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Original Article

Immunomodulatory and Acute Toxicity Tests of Rhizome Ethanol Extract of *Etlingera Flexuosa* Poulsen (Zingiberaceae) on Male Mice (*Mus Musculus*)

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ABSTRACT

Introduction: Immunomodulators are molecules of synthetic or biological origin that help to regulate the immune system. Many studies have focuses on exploring for phytochemical compounds that used as immunomodulatory properties in Indonesia, as well as in Sulawesi. The immunomodulatory activity of rhizome extract of *E. flexuosa*, an endemic flowering plant of Sulawesi on male mice were studied. **Methods:** 25 male mice (*Mus musculus*) used were randomly divided into 5 groups and *Staphylococcus aureus* (ATCC 25923) was used as inducer. The negative control group was given 0.5% Na-CMC (Carboxymethyl Cellulosa Sodium), positive control group was given stimuno® and treatment groups were an ethanol extract of *E. flexuosa* with successive doses of 200, 400 and 800 mg/kg body weight (BW) respectively. Each group was given the preparation orally for 7 days and on the 8th day the test animals were induced by *Staphylococcus aureus* bacteria intraperitoneally. The mice were dissected and the peritoneal fluid was taken to determine the activity of the macrophage cells. Meanwhile, Thomson and Weil method was used to study the acute toxicity test and determine the lethal dose 50 (LD_{50}). **Results:** The percentage of macrophage activity in each group of negative control, positive control, extract doses of 200, 400 and 800 mg/kg BW respectively were 40.40%, 82.65%, 53.05%, 69.38% and 82.06%. Based on the results obtained, it was shown that the *E. flexuosa* rhizome extract has an optimum dose of 800 mg/kg BW, which was not significantly different from the positive control. Meanwhile, the symptoms of toxicity began to appear from a dose of 600 mg/kg BW to a dose of 2400 mg/kg BW including decreased motor activity, tremor, ataxia, lids and writhing. LD_{50} expressed in LD_{50} within the criteria of being practically non-toxic. **Conclusions**: The *E. flexuosa* rhizome ethanolic extract showed the immunomodulatory activity at optimum dose of 800 mg/kg BW by the increasing of macrophage phagocytosis activity. Moreover, the extract was also practically non-toxic based on LD_{50} value.

Key words: *Etlingera flexuosa*, Phagocytosis, Macrophages, Immunomodulators, Immunostimulants, Lethal Dose 50, Toxicity.

INTRODUCTION

Human beings have been subject to numerous infectious diseases, despite the existence of an immune defense system capable of fighting pathogenic agents, such as bacteria, viruses, fungi, and protozoa[1, 2]. The immune system of human being plays a pivotal role in the maintenance of ordinary physiological and immunological functions as well as internal environment[3] .

Immunomodulators are molecules of synthetic or biological origin that help to regulate these effects on the immune system. They are able to modulate, suppress, and stimulate the pathophysiological processes within the body[4]. According to how they influence the efficiency of the immune system, they can be categorized as immunosuppressants, immunostimulants, and immuno-adjuvants[5,3] .

Since ancient times, human beings have learned to utilize natural plants, with the aim of alleviating symptoms or even curing the most varied ills [6]. Natural plants and their active metabolites play an imperial role in the management of immunological diseases by modulating immune responses. Recently, phytochemicals have gained a great interest due to their multi-pharmacological activities such as immunomodulatory and antioxidant activities[4]. Phytochemicals or secondary metabolites are commonly produced in order to response of external stimuli including infection, nutrition or alteration of climatic conditions. Additionally, these compounds are synthesized in specific parts of the plants. Till date, about 4000 phytochemicals have been identified .

Many studies have focuses on exploring for phytochemical compounds that used as immunomodulatory properties in Indonesia[7,8], as well as in Sulawesi[9,10].

Etlingera flexuosa (Zingiberaceae) is a native flowering plant to the island of Sulawesi that was firstly described and collected by Poulsen[11] from montane forest of Lore Lindu National Park Central Sulawesi Indonesia It is naturally distributed in Central and South Sulawesi. *E flexuosa* was known as "karondo" by local people in Sedoa village, Poso District, Central Sulawesi Indonesia. In previous study reported that *E flexuosa* contains some secondary metabolites such as flavonoids, tannins, saponins, terpenoids, alkaloids and steroids, and also shows antioxidant activity[11] and essential oil[12].

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Conclusively, the extract of *E. flexuosa* could be used as inhibiting agent for the growth of *Candida albicans* yeast has ability can be used as antifungal[12], antibacterial[13]. Besides, the ethanol extracts of the leaves, pseudostems, and rhizomes parts of the species has antiviral activity of HIV-infected MT-4 cells[14] .

This study aims to determine the immunomodulatory activity of *E. flexuosa* rhizome extract on phagocytic activity of macrophages on male mice and to obtain the optimal dose

MATERIAL AND METHODS

Ethical Clearance

This research was conducted after obtaining ethical approval from the Medical and Health Research Ethics Committee, Faculty of Medicine, Tadulako University Number: 7691/UN 28.1.30/KL/2022.

Plant Material

Etlingera flexuosa sample was obtained from the montane forest of Lore Lindu National Park (LLNP), an important protected area in Central Sulawesi[15-17] .

The collection of plant material was permitted by the park authority (Research Permitted No. SI 22/IV-T5/ BIDTEK/6/2023) and then identified at the Academic Support Unit Biodiversity of Sulawesi, Herbarium Celebense (CEB) Tadulako University by the author (Ramadanil Pitopang). Herbarium specimen (RP. 10041) was keep at the CEB Tadulako University Palu.

Plant extraction

Plant extraction was conducted at the Laboratory of Pharmacognosy-Phytochemistry, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Tadulako University. The extraction of the sample was carried out using known standard procedures[18,19] .

E. flexuosa rhizome was cleaned by washing with running tap water to remove adhering dirt, then sliced to 3 cm length, washed more, and then kept in flowing tap water for five minutes then dried using an oven with a temperature of 45^0 C. Cleaned dry plant sample was blended until smooth to a simplicial powder by a mixer grinder. These powder materials was then used for extraction of dyes. The extract was prepared by the soxhletation method using 70% ethanol as solvent. The obtained filtrate was concentrated using a vacuum rotary evaporator at a temperature of 60-65°C to obtain the crude extract[19].

Bacterial culture and animals

Bacterial culture *Staphylococcus aureus* (ATCC 25923) was obtained from the laboratory of Microbiology, Department of Biology Faculty of Mathematics and Natural Sciences, Tadulako University, Palu and then maintained on Nutrient Agar medium (NA).

Immunomodulator Activity Testing

The test animals were 25 male mouse divided into 5 groups. Group I, a negative control was given 0.5% CMC-Na, group II as a positive control was given Stimuno® containing commercial *Phyllantus niruri* L. (Phyllantaceae) extract at a dose of 4.5 mg/kg body weight (bw), group III, IV and V were given an ethanol extract of *Etlingera flexuosa* with successive doses of 200, 400 and 800 mg/kg BW respectively. Each group was given the preparation orally for 7 days and on the 8th day. Each test animal was infected induced with 0.5 mL of by *Staphylococcus aureus* bacteria suspension intraperitoneally and then left for 1 hour. Animals were anesthetized by using ketamine at a dose of 6.5-13 mg/kg BW, then the abdomen was dissected using a scalpel and sterile tweezers. If the peritoneal fluid in the rat's stomach was found in small quantities, then 1-2 mL of phosphate buffered saline (PBS) steril solution with pH 7.8 was added and shaken slowly. The peritoneal fluid was taken from the peritonial cavity by using 1 mL syringe. Peritoneal fluid was stained on the glass slide, fixed with the addition of methanol for 5 minutes, stained with 10% Giemsa stain, left for 20 minutes and then rinsed with running water. After the glass slide dried, the sample was dripped with immersion oil and viewed under a microscope (Olympus CX23 LED, Olympus) using magnification of 1000× [20]. Meanwhile, the blood was also taken from each animal via intracardiac section and put into Eppendorf tube that contain EDTA. Blood was centrifuged with a centrifuge (Series C2®) for 15 min at 3000 rpm to collect plasma. The collected plasma was then put into a microtube and stored in a container (-20°C) to be tested for IFN-γ and TNF-α levels using an ELISA Reader at a wavelength of 450 nm according to manufacturer's instructions. The results were expressed as picograms of cytokine per milliliter of protein. The value of phagocytosis activity is the percentage of macrophage cells that actively carry out the process of phagocytosis among 100 macrophage cells[21]. Calculation of the number of macrophage cells using the ImageJ application.

The phagocytic activity of macrophages in the peritoneal fluid was calculated by using formula :

% A = B / C x 100 %

A=Phagocytic activity; B=number of active macrophages; C=number of observed macrophages [9]

Phagocytosis Index (IF) was calculated for each test group compared to the negative control group. Phagocytosis Index was calculated by using the formula below[22, 23]

Phagocytosis Index = (% Phagocytosis Activity of Mouse X)/(% Phagocytosis Activity of Negative control)

Statistical Analyses

The Data were analyzed statistically using Statistical Product and Service Solution (SPSS) version 26. he phagocytotic activity of macrophages and TNF-α level were analyzed by using one way ANOVA test, followed by Post Hoc Duncan test, Meanwhile, the IFN-γ level was analyzed by using the Kruskal-Wallis test, followed by the Mann-Whitney test. Analysis was performed with a significance value of 95% ($p \leq 0.05$)[20]. The data of Acute toxicity test and Lethal Dose 50 (LD_{50}) were analyzed using T-Test, D0 and D14, and then LD50 calculated using Thompson and Weil method.

RESULT AND DISCUSSION

Etlingera flexuosa, one of endemic *Etlingera* of Sulawesi. It is a terrestrial herb species which can reach 5 m in height in its natural habitat. It was very easy to recognize in their habitat due to leaves sheath color from yellowish to purple. The flowering shoot is arising from rhizome with flowers pale pink in color. The main characteristic of the species is the labellum bends outwards with age. Locally, it is utilized extensively by local people for a wide variety of cultures uses. The fruit is an important source for cooking fish dishes such as to enhance flavor of food. The young shoots are edible as vegetable while the leaves are used as roofing material[13]. The morphological picture of the species is provided in Figure 1 below:

The rhizomes of the *E. flexuosa* plant were used in this experiment because it has been utilized empirically in medicine purpose. Besides, the rhizome contains secondary metabolites which are suspected to be immunomodulatory such as flavonoids and alkaloid compounds, both of which have immunomodulatory activity[11, 8] and volatile compounds sesquiterpenes[12].

The rhizome extract of plant was prepared by the soxhlation method using 70% ethanol at temperature 70°C. The soxhlation method

Figure 1. *Etlingera flexuosa* Poulsen (A), *E. Flexeusa* in its habitat (B), Rhizome (C) and Flower (D).

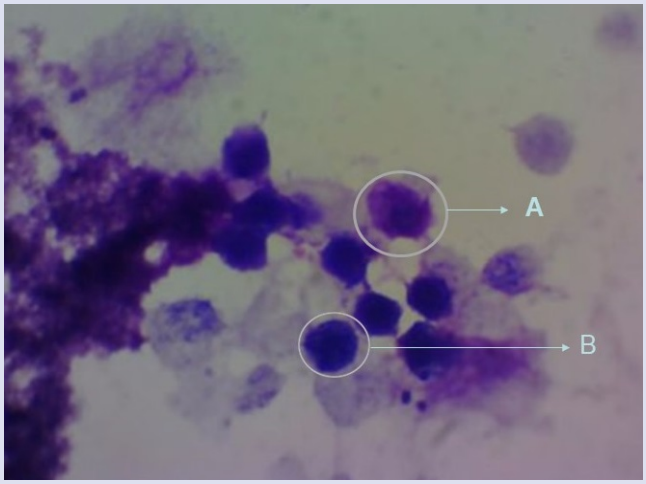

Figure 2. Peritoneal fluid smear with 1000x magnification (A) Active macrophages and (B) Inactive macrophages.

was chosen because in this method the extraction process occurs continuously, the sample is extracted by pure solvent resulting from condensation so as to minimize the solvent used and does not require much time compared to maceration, and the extracted sample is an ideal sample for soxhlet extraction because it is a dry solid and been refined[24] .

Macrophages Phagocytosis Activity

The phagocytosis macrophages activity was characterized by the shape and size of active macrophages that are getting bigger. The phagosome appears as the membrane becomes more tortuous, the number of lysosomes increases, the Golgi apparatus increases in size and the rough endoplasmic reticulum develops, while in-active macrophages have a smaller shape and size than active macrophages[25, 20]. The differences between active and inactive macrophages can be seen in Figure 2.

Immunomodulatory testing was performed by calculating the phagocytosis activity of mice peritoneal macrophages. The value of macrophage phagocytosis activity can be calculated from the macrophages that actively carry out phagocytosis among the total number of cells expressed in percent. The phagocytosis index (PI) can be a reference for the classification of immunomodulatory activity. If the value of PI> 1 is classified as an immunostimulant compound, which means it can increase the body's resistance, while the value of PI <1 is classified as an immunosuppressant compound, which means it can suppress the immune system's excessive response. Macrophage phagocytosis activity can be seen in Table 1.

Acute Toxicity Test and Lethal Dose 50 (LD_{50})

The results of observations of signs of toxicity that appear in test animals 24 hours after treatment are presented in the table 2.

Toxicity Test Results Based on Number of Deaths

The results of the acute toxicity test of ethanol extract of *Etlingera flexuosa* Poulsen rhizome on male mice are presented in table 3.

The results based on the data in table 3, it was obtained the value of r (1,0,0,0). These results can be ignored because only 1 mice experienced death (did not reach 50%). According to Chinedu et al[26], if the maximum dose does not cause the death of test animals, then the LD50 is expressed as pseudo LD50 by taking the maximum dose. So in this study, LD50 is known as pseudo LD50, which is 2400 mg/kgBB. If at the maximum dose there is no death in experimental animals, it is clear that the compound is included in the criteria of practically non-toxic.

Mice Body Weight Results

The average weight of mice for 14 days is presented in Figure 3.

DISCUSSIONS

This experiment divided into 5 groups, namely; negative control group which was given 0.5% Na-CMC and other comparison groups. Na-CMC have not a pharmacological effect on tested animals, but it just was used as as a suspension in the test preparation because it has inert properties and produces a stable suspension[20]. Na-CMC suspension also has advantages in terms of viscosity and sedimentation volume along with a lower flow rate[27]. The second group is a positive control, which was given stimuno® as a standard immunomodulator. The Stimuno® contains "meniran" extract (*Phyllantus niruri L.*) that can used as immunomodulator because it contains various compounds, especially flavonoids that can increase the immune system[28] .

Another treatment is a variation of 3 different doses, namely 200 mg/kg BW, 400 mg/g BW and 800 mg/kg BW that aims to determine the optimal dose following research conducted by Wahyuni et al[20] that use the same genus plants *Etlingera elatior*, where a dose of 400 mg/kg BW shows the best immunomodulatory activity. The treatment given

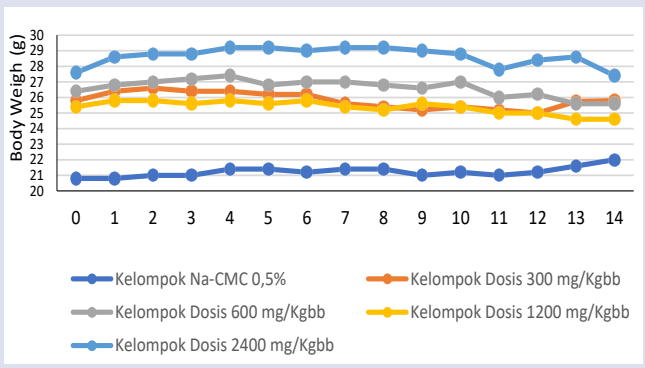

Figure 3. Body weight of mice for 14 days

Table 1. Percentage of macrophages phagocytosis activity at each treatment and its Phagocytosis Indices (PI).

Treatment	%age of Macrophages phagocytosis activity	Phagocytosis Indices
Negative Control	40,40±5,82[a]	-
Positive Control	82,65±2,78[d]	2,05
Dose 200 mg/kg BW	53,05±2,98[b]	1,31
Dose 400 mg/kg BW	69,38±5,67[c]	1,72
Dose 800 mg/kg BW	82,06±2,81[d]	2,03

Values are means and SD of three replicates of each treatment. Bars for each treatment with the same letter indicated not significant different (P<0.05).

Table 2. Toxicity Symptoms in Test Animals.

Observed symptoms	Sodium-CMC 0,5 %	300 mg/ KgBW %	600 mg/ KgBW %	1200 mg/ KgBW %	2400 mg/ KgBW %
Motoric Activity	0	0	0	40	60
Straub	0	0	0	0	0
Tremor	0	0	40	40	60
Convulsion	0	0	0	0	0
Ataxia	0	0	0	20	0
Reflex Reaction	0	0	0	0	0
Muscle Tone	0	0	0	0	0
Palpebra	0	0	0	40	0
Salivation	0	0	0	0	0
Stretching	0	0	20	40	60
Piloerection	0	0	0	0	0
Skin Discoloration	0	0	0	0	0
Sweating	0	0	0	0	0

Table 3. Number of Deaths of Test Animals After 14 Days.

No.	Groups	Amount of Mice	Extract dose	Amount of Death
1	II	5	300 mg/KgBW	1
2	III	5	600 mg/KgBW	0
3	IV	5	1200 mg/KgBW	0
4	V	5	2400 mg/KgBW	0

to each group was carried out for seven consecutive days once a day orally with the aim of stimulating the immune system of each group of test animals. On the eighth day, each test animal was infected with 0.5 mL of *Staphylococcus aureus* ATCC 25923 bacterial suspension intraperitoneally with the aim of triggering infection[20]. *S. aureus* bacteria was used with consideration because this species is the most invasive and is easily phagocytosed with sufficient antibodies because it does not contain protein A which is antiphagocytic. This bacterium is also a gram-positive bacterium, which is able to absorb Giemsa staining so that it is clearly easy to observe under a microscope[29, 30] .

Based on Tabel 1, it can be seen that the value of mean and SD of macrophage phagocytosis activity on the experimental animal were differ among the treatment. There were 40,40% on the negative control, 82,65% positive control, and 53.05%, 69.38% and 82.06% at dose 200 mg/kg BW, dose 400 mg/kg BW and dose 800 mg/kg BW respectively. The highest percentage of macrophage phagocytosis activity was found in the positive control group of 82.65%, then followed successively by the group with dose 800 mg/kg BW (82.06%), 400 mg/kg BW (69.38%) , and 200 mg/kg BW (53.05%) respectively. The lowest percentage of macrophage phagocytic activity was found in the negative control group, namely 40.40%. These results indicate that the percentage of macrophage phagocytosis activity is directly proportional to the increase in the dose of the ethanol extract of *E. flexuosa* .

The results of the one-way ANOVA test (sig <0.05) indicated that that the ethanol extract of *E. flexuosa* rhizome gave a significant difference in increasing macrophage phagocytosis activity. Based on Duncan's Post Hoc test, it can be seen that there is a significant difference between the positive control group and the extract dose groups compared to the negative control group. It is due to the negative control was only given 0.5% Na-CMC suspension which did not provide an immunomodulatory effect, whereas the treatment with the three extract doses had potential as an immunomodulator because there was a difference in the amount of macrophage phagocytic activity when compared to the negative control. The negative control still showed phagocytic activity due to the innate (natural) immune response from macrophage cells which provide the body's defense against antigens that enter the body. Whereas in the positive control group there was an increase in macrophage phagocytosis activity due to administration of Stimuno® containing meniran extract which was used as a standard immunomodulator. The increase in phagocytic activity in the extract dose treatment group was due to, apart from the natural immunity of macrophage cells, it also came from the active ingredients contained in the ethanol extract of *E. flexuosa*. There was a significant differences immunomodulatory activity among the three doses of the extract, and the 800 mg/kg BW dose had the highest immunomodulatory activity, and did not differ significantly from the positive control group, so it can be said that there was no difference phagocytic activity of macrophages between the 800 mg/Kg BW extract group and the positive control group. *Etlingera flexuosa* contains some secondary metabolites such as flavonoids, tannins, saponins, terpenoids, alkaloids and steroids[11]. Several studies reported that specific secondary metabolites meditate the immunostimulatory activity of the medicinal plant[31, 10]. Flavonoids have been shown to increase IL- 2 and lymphocyte proliferation which in turn, will affect CD4+ cells and activate Th1 cells. Furthermore, the activation of Th1 cells affects specific macrophage-activating factors. Flavonoids also activate NK cells to stimulate the production of TNF-α and IFN-γ[31]. Phenolic compounds were reported to activate β cells and increase the killing activity of NK cells[32]. Meanwhile, tannin compounds are antibacterial by stimulating cells to phagocytize bacteria. Besides, *E. flexuosa* contains the essential oils such as ; monoterpenes, Diterpenes, triterpenes and sesquiterpenes. Pitopang et al[12] reported 76 and 39 essential oil compounds were analyzed using GC-Mass Spectrophotometer by extraction and hydro distillation method respectively. Essential oils (EOs) are a mixture of natural, volatile, and aromatic compounds obtained from plants that can be utilized as immunomodulatory activity[34, 35] .

Based on the results of the calculation of the phagocytosis index, it shows that the phagocytosis index will increase along the increasing dose of the extract. The phagocytosis index of the three different doses of the extract had PI values > 1, its mean that the ethanol extract of *E. flexuosa* rhizome is an immunomodulator belonging to the immunostimulant category. Immunostimulants are compounds that can increase the function and activity of the immune system. The general mechanism of immunostimulants is to correct the imbalance of the immune system by increasing specific and non-specific immunity. Immunostimulants can increase the number of phagocytic cells and increase their phagocytic activity[35] . According to Azizah and Winata[23] that the phagocytosis index was calculated after obtaining the percentage value of macrophage phagocytosis activity from each test group. If the phagocytosis index value is greater than 1 (PI> 1), then the test substance has the ability to act as an immunostimulant

In this study, an acute toxicity test was carried out by looking at the toxic effect of ethanol extract of *Etlingera flexuosa* Poulsen rhizome which can be seen from the LD50 value. Toxicity tests using the Thomson and Weil methods because this method requires fewer test animals and has a high level of accuracy because the data analysis uses the Weil table data list[36]. The test animals used are mice because mice have physiological properties that are almost the same as humans and

the handling is quite easy. This study uses male mice because hormonal conditions in male mice are more stable than female mice which can experience changes in hormonal conditions in the ovulation cycle and more often experience stress than male mice[37] .

This study was conducted using 25 mice that had previously been acclimatized for 7 days which aims to make the test animals accustomed to a new place of residence and not stressed. The test animals were divided into 5 groups with each group consisting of 5 mice which were given ethanol extract of Etlingera flexuosa Poulsen rhizome in graded doses. Taking the initial dose according to regulation of Indonesian Agency of Drugs and Food[38] when there is no information regarding the test material, the recommended initial dose is 300 mg/ kgBW to consider the welfare of the test animals. So the doses used are 300 mg / kgBW, 600 mg / kgBW, 1200 mg / kgBW and 2400 mg / kgBW.

The observation of clinical symptoms observed after 24 hours of treatment with several parameters such as motor activity, straub, tremor, convulsions, ataxia, reflex reactions, muscle tone, palpebra, salivation, writhing, piloerection, skin color changes, sweating. Sodium-CMC 0.5% group did not experience symptoms of ketoxicity and looked normal. In the 300 mg/KgBW dose group did not experience symptoms of ketoxicity and looked normal. The 600 mg/ KgBW dose group experienced ketoxic symptoms such as 20% tremor and 20% writhing. The 1200 mg/KgBW dose group experienced ketoxic symptoms in the form of decreased motor activity 40%, tremor 40%, ataxia 20%, palpebra 40% and writhing 40%. The 2400 mg/KgBW dose group experienced symptoms in the form of decreased motor activity 60%, tremor 60%, and writhing 60% (Table 2).

This study shows that the administration of *Etlingera flexuosa* Poulsen extract observed from day 1 to day 14 obtained mortality data based on Table 3. In the 300 mg/KgBW dose group there was death in 1 mice, the 600 mg/KgBW dose had no mice deaths, the 1200 mg/KgBW dose had no mice deaths, and at the 2400 mg/KgBW dose there were no deaths. Observation of delayed toxic effects for 14 days was carried out to determine toxic effects that were not present in the previous 24-hour observation and the number of deaths of test animals as a parameter of the LD50 value[26.]

Determination of the LD50 value was obtained using the Thompson and Weil formula. This method was chosen because it has a fairly high level of confidence and is the most frequently used method, this method also uses a list of LD50 calculations so that the results obtained are more accurate[39]. The LD50 calculation shows the absence of the R value in the Weil table because only 1 mice died in the 300 mg dose group so that the R value obtained is (1, 0, 0, 0). These results can be ignored because it only occurs in 1 mice (not reaching 50%), which may be caused by other factors outside the effects caused by the preparation of *Etlingera flexuosa* Poulsen rhizome extract. These deaths can be caused by errors at the time of administration of the preparation. If the maximum dose does not cause the death of the test animals, the LD50 is expressed as pseudo LD50 by taking the maximum dose. So in this study LD50 is known as pseudo LD50, which is 2400 mg/kgBW. If at the maximum dose there is no death in experimental animals, it is clear that the compound is included in the "Practically Non-Toxic" criteria[40].

The next toxicity parameter is the observation of body weight for 14 days. Observation of body weight for 14 days aims to determine the relationship of *Etlingera flexuosa* Poulsen rhizome extract to changes in the average body weight of mice for 14 days. The statistical results showed that the body weight data of male mice before and after treatment had no significant difference because the P>0.05 value was obtained. This means that the increase in body weight of male mice in different groups has almost the same increase. However, there are many factors or variables that affect changes in body weight such as stress, movement space, and feed intake given[41.]

CONCLUSIONS

Based on the results obtained, it was shown that the *E. flexuosa* rhizome extract has an optimum dose of 800 mg/kg BW, which was not significantly different from the positive control, so it has the potential to be developed as a scientific immunomodulator with immune stimulating activity. Besides, the symptoms of toxicity began to appear from a dose of 600 mg/kg BW to a dose of 2400 mg/kg BW including decreased motor activity, tremor, ataxia, lids and writhing. LD_{50} expressed in LD_{50} within the criteria of being practically non-toxic.

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