $CD4^+$ cells themselves as well as the quantity of other T cells like $CD8^+$ will both increase with a higher $CD4^+$ count (Rachmawati & Rifa'i, 2014) .

In contrast to the negative control group, the results demonstrated that the administration of CAE dosages of 100 mg/kgBW, CAE dosages of 200 mg/kgBW, and CAE dosages of 400 mg/kgBW increased the $CD4^+$ counts but decreased the $CD8^+$ counts (Figure 2). Normal cells shouldn't overexpress the corresponding response since helper T cells ($CD4^+$) and cytotoxic T cells ($CD8^+$) need to experience apoptosis in order to maintain the body's homeostatic parameters (Bolivar-Wagers et al., 2022).

The immune system can quickly control infection, clear pathogens, and inhibit host tissue damage in cases of infection. In preventing such tissue from being damaged and inhibiting the rate and duration of inflammation, cells regulate both innate cells (macrophages, dendritic cells) and the adaptive immune system. When pathogens like viruses invade the body, innate immunity cells will react swiftly by identifying their molecular patterns. This is especially true when viruses are present. In other circumstances, adaptive immune cells play a major role in mediating immune responses that lead to resistance and the development of immunological memory against pathogens. T cells are important components of the adaptive immune response and function as cells that combat infections. Through the expression of adhesion molecules and cytokines, T cells also "help" other cell types. $CD8^+$ mediates the major role of these cells, whereas $CD4^+$ mediates their secondary function (Egawa, 2015).

T cells develop from multipotent progenitors (MPPs) or common lymphoid precursors (CLPs) in the thymus (Pankow & Sun, 2022). T cell differentiation produces subcells namely $CD4^+$ helper cells (Th1 and Th2), cytotoxic CD8+ (CTL/Tc/Ts/Tr/Th3), and memory T cells (Baratawidjaja & Rengganis, 2014).

MPPs or CLPs in the thymus give rise to T cells (Pankow & Sun, 2022). T cell differentiation produces subcells namely $CD4^+$ helper cells (Th1 and Th2), cytotoxic $CD8^+$ (CTL/Tc/Ts/Tr/Th3), and memory T cells (Baratawidjaja & Rengganis, 2014). Because they attach to MHC II and MHC I and activate the TCR, helper T cells and cytotoxic T cells have a significant effect on the immune response. Interactions between dendritic cells and $CD8^+$ cytotoxic T lymphocytes stimulate the latter's proliferation and enhanced synthesis of TNF and IFN-γ, which can kill intracellular antigen-infected cells. Conversely, $CD4^+$ helper T cells control B cells, cytotoxic T cells, and innate immune responses via generating a variety of cytokines (Egawa, 2015).

By encouraging B cells to multiply and develop, $CD4^+$ cells contribute to humoral immunity. As inflammatory mediators, $CD4^+$ Th1 cells secrete TNF, IFN-γ, and IL-2, which aid in the start of the inflammatory process. It has been demonstrated that $CD4^+$ Th2 cells release cytokines such IL-3, IL-4, IL-5, IL-10, and IL-13, which in turn promote and increase the production of T cells and B cell antibodies. Th2 cells are required for activation of B cells by soluble proteins. In addition to Th1 and Th2, other helper T cells have been found, namely Th2, Th9, Th17, Th22, and Follicular Th (Tfh) with diverse roles and functions. Th9 plays a role in the pathophysiology of airway allergies. Th17 secretes

IL-17 which plays a role in neutrophil activation. Th22 plays a role in inflammation in the epidermal cell lining. Tfh is closely related in the regulation of B cell growth. $CD8^+$ cells work intracellularly by triggering apoptosis of infected cells, malignant cells, and compatible histoin cells, which are cells that cause transplant rejection. The $CD8^+$ cells can also directly destroy infected cells under certain conditions (Baratawidjaja & Rengganis, 2014).

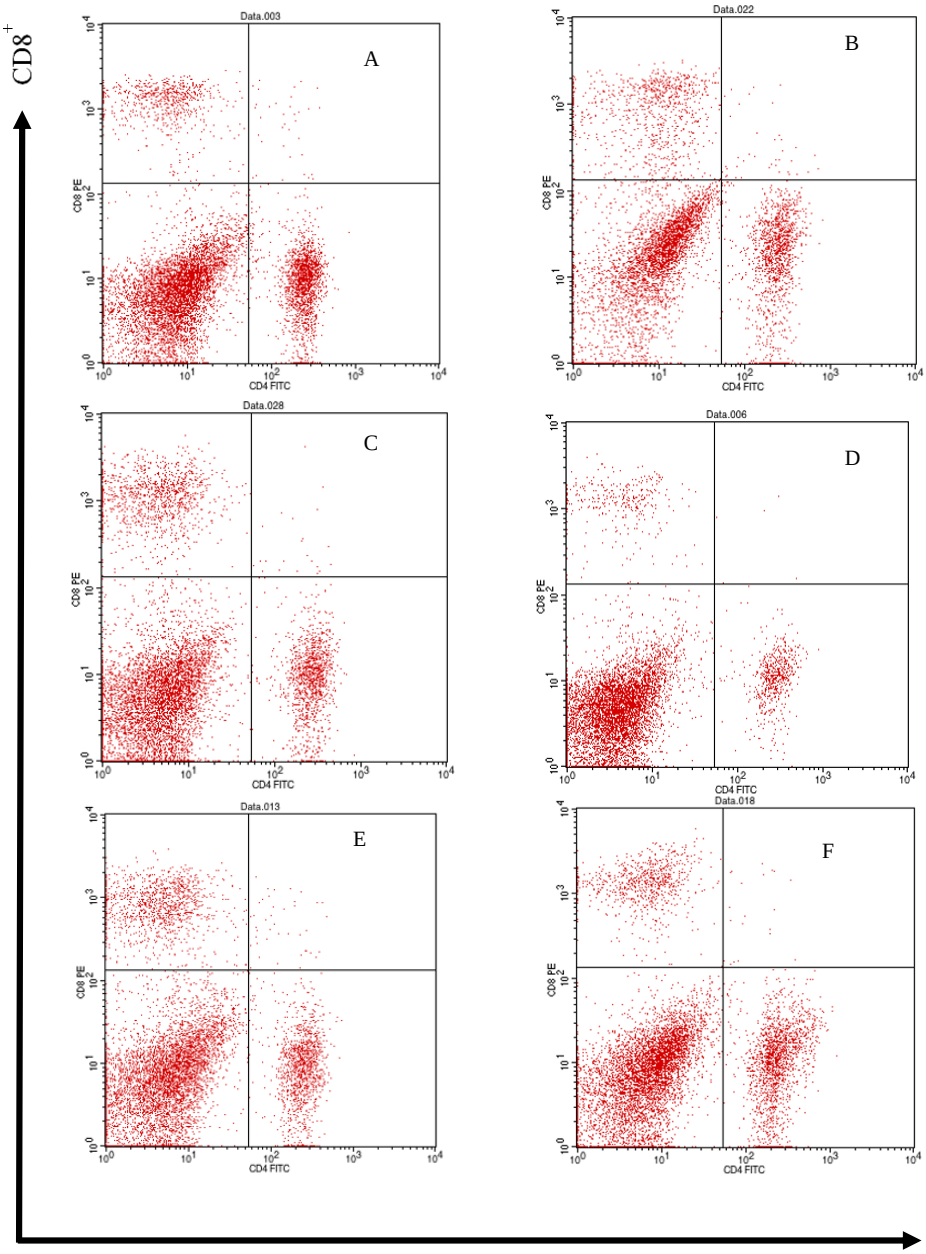

Figure 1. Flow cytometric chart $CD4^+$ dan $CD8^+$ expression post treatment, normal mice (A), negative control (B), positive control (C), CAE dose of 100 mg/kgBW (D), CAE dose of 200 mg/kgBW (E) and CAE dose of 400 mg/kgBW (F)

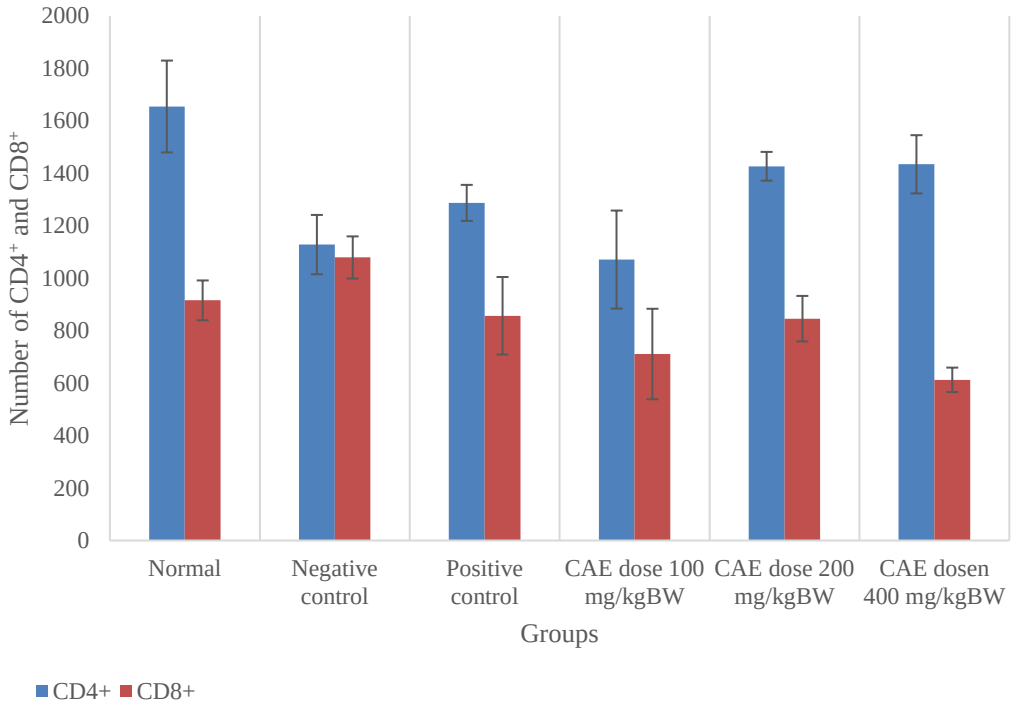

Figure 2. Post-treatment CD4$^+$ and CD8$^+$ expression features analyzed by flow cytometry method in lymph organs

In peripheral blood, the ratio of CD4$^+$/CD8$^+$ in mice and healthy individuals is approximately 2:1; deviations from this ratio may signify immune-related or autoimmune disorders. An immune system malfunction is indicated by a CD4$^+$/CD8$^+$ ratio of less than 1/1, or inverted. Conversely, a greater CD4$^+$/CD8$^+$ ratio is associated with improved immunological activity. A reduction in the ratio suggests a decline in immune system performance as well (Bradshaw et al., 2020). It can be observed from the study results that CAE dosages of 100 mg/kgBW, CAE dosages of 200 mg/kgBW, and CAE dosages of 400 mg/kgBW can raise the expression ratio of CD4$^+$ and CD8$^+$. Notably, as compared to negative controls, the administration doses of 400 mg/kgBW of CAE showed significantly different increases ($p<0.05$) (Table 1). This shows that giving CAE doses of 400 mg/kgBW is very potential to improve immune function.

Table 1. The post-treatment ratio values expression of CD4$^+$ and CD8$^+$ in *Salmonella typhimurium* infected mice

Treatment Groups	Mean CD4$^+$/CD8$^+$ Ratio±SE Values
Normal	1.87±0.30
Negative control	1.10±0.20
Positive control	1.70±0.28
CAE dose 100 mg/kgBW	1.88±0.27
CAE dose 200 mg/kgBW	1.75±0.17
CAE dose 400 mg/kgBW	2.36±0.15*

* Considerably different ($p<0.05$) from the negative control group

Twenty phytochemical substances with a range of medicinal effects are found in CAE, according to its phytochemical study. These substances include the widely distributed 1,2,3-Propanetriol and its

esters, n-Octadecanoic acid, n-Hexadecanoic acid, n-Octacosane, l-(+)-Ascorbic acid-2,6-dihexadecanoate, and 9-Octadecenoic (Z) acid (Omotoso et al., 2024). CAE has high anti-inflammatory activity, can reduce TNF-α expression by 39.78% and IL-6 expression by 97.81% where TNF-α is produced as much as 46% and IL-6 is produced as much as 48.38% in macrophages stimulated by lipopolysaccharides (LPS). This indicates that the bioactive component composition of the extract has the potential to exhibit antioxidant and anti-inflammatory properties in vitro (Us Medina et al., 2019). mTOR, especially in T cells, is a crucial modulator of metabolism and the immune system. Flavonoids have the ability to inhibit mTOR activity, which in turn can trigger a portion of T regulation (Hosseinzade et al., 2019).

CONCLUSION

According to this study, 400 mg/kgBW of CAE may one day be produced as an immunomodulatory drug to improve immune function.

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Combination of polyherbal *Phyllanthus reticulatus* with *Zingiber officinale* and *Cymbopogon citratus* to optimize the antioxidant capacity

Elizabeth Betty Elok Kristiani [1*], Sri Kasmiyati[1], Yohanes Martono[2]
[1]Study Program of Biology, Faculty of Biology, Satya Wacana Christian University
Jl. Diponegoro 52-60, Salatiga 50711, Central Java, Indonesia
[2]Study Program of Biology, Faculty of Sains and Mathematic, Satya Wacana Christian University
Jl. Diponegoro 52-60, Salatiga 50711, Central Java, Indonesia

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ABSTRACT

Currently, the public is interested in polyherbal-based foods and beverages as a source of natural antioxidants. The aim of the study is to evaluate the antioxidant properties and the phenolic and flavonoid compounds of formulations containing *Z. officinale*, *C. citratus*, and *P. reticulatus* (ZCP). Each sample was extracted using the maceration process in an ethanol solvent at room temperature for three 72-hour periods. There were fourteenth formulation of *Z. officinale* rhizome, *C. citratus* leaves, and *P. reticulatus* fruit which used Design of Expert (DoE). The DPPH method was used to determine the power of antioxidants. The flavonoid content of the extract was measured using the colorimetric method and $AlCl_3$ reagent, while phenolics content using Folin-Ciocalteu. The formulations ZCP 1:0:0, 0:0:1, and 1:1:1 showed the antioxidant capacity in a strong categorization, with an IC_{50} value less than 50 µg/ml, while ZCP 0:1:0 was in a weak categorization (IC_{50} > 250 µg/mL). Another ZCP formulation was in a medium category. The ZCP 1:1:1 formulation was suggested as the best one for this investigation, which contains three plant samples. This formulation is interesting for further toxicity studies and in vivo testing so that it can be applied as an antioxidant-rich supplement product.
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Keywords: antioxidant capacity, polyherbal formulation, Zingiber officinale, Cymbopogon citratus, *Phyllanthus reticulatus*

Corresponding author:
Elizabeth Betty Elok Kristiani
Study Program of Biology, Faculty of Biology, Satya Wacana Christian University
Jl. Diponegoro 52-60, Salatiga 50711, Central Java, Indonesia
Email: betty.elok@uksw.edu

INTRODUCTION

The natural world has historically been a fantastic source of therapeutic substances, offering a wide range of medicinal plants that produce useful phytochemicals (Foghis et al., 2023). According to WHO, traditional medicine is defined as a number of health behaviors, techniques, information, and traditions that include manual methods, physical activity, religious treatments, and substances derived from organisms such as animals, plants, or mineral content that are used singly or in combination to encourage health and prevent disease (Mukherjee & Karati, 2023). One function that is currently being studied a lot is antioxidant capacity which is closely related to the body's immunity.

Antioxidant compounds are beneficial for maintaining a healthy body because they are able to maintain a balance between oxidation and anti-oxidation in the body, preventing oxidation in the body which can trigger various diseases (Wang et al., 2014; Sindhi et al., 2013). Several secondary metabolites were reported to have antioxidant abilities such as polyphenols (phenolic acids, flavonoids, anthocyanins, lignin, and stibine), carotenoids (xanthophylls and carotene), and ascorbic acid, tocopherol, and also tannin (Irfan et al., 2022; Baiano & Nobile, 2016; Xu et al., 2017; Wafa et al., 2016). Fruit and vegetable are known as sources of exogenous antioxidants because of their antioxidant compounds contents (Bouayed & Bohn, 2010). Since the time of their forebears, the community has used ginger (*Zingiber officinale*) and lemongrass (*Cymbopogon citratus*) as components of herbal medicine and traditional medicine. Several scientists (Begum et al., 2006; Jamal et al., 2008) have studied Mangsian, the Indonesian name for *Phyllanthus reticulatus*, but the people there is yet to utilize it as a source of antioxidants.

A lot of people think that preparations from multiple plants are only useful as medicines. While many polyherbal extracts still need to be researched, some have been scientifically shown to be effective in the treatment of oxidation stress-related diseases (Adejoh et al., 2016). The existence of many antioxidant molecules in raw materials can have interactions with each other that might be additive, antagonistic, or synergistic (Gupta et al., 2021; Sindhi et al., 2013; Bouayed & Bohn, 2010). Positive interactions, sometimes referred to as synergistic interactions, occur when two or more substances are combined and the result exhibits a greater action than the total number of chemicals. Researchers alike use the concept of synergism in their research on antioxidants, antimicrobials, antifungals, and novel therapeutic formulation (Blesson et al., 2015). On the other hand, antagonism is a result that is diminished when combined. Findings that are both positive nor negative are characterized as being indifferent, and the whole effect of the combination is additive.

Several studies in vitro and in vivo have documented antioxidant properties of *Z. officinale*, *C. citratus*, and *P. reticulatus* either by itself or in combination with other plants. Numerous investigations showed the phytopharmaceutical properties of ginger extract under specific circumstances, including considerable superoxide radical scavenging action, reducing stresses and damage to cells and anti-inflammation (Mustafa & Chin, 2023; Rostamkhani et al., 2022; Adejoh et al., 2016; Morakinyo et al., 2012). The aqueous and ethanol extracts of *Z. officinale* from Nigerian may be useful in the treatment of conditions oxidative stress-related diseases like atherosclerosis and diabetes mellitus (Morakinyo et al., 2012). The study of drying methods on ginger's antioxidant found that water-ethanol mixtures are effective for extracting polar antioxidants whereas sun-dried ginger showed the highest recovery of phenolic compounds contents (Mustafa & Chin, 2023). In the study of lemongrass leaves, extraction using the maceration technique showed that the 70% ethanolic concentration had the highest levels of scavenging activity compared to the 50% ethanolic concentration and the 70% acetone concentration (Irfan et al., 2022). (Wuryatmo et al., 2021) stated that lemongrass has the potential to be employed as a natural food preservative, especially in high fat food products, as evidenced by its high antioxidant activity. Its study showed that the ethanol extract of stem *C. citratus*, total phenolic content of 19.31 mg GAE/g and flavonoids at 3.31 mg GAE/g. This outcome was connected to the extract's 79.96% antioxidant activity. At about mangsian (*P. reticulatus*), although that plant has been researched by some researchers (Maruthappan & Shree, 2010; Begum et al., 2006), the general public has not yet exploited it as a source of antioxidants. Many researchers studied about the combination polyherbal about ginger, lemongrass, and mangsian. The combination of ginger and sappan wood also increases

antioxidant activity better than being separated reported (Widyapuspa et al., 2022). The analgesic and anti-inflammatory activity of a combination of *Phyllanthus reticulatus* and *Mimosa pigra* (Akhter et al., 2018). A study of the three species of *Z. officinale* with *Cinnamomum burmannii* and *Caesalpinia sappan* showed that the combination of ginger had either higher total phenolic or antioxidant activity than the non-combination (Mahmudati et al., 2022). Study on a combination of herbs, named Trikatuk, in many combinations, resulting in most of the combinations investigated in the present study also exhibiting additive or synergistic effects (Nutmakul & Chewchinda, 2023).

The research of the relationship between the antioxidant properties and antioxidant components of blends of *Z. officinale, C. citratus*, and *P. reticulatus* has not yet been reported. The time difference from standard practice has not been determined by other investigations. Finding the best formulations for health supplements, especially those that are providers of antioxidants, through the research of polyherbal is interesting. So, the purpose of this study is to develop a composition containing *Z. officinale* rhizome, *C. citratus* leaves, and *P. reticulatus* fruits and to evaluate the antioxidant properties and their phenolic and flavonoid compounds.

MATERIALS AND METHOD

Materials

The design of research using method of experimental design. The plant samples were included with *Zingiber officinale*, *Cymbopogon citratus*, and *Phyllanthus reticulatus*. The plants collection (No. 001 until No. 004/2014/FPBUKSW/Koleksi) were preserved in *Biologi Dasar* Laboratory, Faculty of Biology, Satya Wacana Christian University, Salatiga, Indonesia. The parts of the plant used are the rhizome of *Z. officinale*, the leaf of *C. citratus*, and the fruit of *P. reticulatus*.

Methods

Extract and formulation preparation

Every research material (Figure 1) was prepared as 5 pieces as a replicate of the study. With the exception of *P. reticulatus*, every material was broken up into tiny pieces and air dried before being baked at 50^{o}C. The dry sample was then counted using a mixer. Using the maceration procedure, ethanol (1: 1.5 w/v) was used as the solvent to create the extracts. Three times of macerations, duration of 72 hours each of them was performed. After each maceration process, the macerate is filtered, and the supernatant is then subjected to another maceration. The mixed filtrate was dried at 40^{o}C in a Rotavapor R114 Buchi rotary evaporator under vacuum (Eyela A-1000S).

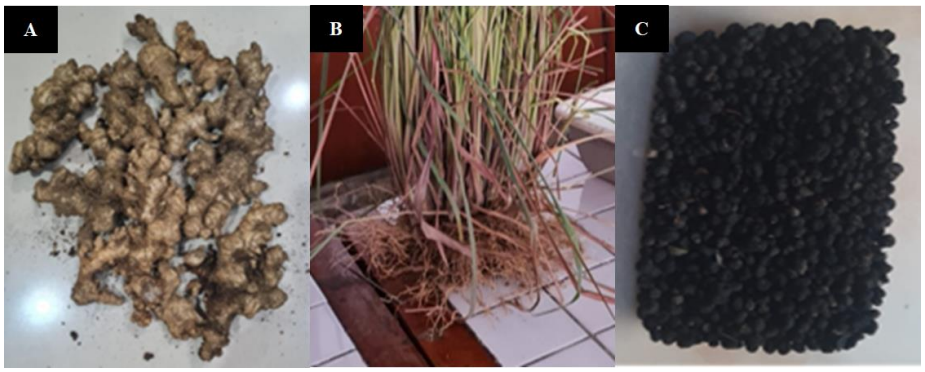

Figure 1. The samples of research. (A) Rhizome of *Z. officinale* (B) Leaves of *C. citratus*, (C) Fruit of *P. reticulatus*

The compositions of extract were designed using the Desain of Experiment was used to create the extract formulation. (DoE) (Table 1).

Table 1. Composition of polyherbal, a mixture of ethanol extracts of ginger rhizome (*Z. officinale*), lemongrass leaves (*C. citratus*), and mangsian fruit (*P. reticulatus*) using Design of Expert (DoE) (Design-Expert 13.0 trial version)

Polyherbal formulation	Percentage of compotition (%)		
	Z. officinale	*C. citratus*	*P. reticulatus*
ZCP 1:0:0	100.00	0.00	0.00
ZCP 1:1:4	16.67	16.67	66.67
ZCP 1:4:1	16.67	66.67	16.67
ZCP 1:0:1	50.00	0.00	50.00
ZCP 0:0:1	0.00	0.00	100.00
ZCP 0:1:0	0.00	100.00	0.00
ZCP 1:1:0	50.00	50.00	0.00
ZCP 4:1:1	66.67	16,67	16.67
ZCP 1:0:0	100.00	0.00	0.00
ZCP 1:1:0	50.00	50.00	0.00
ZCP 0:1:0	0.00	100.00	0.00
ZCP 0:0:1	0.00	0.00	100.00
ZCP 0:1:1	0.00	50.00	50.00
ZCP 1:1:1	33.33	33.33	33.33

Antioxidant activity assay

The ability of antioxidants was evaluated using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method (Senja et al., 2016) with slight modification. The solvent used is methanol. Sample concentration series for the determination of IC_{50} values in the range of 50, 100, 150, and 200 µg/mL. At amount of 1 mL of sample was added with 1 mL of DPPH, without adding ethanol up to 5 mL. The time incubation of the mixture was 30 minutes in the dark condition. In the measurement of absorbance at wavelength of 517 nm, a Shimadzu UV mini1240 UV -Visible spectrophotometer was used.

Phenolic content assay

Using the Folin-Ciocalteu reagent with a colorimetric technique, the extract's total phenolic acid content was determined Almey et al. (2010) with slight modification. Applying gallic acid as a standard phenolic compound in concentration series 20, 40, 60, 80, and 100 µg/mL. The extract concentration analyzed was 100 µg/mL. The spectrophotometer used for mixture absorbance measurement is UV-Visible spectrophotometer (Shimadzu UV mini1240), at a wavelength of 550 nm. Phenolic acid content (mg GAE/g extract) = c (V/m). c: gallic acid concentration based on gallic acid linear regression equation (mg/l); V = extract volume (l); m = mass of extract (g).

Flavonoid content assay

The colorimetric technique with $AlCl_3$ reagent were used to determine the extract's flavonoid content (John et al., 2014) with slight modification. Absorbance measurement of the sample was carried out at a wavelength of 415 nm using UV-Visible spectrophotometer (Shimadzu UV mini1240), at a wavelength of 415 nm. As a reference substance, quercetin was utilized at concentrations of 20, 40, 60, 80, and 100 mg/l. Flavonoid content (mg QE/g extract) = c (V/m). c: flavonoid concentration based on quercetin linear regression equation (mg/l); V = extract volume (l); m = mass of extract (g).

Data Analysis

The data were analyzed statistically using the analysis of variance test to determine the significant difference in values between extracts and test parameters and continued with the Tukey test to determine the correlation between the concentration of the test compound and the antioxidant ability of the sample.

RESULT AND DISCUSSION

Antioxidant activity

The assay of the antioxidant ability of each formulation was done using the DPPH method. The test using DPPH is a non-enzymatic in vitro test that is widely used to measure the antioxidant capacity of a compound. This method is widely used because its implementation is very simple, fast using an ultraviolet-visible spectrophotometer, and low cost (Dontha, 2016). Using the DPPH technique, the inhibitory potential of each formulation was examined at concentrations of 50, 100, 150, and 200 µg/mL. A linear regression equation was developed from the data already collected, and the formulation concentration at 50% inhibition (IC_{50} value) was calculated (Table 2).

Table 2. The phenolic and flavonoid content, and IC_{50} values of ethanol extracts of ginger (*Z. officinale*) rhizome, lemongrass (*C. citratus*) leaves, and mangsian (*P. reticulatus*) fruit in various compositions

Polyherbal formulation	Flavonoid content (µg/g extract)	Phenolic content (µg/g extract)	IC_{50} value of antioxidant (µg/mL))	Categories of antioxidant activity*
ZCP 1:0:0	13.82 ± 2.30^{cde}	18.92 ± 1.01^{a}	64.4 ± 6.1^{l}	Strong
ZCP 1:1:4	16.67 ± 1.57^{bcd}	10.79 ± 0.44^{ef}	101.8 ± 6.5^{h}	Medium
ZCP 1:4:1	27.18 ± 3.68^{a}	6.52 ± 0.45^{h}	205.8 ± 30.0^{c}	Medium
ZCP 1:0:1	14.13 ± 1.60^{cde}	16.02 ± 1.00^{b}	61.4 ± 4.3^{k}	Strong
ZCP 0:0:1	15.75 ± 0.53^{bcde}	11.68 ± 0.64^{de}	71.6 ± 2.1^{j}	Strong
ZCP 0:1:0	19.68 ± 1.10^{b}	2.08 ± 0.08^{i}	396.3 ± 33.0^{b}	Weak
ZCP 1:1:0	27.90 ± 1.24^{a}	9.03 ± 0.57^{fg}	150.0 ± 8.2^{d}	Medium
ZCP 4:1:1	12.22 ± 2.59^{de}	11.91 ± 0.34^{de}	111.2 ± 8.1^{g}	Medium
ZCP 1:0:0	16.28 ± 1.73^{bcde}	14.87 ± 0.79^{bc}	81.7 ± 4.6^{i}	Strong
ZCP 1:1:0	11.32 ± 1.68^{ef}	10.66 ± 0.43^{ef}	139.6 ± 13.4^{de}	Medium
ZCP 0:1:0	20.77 ± 0.36^{b}	2.39 ± 0.09^{i}	497.0 ± 74.7^{a}	Weak
ZCP 0:0:1	16.02 ± 0.82^{bcde}	13.48 ± 0.75^{cd}	63.8 ± 11.8^{jk}	Strong
ZCP 0:1:1	18.13 ± 0.72^{bc}	8.69 ± 0.42^{g}	116.9 ± 4.4^{f}	Medium
ZCP 1:1:1	7.00 ± 0.00^{f}	11.73 ± 0.52^{de}	117.8 ± 5.5^{f}	Medium

Note: Z: *Z. officinale*; C: *C. citratus*; P: *P. reticulatus*. *Based on the IC_{50} (µg/mL) value. Very Strong= $IC_{50} \leq 50$ µg/mL; Strong = $50 < IC_{50} \leq 100$; Medium = $100 < IC_{50} \leq 250$; Weak = $250 < IC_{50} \leq 500$; Not Active = $IC_{50} > 500$ (Molyneux, 2004). The value is the mean ± standard deviations (SD) (n = 3); Duncan's test was used to assess the mean difference between samples ($p > 0.05$) after one-way ANOVA to determine whether mean values in the same column with different letters differed significantly

Based on the IC_{50} value, it appears that under single conditions, *Z. officinale* and *P. reticulatus* extracts have strong antioxidant abilities, while *C. citratus* is classified as weak. This shows that mangsian fruit has the potential to be developed as a source of natural antioxidants. When ginger and mangsian are combined 1:1 (Formulation ZCP 1:0:1), the antioxidant activity is still powerful, which is at a strong categorized ($IC_{50} < 100$ µg/mL). On the other hand, when the mixture also contains three other types of components, it loses strength and falls into the medium category regardless of how the three ingredients are combined (IC_{50} value in the range of 101–250 µg/mL).

Exogenous antioxidants mainly come from food and medicinal plants such as fruits, vegetables, flowers, spices and traditional herbal medicine (Bouayed & Bohn, 2010). Consuming fruit and vegetables with antioxidant capacity, as well as medicinal plants and herbs that have these properties can provide optimal health and nutritional outcomes. It is based on quality nutrition that may have beneficial effects in the prevention of some chronic and degenerative diseases such as cancer that are prevalent in society. This study combined ginger rhizome, lemongrass leaves, and mangsian fruits to obtain the combination that has the optimal antioxidant activity.

The ZCP 1:0:0 formulation showed strong antioxidant capabilities with an IC_{50} value < 100 µg/mL. The results of the study show that the ethanol extract of *Z. officinale* has a high antioxidant capacity, in accordance with several existing reports. The study by Mustafa & Chin (2023) showed that ethanol extracts had the best free radical scavenging activity with IC_{50} values < 50 µg/mL, with IC_{50} values of 15.23 µg/mL for sun-dried ginger samples and 22.10 µg/mL for oven-drying. The ultrasonic approach produced an extract from ginger that had a very high antioxidant capacity and an IC_{50} value of 51.46 0.31 µg/mL (Sarastri et al., 2023). There were the same strength categories for antioxidant ability: very strong antioxidant ability. With an IC_{50} of 18.4 g/mL, the methanol extracts of *Z. officinale* demonstrated a very strong antioxidant capacity (Adejoh et al., 2016). The strong antioxidant capacity was also shown in the ZCP 0:0:1 formulation. When compared to ethanolic extracts, *P. reticulatus* whole plant extracts have stronger antioxidant activity (Maruthappan & Shree, 2010). Antioxidant activity of plant powder of *P. reticulatus* was 82.4%, exhibits good activity when compared to Butylated Hydroxy Toluene (BHT) of 85%, each at a concentration of 400 g/ml. In this study, 200 µg/ml of a fruit extract (ZCP 0:0:1 formulation) had a 99% inhibitory antioxidant. From both studies, it is possible to employ this plant as a powerful source of natural antioxidants, although people have not used this plant much for health. When the two chemicals coexist, they show a certain pattern. When the difference in phenolic and flavonoid concentrations between *Z. officinale* and *P. reticulatus* is 5 µg/ml, these conditions provide the extract with strong antioxidant properties, as seen in the ZCP 1:0:0, ZCP 0:0:1, and ZCP 1:0:1 formulation.

Different from *Z. officinale* and *P. reticulatus*, the antioxidant ability of single lemongrass leaves (ZCP 0:1:0 formulation) was classified as weak, with an IC_{50} value of 447 µg/mL. With a difference of 18 µg/ml, this formulation's flavonoid level is significantly higher than its phenolic amount, making it apparent that flavonoids can reduce antioxidant activity. These findings match those of (Wuryatmo et al. (2021), who discovered that an ethanol extract of lemongrass stems and leaves had an inhibitory activity of 80% and 67%, respectively, at a concentration of 20,000 µg/mL. This indicates that it is far less effective as an antioxidant source than ginger and mangsian.

In combination with a greater composition of *C. citratus* (ZCP 1:4:1) and a composition of ginger with the same of *Z. officinale* and *C. citratus* (ZCP 1:1:0), the mixture formed contained 21 µg/mL more flavonoids than phenolics, but the antioxidant capacity was classified as medium. This may occur because the effect of antioxidant compounds in ginger is different from that in lemongrass, even though they are in the same compound group. Although *Z. officinale* and *P. reticulatus* are powerful antioxidants, combined together with *C. citratus*, the antioxidant power is drop to medium capacity. The ZCP 4:1:1 combination further demonstrates the antagonistic nature of the lemongrass presence in the combination; despite the ginger proportion being higher than the lemongrass and mangsian proportions, the mixture's antioxidant capacity only reaches a medium level. Poh et al. (2018) found that the 1:1 ratio of lemongrass-curry leaf extracts, lemongrass-turmeric extract, and lemongrass-ginger extract showed antagonistic interaction effects. ZCP 1:0:0, ZCP 0:0:1, and ZCP 1:0:1 formulation, which components without lemongrass, has an antioxidant capacity that belongs in the high category (IC50 were between 60 - 80 µg/mL). This demonstrates how the mixture's antioxidant capacity is lowered by the inclusion of lemongrass. As a result, we suggest that formulations ZCP 1:0:0, ZCP 0:0:1, and ZCP 1:0:1 is the best formulation for this investigation.

Bioactive compounds content

The antioxidant capabilities of secondary metabolites, such as polyphenols and anthocyanins, are well documented (Shi et al., 2018; Lauro et al., 2016). In this study, flavonoids and phenolics were the bioactive substances that were quantified. Figure 2 demonstrates that the flavonoid and phenolic levels were similar throughout the five formulations, including the one that had just *Z. officinale* and *P. reticulatus* and the one that included ginger and mangsian mixed 1:1.

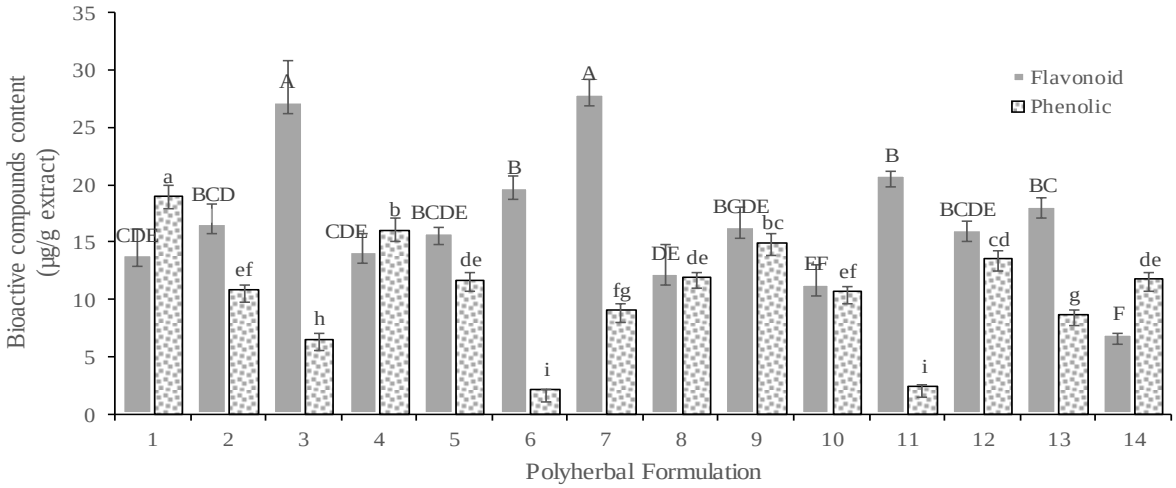

Figure 2. The flavonoids and phenolics content of ethanol extracts of ginger (*Z. officinale*) rhizome, lemongrass (*C. citratus*) leaves, and mangsian (*P. reticulatus*) fruit in various compositions. The composition of ZCP is 1= 1:0:0; 2= 1:1:4; 3= 1:4:1; 4= 1:0:1; 5= 0:0:1; 6= 0:1:0; 7= 1:1:0; 8= 4:1:1; 9= 1:0:0; 10= 1:1:0; 11= 0:1:0; 12= 0:0:1; 13= 0:1:1; 14= 1:1:1. The value is the mean standard deviations (SD) (n = 3); Duncan's test was used to assess the mean difference between samples (p > 0.05) after one-way ANOVA to determine whether mean values in the same color of the bar with different letters differed significantly

Researchers have extensively examined flavonoids and phenolic chemicals for their potential as antioxidant sources. A group of chemicals known as phenolic compounds has a variety of structures and phytopharmaceutical properties. Numerous studies' in vitro testing demonstrated that phenolic substances, even at low quantities, have strong antioxidant properties (Kruk et al., 2022). According to Santos-Sánchez et al. (2019), the process by which phenolic compounds exert their antioxidant effect involves the transfer of hydrogen ions from phenol to free radicals, resulting in the establishment of an H-O transition condition. Plants are accessible to a wide variety of flavonoids. Many plants contain a class of chemicals called flavonoids that share the basic structure of polyphenols. Flavanols, flavones, flavonones, isoflavones, flavonols, and flavanonols are the several categories of phenolic chemicals (De Luna et al., 2020). Flavonoids may inhibit the activation of free radicals in four ways that were blocking the activity of nitric-oxide synthase, blocking the activity of xanthine oxidase, altering channel pathways, or interacting with other enzyme systems (Kumar & Pandey, 2013).

All compositions contain phenolics and flavonoids in varying levels (Table 2). Antioxidant ability appears to be strongly influenced by the levels of phenolic compounds. In compositions with strong levels of antioxidant ability (ZCP 1:0:0, ZCP 0:0:1, and ZCP 1:0:1), statistically high levels of phenolics were detected (codes a, b, bc, cd, and de behind the test value) even though the phenolic content at the level is not statistically high (codes bcde and cde behind the test value). On the other hand, one composition at the weak level, namely ZCP 0:1:0, has the lowest phenolic content statistically (code i behind the test value) even though the phenolic content is statistically high (code b behind the test value). Compositions with medium

antioxidant capacity were detected to contain statistically low phenolics even though flavonoid levels were statistically high.

Extraction is a crucial process in this research because it is intended to extract bioactive compounds using a solvent (Le et al., 2018; Othman et al., 2015). In their investigation, Irfan et al. (2022) performed maceration on lemongrass leaves for 24 hours using 50% and 70% ethanol to produce a yield of around 20%. The yield appears to be the same as the study's findings, with a shorter time investment. In contrast to the time needed in this investigation, which was up to 3 x 72 hours, it appears that heating the maceration conditions to a temperature of 40ºC can speed up the compound extraction process. The study on *Fortunella polyandra* showed that extraction at a temperature of 30 °C resulted in an extract with optimum antioxidant activity (Elias et al., 2023). Maceration of *Z. officinale* using maceration method done 3 x 24 hours yield the 7.59% of extract (Andriyani et al., 2015). This study's reduced yield value demonstrated how the passage of time affects the outcomes of extraction. The yield obtained increases with longer extraction times.

Oxidation-reduction reactions are a pairs important processes that in occur all the time in the cell. Free radicals are one of caused of oxidation reaction. However, certain oxidation processes become harmful to cells because of its negative effects. Some compounds have the ability to bind free radicals so that oxidation reactions do not occur and prevent damage to healthy cells, known as antioxidants (Dontha, 2016). In assessing the antioxidant ability of natural ingredients, before carrying out the in vivo test, the researchers conducted an in vitro test first. It is assumed that compounds that have high antioxidant abilities in vitro will also have high abilities in vivo (Tukun et al., 2014).

The correlation of phenolic, flavonoid content, and antioxidant activity

The antioxidant ability of a plant extract is influenced by the compound content present in the extract. The relationship between antioxidant activity and flavonoid and phenolic concentration was displayed in Table 3.

Table 3. The correlation between phenolic, flavonoid content, and antioxidant activity of ethanol extracts of ginger (*Z. officinale*) rhizome, lemongrass (*C. citratus*) leaves, and mangsian (*P. reticulatus*) fruit in various compositions

	Flavonoid	Phenolic	IC_{50}
Flavonoid		-0.52043	0.40909
Phenolic	-0.52043		-0.88239
IC_{50}	0.40909	-0.88239	

The ratio of flavonoid to phenolic content is inversely connected, meaning the higher the concentration of these compounds, the lower the concentration of phenolic. These two categories of substances exhibit various relationships with the IC_{50} value, which also indicates antioxidant capacity. The phenolic content and the IC_{50} value are inversely correlated, meaning that the higher the phenolic content, the lower the IC_{50} value. The lower the IC_{50} value, the greater the antioxidant ability. Therefore, the ability to act as an antioxidant increases with phenolic concentration. In contrast, a positive association between flavonoid concentration and the IC_{50} value indicates that the capacity of an antioxidant is lower if the flavonoid content is higher.

Various factors contribute to the antioxidant ability of plant extracts, but one crucial determinant is the compound content present in the extract (Bavisetty & Venkatachalam, 2022; Shekarchian & Soleymani, 2019). This study measures the quantity of flavonoid and phenolic compounds prior to examining the relationship between these two substances and each composition's antioxidant capability. Quercetin was used as a standard flavonoid compound while gallic acid was used as a standard phenolic compound. Both compounds were found abundant in several fruits and medicinal plants (Xu et al., 2019; Kahkeshani et al., 2019) . Based on statistically analysis, phenolics have a stronger impact on antioxidant capacity than either of these two substances. The extract's capacity as an antioxidant increases with its phenolic concentration. The flavonoid content, however, exhibits

opposite trend. An et al. (2016) stated that DPPH demonstrated a significant correlation with total phenolic concentration, but a less correlation with total flavonoid concentration. According to the research on *Polygonum minus*, *Z. officinale*, and *Curcuma longa*, plant extracts with higher total phenolic content had increased antioxidant activity, suggesting that phenolic chemicals are important factors of antioxidant activity (Maizura et al., 2011).

CONCLUSION

The antioxidant capacity of single extracts of *Z. officinale* and *P. reticulatus* was strong, while that of *C. citratus* was weak. The composition with three plant samples ZCP 1:0:0, ZCP 0:0:1, and ZCP 1:0:1 was recommended as the ideal compotition. Further toxicity research and in vivo testing on that formulation are interesting in order to use it as an antioxidant-rich dietary supplement. Phenolic substances play a major role in antioxidant action.

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Ethnopharmacology study of medicinal plants utilization for antidiarrheal remedies by Tengger tribe in Tosari District, Indonesia

Indah Yulia Ningsih[1]*, Virda Fitra Mandasari[1], Antonius Nugraha Widhi Pratama[2], Mochammad Amrun Hidayat[1]
[1]Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, University of Jember, Jl. Kalimantan I/2 Kampus Tegalboto, Jember 68121, East Java, Indonesia
[2]Department of Clinical and Community Pharmacy, Faculty of Pharmacy, University of Jember, Jl. Kalimantan I/2 Kampus Tegalboto, Jember 68121, East Java, Indonesia

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ABSTRACT

Tengger is one of the tribes in East Java Province, Indonesia practising traditional medicine by using mantras and medicinal plants. A disease with a high incidence rate and widely treated with medicinal plants in Indonesia, including in Tosari District, Pasuruan Regency, is diarrhoea. To conserve traditional medicine, mainly the utilization of medicinal plants as anti-diarrhoea agents, it is necessary to develop a database that keeps up with technological advances. The study aimed to determine medicinal plants utilization for antidiarrheal remedies by the Tengger tribe in four villages of Tosari District, Pasuruan Regency, namely Wonokitri, Tosari, Ngadiwono, and Podokoyo. The study employed the snowball sampling method, which involved conducting semi-structured interviews. The result showed nine medicinal plants for traditional antidiarrheal remedies, with Musaceae (23%) as the most widely used plant family. Most informants used immature plant (56.25%) and fruits (89.58%). In addition, most plants were administered orally (98%) without specific compounding methods (76%). The value of Factor of the informant's consensus (Fic) of plants used for diarrhoea was 0.74. The highest Fidelity Level (FL) and Choice Value (CV) were obtained from *Elaeocarpus longifolius* Blume at 69% and 2.4, respectively. Based on the findings of the study, *E. longifolius* has the potential to be further investigated for development in antidiarrheal treatment.

Keywords: antidiarrheal remedies, tengger tribe, factor of the informant's consensus, fidelity level, choice value

Corresponding author:
Indah Yulia Ningsih
Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, University of Jember
Jl. Kalimantan I/2 Kampus Tegalboto, Jember 68121, East Java, Indonesia
Email: indahyulianingsih.farmasi@unej.ac.id

INTRODUCTION

Diarrhoea is defined as an alteration in the shape and consistency of faeces from soft to liquid, accompanied by an increase in bowel movements frequency, occurring at least three times within 24 hours (Hemalatha & Laloo, 2011). Clinically, diarrhoea is frequently caused by infection (bacterial, viral, or parasitic infestation), malabsorption, allergies, poisoning, and immunodeficiency. The main causes of diarrhoea are infection and poisoning (Sari, 2018). Diarrhoea is an endemic disease in Indonesia, occasionally accompanied by mortality. The Riskesdas report in 2018 confirmed that the highest prevalence of diarrhoea in Indonesia occurred in the 1-4 years age group at 11.5%. The prevalence of diarrhoea in East Java was 6.5%, with the prevalence of diarrhoea in toddlers being 9.9% (Kemenkes RI., 2019).

Though using a rehydration solution does not reduce the incidence of diarrhoea, over 90% of dehydration associated with diarrhoea is effectively accomplished with an oral rehydration solution. Meanwhile, antibiotics are only indicated for bloody diarrhoea or cholera (Agtini & Puspandari, 2027). Over the past few decades, there has been a worldwide movement towards back to nature. In all types of traditional medicine, the diversity of plants is utilized to treat and prevent various diseases (Jaradat et al., 2017). Based on the Riskesdas Report in 2018, it is known that the treatment of diarrhoea in toddlers using medicinal plants in East Java was 7.5%, especially in the 24-35 months age group, namely 13.6% (Kemenkes RI., 2019).

The utilization of medicinal plants is an essential component of traditional medicine found within the customs and traditions of a country's population, particularly in developing nations (Amiri & Joharchi, 2012). One of the traditional remedy cultures in Indonesia existed in the Tengger tribe. The tribe has a unique therapeutic culture, namely traditional medicine and mantras passed down through generations. Around 98 types of traditional medicinal plants were used by the Tenggerese community (Batoro, 2012).

The Tengger tribe is located around the Tengger Mountains, East Java Province, Indonesia, which comprise four regencies: Probolinggo, Pasuruan, Malang, and Lumajang (Putri et al., 2022). Most Tenggerese community in Pasuruan lives in Tosari District (Sutarto, 2007). The total area of Tosari District is 9,214.753 hectares. Moreover, the district comprises eight villages: Wonokitri, Tosari, Ngadiwono, Podokoyo, Mororejo, Sedaeng, Baledono, and Kandangan (Galba et al., 1989). However, due to an alteration in value orientation, most Tenggerese community in Tosari District only inhabit four villages: Wonokitri, Tosari, Ngadiwono, and Podokoyo (Sutarto, 2007). According to the epidemiological report on the top 20 diseases in Tosari District by Tosari PHC, public health center (*Puskesmas*), the incidence of diarrhoea has decreased over the years. In 2013, there were 533 cases; in 2014, there were 408 cases; and in 2015 there were 321 cases. Nevertheless, in 2016, the incidence of diarrhoea in Tosari District increased to a total of 471 cases. Due to the common use of traditional medicine by the Tengger tribe, it is necessary to conduct an inventory of medicinal plants commonly employed to alleviate diarrhoea symptoms by the Tenggerese community located in four villages of Tosari District.

MATERIALS AND METHOD

Study area

This research was preceded by a preliminary survey followed by data collection in Wonokitri, Tosari, Ngadiwono, and Podokoyo Villages, located in Tosari District, Pasuruan Regency, East Java Province, Indonesia at an altitude range of 1681-2597, 887-2599, 1099-2198, and 1820-2488 m above sea level, respectively. The researchers obtained authorization from the National Unity and Politics Agency (*Bakesbangpol*) of Pasuruan Regency (No. 072/559/424.104/SUR/RES/2017) to perform the study.

Population and sample

The population of the study was the Tenggerese community in Wonokitri, Tosari, Ngadiwono, and Podokoyo Villages. The sample consisted of the Tenggerese community in the four villages who met inclusion criteria, namely being native descendants of the Tengger tribe aged over 17 years, living in the four villages, possessing traditional Tengger treatment knowledge and using medicinal plants to treat

diarrhoea. The exclusion criteria of the study were those who used modern medicine and used plants which were not cured of diarrhoea.

Data collection

The study was a cross-sectional research using a combination of qualitative and quantitative methods. Qualitative study was performed through semi-structured interviews using open-ended questions, respondent investigation, observation, and identification. Plants with medicinal properties were identified by their scientific names at Faculty of Mathematics and Natural Sciences, University of Jember. Quantitative data was presented by measuring Fic, FL, and CV. The determination of the sample was performed using snowball sampling method, which is a sampling technique that begins by selecting a small group and asking them to identify their acquaintances. These acquaintances then nominate further acquaintances, forming a snowball (Nasir et al., 2011).

Data Analysis

Data analysis employed in the study is a quantitative descriptive analysis using the Fic, FL, and CV analytical methods. The method was used as a reference for assessing the potential medicinal plants for use as antidiarrheal agents.

Factor analysis of informant consensus (Fic)

Data analysis of Fic aims to demonstrate the consistency of information obtained from informants regarding the most effective medicinal plants in treating specific diseases. Fic value is low (close to 0) when plants are selected randomly or when informants do not provide information on the species utilization in treating diarrhoea. A high Fic value (close to 1) is obtained when there are well-defined selection criteria established by the community and/or when informants exchange information on the use of the species in treating diarrhoea. Fic is determined by applying the subsequent formula (Karaköse et al., 2019):

$$Fic = \frac{\text{Nur}-\text{Nt}}{\text{Nur}-1} \quad \text{(1)}$$

Nur represents the number of informants who possess knowledge about and utilize the plant species for their antidiarrheal properties, while Nt denotes the count of plant species that are employed as antidiarrheals.

Fidelity Level (FL)

FL analysis enables the determination of the number of informants who affirm employing a particular plant for equal primary treatment purposes. FL is characterized as the proportion of informants who assert that a particular plant species is used to treat diarrhoea. The formula for determining FL is as follows (Khan et al., 2014):

$$FL = \frac{\text{Ip}}{\text{Iu}} x100 \quad \text{(2)}$$

Ip represents the count of informants who reported using the plant species to treat diarrhoea, whereas Iu represents the total number of informants.

Choice Value (CV)

Analysis of CV aims to determine the number of plant species utilized as antidiarrheal drugs. CV value is classified from 0 to 100, with a score of 100 indicating a species used as an alternative treatment for specific diseases. CV is determined using the subsequent formula (Jaradat et al., 2017):

$$cv = \frac{\text{Pcs}}{\text{Sc}} \text{.....(3)}$$

Pcs is the number of informants who mention a specific plant species for diarrhoea treatment, and Sc represents the total number of plant species claimed for treating diarrhoea by informants.

RESULT AND DISCUSSION

Characteristics of informants

The local knowledge of using plants as diarrhoea remedies is still widely recognized among the Tenggerese community in Tosari District, Pasuruan Regency. Each village in Tosari District has one traditional shaman and one traditional birth attendant. They use mantras when treating sick people. Meanwhile, plants are utilized as intermediaries for medicinal purposes. This study collected data from 32 informants by interviews in four villages of Tosari Districts. The characteristics of the informants are listed in Table 1. Most informants are ordinary people (75%) from Wonokitri Village (28%) and Tosari Village (28%) with male gender (59.4%). There are more male informants since men were more easily met than women when visiting the Tengger tribe area. Most informants were between 40 and 49 years old (37.5%). Most respondents graduated from primary education (53.1%) and identified as Hindu (59.4%). Those with higher educational backgrounds had less knowledge about medicinal plants utilization since, in order to pursue higher education, the Tenggerese community had to leave their inhabitation. When the Tenggerese community leaves their habitation, they could encounter the impact of urban living. A possible effect is that they have been used to apply modern medical treatments for diarrhoea. The original religion of the Tengger tribe is Hindu. Therefore, the Hindu community still adhere to the indigenous healing practices of the Tengger tribe and have faith in them.

Table 1. Characteristics of informants from four villages in Tosari district

Characteristics of informants		Number of informants	Percentage
Village	Wonokitri	9	28
	Tosari	9	28
	Ngadiwono	8	25
	Podokoyo	6	19
Social status	Traditional shaman	4	12.5
	Traditional birth attendant	4	12.5
	Ordinary people	24	75
Gender	Male	19	59.4
	Female	13	40.6
Age	40-49 years old	12	37.5
	50-59 years old	6	18.8
	60-69 years old	11	34.4
	70-79 years old	2	6.3
	80-89 years old	1	3.1
Education	Not graduated from elementary school	4	12.5
	Graduated from elementary school	17	53.1
	Graduated from junior high school	3	9.4
	Graduated from senior high school	8	25
Religion	Hindu	19	59.4
	Islam	13	40.6

Inventory of medicinal plants used by the Tengger tribe

Based on the information obtained from the informants, there are nine plants for compounding traditional recipes of the Tengger tribe as listed in Table 2. All plants were easily obtained in

nearby yards or forests. The duration of treatment for diarrhoea using herbal remedies often lasts 1-2 days. In previous research, only six plants were found as antidiarrheal remedies by the Tengger tribe, namely *Elaeocarpus longifolius* Blume, *Rubus rosaefolius* Sm., *Centella asiatica* (L.) Urb., *Musa paradisiaca* L., *Areca catechu* L., and *Psidium guajava* L. (Arifin, 2012). Other study stated *Annona squamosa* L., *Coriandrum sativum* L., *Alyxia reinwardtii* Blume, *Colocasia esculenta* (L.) Schott, *Ananas comosus* (L.) Merr., *Casuarina junghuhniana* Miq., *Elaeocarpus longifolius* Blume, *Ocimum citriodorum* Vis., *Psidium guajava* L., *Cymbopogon citratus* (DC.) Stapf, *Rubus rosaefolius* Sm., *Physalis angulata* L. for antidiarrheal treatment of the Tengger tribe (Bhagawan et al., 2023).

Table 2. Medicinal plants utilized by the Tengger tribe for antidiarrheal treatment

Family	Species	Local name	Plant parts	Age of plants	Compounding method	Routes of administration
Acoraceae	*Acorus calamus* L.	Dringu	Leaves	Immature	Pounded	Topical
Apiaceae	*Centella asiatica* (L.) Urb.	Calingan	Fruits	Immature, mature	Raw	Oral
Elaeocarpaceae	*Elaeocarpus longifolius* Blume	Jambu wer	Fruits	Immature, mature	Raw, squeezed	Oral
Lauraceae	*Litsea cubebae* (Lour.) Pers.	Krangean	Leaves	Immature	Decoction	Oral
Musaceae	*Musa balbisiana* Colla	Pisang klutuk	Fruits	Immature	Raw, squeezed	Oral
	Musa paradisiaca L.	Pisang raja	Fruits	Immature, mature	Raw, roasted	Oral
Piperaceae	*Piper betle* L.	Sirih	Leaves	Immature	Decoction	Oral
Rosaceae	*Rubus rosaefolius* Sm.	Grunggung	Fruits	Mature	Raw	Oral
Zingiberaceae	*Curcuma longa* L.	Kunir	Rhizomes	Mature	Squeezed	Oral

Based on the data collection conducted, it is known that Musaceae (23%) is the plant family most commonly used in the treatment of diarrhoea by the Tengger tribe (Table 3). Informants recommended several plants that have differences in the age of the plant parts used. Table 3 compares the age of plant parts used as antidiarrheal remedies, with 56.25% utilizing immature plants and 43.75% using mature plants. This study also revealed that the parts of plants utilized in diarrhoea treatment by the Tengger tribe are fruits (89.58%), leaves (6.25%), and rhizomes (4.17%). Immature fruits were preferred more than other plant parts due to their high astringent properties (Kusuma & Zaky, 2005).

As shown in Table 4, most medicinal plants were used without any specific compounding methods (raw ingredients) (76%). The other methods of preparation used by Tengger tribe for diarrhoea treatment involved squeezed (16%), decoction (4%), roasted (2%), and pounded (2%). Some informants stated that the absence of complex and time-consuming compounding procedures made it easier and more practical. These compounding method were commonly known by the Tenggerese community as reported in the previous study (Bhagawan et al., 2023).

Table 3. Family of plants, age of plant parts, and plant parts of medicinal plants utilized by the Tengger tribe for antidiarrheal treatment

Family	Species	Utilization of family	Age of plant parts		Plant parts		
			Immature	Mature	Fruits	Leaves	Rhizomes
Acoraceae	*Acorus calamus* L.	1	1	-	-	1	-
Apiaceae	*Centella asiatica* (L.) Urb.	1	-	3	5	-	-
Elaeocarpaceae	*Elaeocarpus longifolius* Blume	1	12	10	12	-	-
Lauraceae	*Litsea cubebae* (Lour.) Pers.	1	1	-	-	1	-
Musaceae	*Musa balbisiana* Colla	2	5	-	5	-	-
	Musa paradisiaca L.		2	1	**5**	-	-
Piperaceae	*Piper betle* L.	1	1	-	-	1	-
Rosaceae	*Rubus rosaefolius* Sm.	1	5	5	16	-	-
Zingiberaceae	*Curcuma longa* L.	1	-	2	-	-	2

Table 4. Compounding methods and administration routes of medicinal plants utilized by the Tengger tribe for antidiarrheal treatment

Species	Compounding methods					Administration routes	
	Raw	Squeezed	Decoction	Roasted	Pounded	Oral	Topical
Acorus calamus L.	-	-	-	-	1	-	1
Centella asiatica (L.) Urb.	5	-	-	-	-	7	-
Elaeocarpus longifolius Blume	10	1	-	-	-	12	-
Litsea cubebae (Lour.) Pers.	-	-	1	-	-	-	-
Musa balbisiana Colla	5	2	-	-	-	7	-
Musa paradisiaca L.	5	-	-	1	-	7	-
Piper betle L.	-	-	1	-	-	-	-
Rubus rosaefolius Sm.	13	-	-	-	-	16	-
Curcuma longa L.	-	5	-	-	-	-	-

Table 4 also shows that most traditional medicine usage for antidiarrheal purposes was administered orally (98%). In addition, antidiarrheal plants were also used topically (2%), meaning they were applied to the abdomen of diarrhoea patients. The use of traditional medicine orally was frequently performed individually due to the easy accessibility of the plants. According to information from the Tenggerese community, the oral administration of medicinal plants was believed to promote the healing process of diarrhoea. This method was also employed by the Tenggerese community in other regions to treat diarrhoea (Arifin, 2012; Bhagawan et al., 2023).

Determination of Fic, FL, and CV

The obtained data is quantitatively calculated using the parameters of Fic, FL, and CV for data analysis. Fic value indicates the consistency of information obtained from informants regarding the effectiveness of medicinal plants in treating specific diseases (Karaköse et al., 2019). In this study, Fic value is 0.74, indicating a high level of consistency in the information provided by the informants.

FL value is used to determine the percentage of informants claiming to utilize a plant for the equal main purpose (Khan et al., 2014). Table 5 shows that the highest FL value is obtained for *E. longifolius* (69%), indicating that most informants used this plant as an antidiarrheal treatment. Meanwhile, the lowest value of 3% is obtained for *A. calamus*, *P. betle*, and *L. cubebae*. This indicates that the three plants were not widely used for antidiarrheal remedies by the Tengger tribe community in the four villages.

Table 5. FL and CV value of medicinal plants utilized by the Tengger tribe as antidiarrheal remedies

Plants name	FL (%)	CV
Elaeocarpus longifolius Blume	69	2.4
Rubus rosaefolius Sm.	30	1.1
Musa balbisiana Colla	10	0.6
Centella asiatica (L.) Urb.	9	0.3
Musa paradisiaca L.	9	0.3
Curcuma longa L.	6	0.2
Acorus calamus L.	3	0.1
Piper betle L.	3	0.1
Litsea cubebae (Lour.) Pers.	3	0.1

CV value determines the number of plant species that are relatively used as antidiarrheal agents (Jaradat et al., 2017). The study exhibited that the highest CV value is found in *E. longifolius*, which is 2.4 (Table 5). The value indicates that *E. longifolius* was the most commonly used plant by the Tenggerese community in the four villages as an antidiarrheal. Meanwhile, the lowest CV value of 0.1 was obtained from *A. calamus*, *P. betle*, and *L. cubebae*, indicating that these three plant species were not widely used as antidiarrheal agents. Based on FL and CV values, it is known that the plant most commonly used by the Tengger tribe in the four villages of Tosari District as an antidiarrheal is *E. longifolius*.

A previous study reported that *E. longifolius* had high Species Use Value (SUV) and Fidelity Level (FL) values and contained 25 chemical contents, with peonidin, D-phenylalanine-benzoxazole, 6-shogaol, and piperine as major constituents. The plant also showed antibacterial activity against *Staphylococcus aureus* and *Shigella dysenteriae* (Bhagawan et al., 2023). Peonidin is known to have antibacterial (Jeyaraj et al., 2023), antiinflammation, and antioxidant activity (Bonetti et al., 2017). D-phenylalanine-benzoxazole showed antibacterial activity against *Mycobacterium tuberculosis* (Pepi et al., 2022, 2023). 6-shogaol had antimicrobial, antioxidant (Ghasemzadeh et al., 2018), antiinflammation (Deb et al., 2019), antibiofilm, and antivirulence activity (Lee et al., 2018). In addition, piperine exhibited antimicrobial activity against *Staphylococcus aureus*, *Salmonella* sp., *Proteus mirabilis*, and *Candida albicans* (Alves et al., 2022) . The active ingredient was also known for its antidiarrheal activity (Bajad et al., 2001; Satitsri et al., 2023).

CONCLUSION

The Tenggerese community living in the villages of Wonokitri, Tosari, Ngadiwono, and Podokoyo still used herbal remedies for treating diarrhoea. Based on the results of the 32 informants interviewed, most were men between 40 and 49 years old. They included traditional shamans, traditional birth attendants, and ordinary people. Most of them had completed elementary school and identified as Hindu.

From the nine plants mentioned by those interviewed, it is evident that most informants utilized immature plants, specifically consuming raw fruit orally. In addition, this study demonstrated that *E. longifolius* exhibits promising potential for developing diarrhoea treatment due to its high FL and CV values.

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