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Determination of Total Levels of Secondary Metabolites and Oral Acute Toxicity Testing of Purple Leaf Ethanol Extract (PLEE) in Wistar Rats

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ABSTRACT

Introduction: The use of traditional medicines derived from the active ingredients of several types of plants is preferred by the community in treating several types of diseases. One of the plants that has been used by the community as medicine is purple leaves with the Latin name *Grapthophylum pictum* (L.) Griff. These leaves are traditionally used to treat rheumatism, menstruation, hemorrhoids, urinary tract infections, scabies, swelling, wounds, dermatitis, ear diseases, laxatives, and cancer. The aim of this study was to determine the total levels of secondary metabolites from purple leaf extract ethanol (PLEE) and evaluate the effect of administering PLEE on acute toxicity (LD50) in Wistar rats.

Methods: This research is a laboratory experiment with a post test only controlled group design. The research subjects were 20 male Wistar rats, divided into 1 control group and 3 treatment groups, each consisting of 5 rats. The control group only received distilled water, the Treatment I group (P1) was given a suspension of the purple leaf extract test preparation at a dose of 500 mg/kg. Treatment group II (P2), received a suspension of the test preparation at a dose of 2,000 mg/kg, while Treatment group III (P3) received a suspension of the test preparation at a high dose of 5,000 mg/kg of rats. The test preparation was given orally with only one administration at the beginning of the research period and observation was carried out for 14 days. Data was obtained if the rats died after being given treatment.

Results: The results showed that the total secondary metabolite content of purple leaves was alkaloids (24,725.99 mg/g), saponins (89.191 mg/g), flavonoids (6.332 mg/g) and tannins (0.884 mg/g), while based on toxicity the PLEE classification was mildly toxic with an LD50 value of 3,890 mg/kg in accordance with BPOM RI's acute toxicity potential at toxicity level 4 (oral LD50 500 – 5,000 mg/kg).

Conclusion: PLEE showed the highest total content of secondary metabolites in alkaloids, and consumption of PLEE in high doses caused the death of rats with a mild toxic classification.

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INTRODUCTION

Traditional treatment using medicinal plants is much sought after by the public as an alternative treatment because it has several advantages compared to medical treatment, such as relatively lower side effects, is easy to obtain, does not require high costs, and can be planted and mixed yourself. Medicinal plants can generally be found

growing wild in nature, but some medicinal plants can be obtained from cultivation. One example of a wild plant that can be used as a medicinal plant is the purple plant (1). Graphtophyllum pictum (L.) Griff are also known as purple leaves which are known to contain various chemical compounds, including alkaloids, pectin, formic acid, steroids, saponins, tannins, flavonoid and alcohol. Purple leaves are one of Popular traditional medicines that are widely used in Indonesia. There are many pharmacological functions possessed by green leaf plants, such as antioxidant, anti-inflammatory, antidiabetic, analgesic, photoprotective, immunomodulatory, nephroprotective, antihemorrhoidal, and antibacterial (2).

Biomolecules known as secondary metabolites can be used as basic materials for the discovery and development of new drugs. In plants there are common secondary metabolite compounds, namely alkaloids, flavonoids, steroids, saponins, terpenoids and tannins (3). Most reports of toxic effects are consequential use of herbal medicines associated with hepatotoxicity, despite reports of other toxic effects on kidneys, blood and heart have documented (4).

Currently, the number of medicinal substances and chemicals used by humans has increased significantly (5). Acute toxicity is defined as the unwanted effect (s) that occur either immediately or at a short time interval after a single or multiple administration of these chemicals or pharmacological substances or when large quantities capable of eliciting immediate toxic effect are used, which may result in chronic toxicity in the living system when used over a long period of time. These effects may be mild or severe, depending on the nature of the substance 6). However, studies of acute toxicity usually uncover possible dose-dependent unwanted (or adverse) effects, and these include all the information important for evaluating acute toxicity, including mortality. Currently, evaluation of the lethal dose (LD50), namely the dose that kills 50% of the rat population tested, is used as the main parameter in measuring acute toxicity and as the first step to carry out general chemical and pharmacological screening (7).

Toxicity tests are needed to determine the level of safety and side effects of medicinal plants. Herbal remedies which are commonly used for self-medication, drugs and toxins represent potential hepatotoxic agents because of the many idiosyncrasies and unpredictable toxicity. It should always be considered in the pathology of specimen evaluation (8). The preparations tested were given to experimental animals at different doses, then observed for 14 days. The aim of the acute toxicity test is to detect the intrinsic toxicity of a substance and obtain information regarding the LD50 value, which is a value that shows the dose of the test substance given causes 50% death in acute test animals (9).

In the research of Hilmarni, et.al (2016) a toxicity test of purple leaf extract ethanol (PLEE) was carried out on the blood profile or hematology of wistar rats and it was found that variations in extract doses of 50 mg/kg, 150 mg/kg, and 450 mg/kg did not affect the blood profile values. Rats so it can be said that toxicity testing by administering purple leaf extract ethanol (PLEE) to rats is not toxic to their blood or hematological profile (10). In several other studies, purple leaf extract ethanol (PLEE) has anti-inflammatory properties in healing hemorrhoids (11), and have the strongest antioxidant effect (12). Research by Sari, et al in 2021, stated that purple leaf extract ethanol (PLEE) had the effect of reducing blood glucose in rats induced by streptozotocin at varying doses of 150, 200 and 250 mg/kg (13).

Therefore, researchers conducted research to determine the total levels of secondary metabolites from purple leaf extract and determine the potential for acute toxicity based on LD50 and assess the clinical symptoms of purple leaf extract. It is hoped that the research results will provide information for further research regarding the potential toxicity and safety of purple leaves.

METHOD

This research was an experimental study using a completely randomized design with 20 male Wistar rats divided into 4 groups. This experimental research tries to examine whether there is a cause-and-effect relationship. The method is to compare one or more experimental groups that were treated with one or more comparison groups that did not receive treatment. Simple Random Sampling is randomization of groups, not individual subjects. In this experiment, this technique is used for grouping control group and experimental group were randomized.

This study has been approved by Ethics Committee of Tadulako University with ethical approval letter number of 7704/UN28.1.30/KL/2023. This research was conducted in the period June-August 2023, carried out at

the Phytochemistry-Pharmacognosy Laboratory, Instrument Laboratory, and Biopharmaceutical Laboratory of STIFA Pelita Mas Palu.

Tools and Materials

The tools used are surgical tools, glassware, 40 mesh sieve, HVE 5.0 autoclave, stirring rod, maceration vessel, blender, porcelain cup, separating funnel, disposable syringe, incubator, test animal cage, measuring flask, magnetic stirrer, bath water, dropper pipette, micro pipette, rotary evaporator, 3 ml oral probe, UV Vis spectrophotometry, test tube, gram scale, analytical balance, and water bath. The materials used are distilled water, aluminum foil, ammonia, 2 N hydrochloric acid, concentrated hydrochloric acid P, dragendorf LP, purple leaf extract, 96% ethanol, $FeCl_3$, handscoen, HCl, concentrated H_2SO_4, cotton, label paper, chloroform, magnesium P, mask, Na CMC 0.5%, NaCl 10%, and tissue and gloves.

Simplicia Characterization

Examination of simplicia characterization, particularly determining water content and willpower of general ash content material (14).

Preparation of PLEE

Making PLEE become executed using the maceration technique by weighing 2,400 grams of purple leaf simplicia powder after which extracting it the usage of 8 L of ethanol 95% solvent for 3x24 hours blanketed from mild, whilst every so often stirring. The extract is then filtered using filter paper and a filtrate is obtained. Furthermore, it was concentrated using a Rotary Vaccum Evaporator at a temperature of 60℃ and continued with evaporation using a water bath until a thick extract was obtained (15).

Preparation the experimental subject

Male Wistar rats were obtained from the test animal provider, Faculty of Mathematics and Natural Sciences, Tadulako University. With inclusion criteria: male gender, rat weight 150 – 250 grams, around 2 – 3 months old, healthy, active, normal behavior and activities, and macroscopically no visible anatomical and morphological abnormalities. Twenty rats were acclimatized for 14 days and then fasted for 16 hours before being given treatment.

In this study, male wistar rats were divided into 4 groups, each consisting of 5 (five) male white rats that were determined randomly. These groups include: 1) K: Control group, not treated (extract) only received distilled water and 0.5% Na CMC suspension. 2) P1: Treatment Group I, was given a suspension of the PLEE test preparation at a dose of 500 mg/kg. 3) P2: Treatment Group II, received a suspension of the PLEE test preparation at a dose of 2,000 mg/kg. 4) P3: Treatment Group III, given a suspension of the PLEE test preparation at a high dose of 5,000 mg/kg.

Phytochemical Screening Test

Flavonoid test, the ethanol extract of purple leaves was weighed as much as 0.5 grams, put into a test tube, then 10 mL of distilled water was added and heated over a water bath then filtered, then dissolved in 1 mL of ethanol (96%) with the addition of magnesium P powder, after which it was dissolved in 10 mL of concentrated hydrochloric acid P, if the purple color occurs, it indicates the presence of flavonoids and if the yellow color occurs, it indicates the presence of flavones, chalcone and aurone (3).

Alkaloid test, the alkaloid test was carried out with Dragendorf reagent. A positive test for alkaloids is indicated by the presence of an orange-to-yellow precipitate (15).

Saponin test, the Saponin test was carried out with hot distilled water and 2N HCl. Stable foam indicates a positive saponin test, and the foam does not disappear when added with HCl 2 N (16).

Tannin test, weigh 0.5 grams of purple leaf ethanol extract into a test tube, then add 5 mL of distilled water and boil for 5 minutes. Then filtered, the filtrate was added with 5 drops of 1% $FeCl_3$ (w/v). The dark blue or greenish black color that forms indicates the presence of tannin compounds (3).

Determination of Total Secondary Metabolic Levels

Total Flavonoid Test

Weighed 10 mg of the extract, put it into a 25 ml volumetric flask then add 96% ethanol to the mark. Take 1.5 ml of the solution made previously, put it into a 10 ml volumetric flask then add 0.1 ml of 10% $AlCl_3$ and 0.1 ml of 1M potassium acetate. Next, add distilled water up to the limit mark. Absorbance was measured at a wavelength of 420 nm(17).

Total Alkaloid Test

Weighed 100 mg of extract then put it in a 100 ml measuring flask, add 96% ethanol to dilute to the limit mark and homogenize. Next, read the absorption at a wavelength of 418 nm (18).

Total Saponin Test

Weighed 100 mg of the extract, and added 2 ml of 25% H_2SO_4 then put it in an autoclave for 120 minutes at 110°C, then extracted using ether, then the filtrate was dried. A total of 1 ml of distilled water was added, then stirred using a magnetic stirrer for 5 minutes, added 50 µl of anisaldehyde, shaken and let stand for about 10 minutes. Next, add 2 ml of 50% sulfuric acid and heat in a water bath at 60°C for 10 minutes. Then add distilled water to 10 ml, then read the absorption at a wavelength of 252 nm (3).

Total Tannin Test

Weighed 10 mg of extract then put it into a 10 ml volumetric flask and add distilled water until the mark is reached. Take 1 ml of the previous solution, put it in a 10 ml volumetric flask then add 1 ml of Folin Ciocalteu then let it sit for 3 minutes, then add 1 ml of 20% Na_2CO_3. Next, add distilled water to the limit mark. Then incubate for 40 minutes. Read the absorbance at a wavelength of 756 nm (19).

Observation of clinical symptoms

Observation of clinical symptoms of Wistar rats was carried out intensively starting from the 30's, 60's, 2 hours, 3 hours, 4 hours and at first 24 hours they were counted and recorded. The results of qualitative test observations, in the form of clinical symptoms that appeared with activity parameters; no tremors and convulsions, normal urination, no hypersalivation, no diarrhea and normal eyes for 14 days.

Acute toxicity test of PLEE

The test preparation for all groups was given orally in a single dose according to the dose for each group. Observations were carried out intensively at 30's, 60's, 2 hours, 3 hours, 4 hours and in the first 24 hours they were counted and recorded. This observation was continued for 14 days, including observing clinical symptoms and the number of animals that died (1). This examine used a pure experimental layout in vivo with a simple random sampling method (8).

Data analysis

The observational data obtained was in the form of secondary metabolite levels obtained from UV-Vis spectrophotometry results. The LD50 calculation method is used Thomson and Weil. This calculation is carried out using r-values, namely: The formula used are:

Log LD50 = Log D + d (f +1)

Information:

D = Smallest dose used

d = logarithm of multiples

F = factor obtained from the table

Data was obtained from the number of test animals that died natural 24 hours at each dose and concentration series analyzed using calculations Thomson and Weil. Determination of LD50 is carried out using an

acute toxicity test carried out in accordance with the Acute Toxicity Potential Criteria guidelines by BPOM RI in 2014 (9).

RESULTS

Simplicia Characterization

The results of the characterization of simplicia can be seen in table 1. This table shows that the results of the characterization of purple leaf simplicia meet the requirements in the Indonesian Medical Materials (IMM).

Table 1. Characterization Results of PLEE

No.	Parameter	Average	IMM
1	Water rate	7,67%	< 10%
2	Total ash rate	10,75%	< 12%

Source: primary data 2023

Identification of PLEE

This research used test material from purple leaf plants originating from Pasangkayu District, West Sulawesi in UPT. Then an identification test was carried out at the Sulawesi Biological Resources Center, Tadulako University, with the identification results proving that the purple plant used in this research was the *Graptophyllum pictum* (L.) Griff species, from the *Acanthaceae* family. The PLEE obtained from maceration extraction was 188 grams with a yield of 7.8%.

Phytochemical Screening Test

Phytochemical testing on *Graptophyllum pictum* (L.) Griff leaf extract was carried out to confirm the secondary metabolite compounds contained in the extract. The results of the phytochemical test can be seen in Table 1 below.

Table 2. Phitochemical Test Result for PLEE

No.	Bioactive Compound	Result
1	Flavonoids	+
2	Alkaloids	+
3	Saponins	+
4	Tannins	+

Note: (+): Contains tested compound; (-): Does not contain the compounds tested

Phytochemical screening tests show that the ethanol extract of purple leaves has secondary metabolites, namely, alkaloids, flavonoids, saponins and tannins. These secondary metabolite compounds show that purple leaves have pharmacological effects and have the ability to act as medicinal ingredients.

Total Secondary Metabolic Levels

Testing of total secondary metabolite levels in PLEE was carried out to show the number of active compounds in the extract. The results of the test for total secondary metabolite levels can be seen in Table 3 below.

Table 3. Total Secondary Metabolic Test Results of PLEE

No.	Parameter	Rate (mg/gram)
1	Total flavonoid equivalents of quercetin	6,332
2	Total Alkaloid equivalents of quinine	24.725,99
3	Total saponin equivalents sapogenin	89,191
4	Total Tannin equivalent tannic acid	0,884

Source: primary data 2023

Results of Observation of Clinical Symptoms

Observation of clinical symptoms of Wistar rats was carried out should be done for 30 minutes after administration and every 1 hour's interval for 24 hours. The recording of no mortality should be a confirmation of test result. The results of qualitative test observations, in the form of clinical symptoms that appeared with activity parameters; tremor, convulsions, salivation, urination, diarrhea, piloerection and normal eyes for 14 days, are summarized in table 4 below.

Table 4. Observation of Clinical Symptoms of Wistar Rats over a period of 14 days

Clinical Symptoms	PLEE			
	(Control)	(P1)	(P2)	(P3)
Tremor	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Convulsions	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Salivation	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Urination	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Diarrhea	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Piloerection	100(5/5)	100(5/5)	100(5/5)	100(5/5)
Normal eyes	100(5/5)	100(5/5)	100(5/5)	100(5/5)

Source: primary data 2023

Acute Toxicity Test Results (LD50)

The results of observations of the acute toxicity test for 14 (fourteen) days, in the form of the number of mouse deaths, are shown in table 5 below.

Table 5. Number of deaths during 14 days of administering a single dose of PLEE

Group	Observation Result (Day to)														Total
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
P1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**0**
P2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	**2**
P3	0	1	0	0	0	0	0	1	0	0	1	0	0	1	**4**

Source : primary data 2023

Log LD50 = Log D + d (f +1)
D = Smallest dose used (500 mg)
d = logarithm of multiples (log 4)
F = factor obtained from the table (r) =0,0,2,4 (f)= 1,00

Log LD50 = log 500 + log 4 (1+1)
Log LD50 = 2,69 + 0,9
Log LD50 = 3,59 (antilog)
Log LD50 = 3.890 mg/kg

DISCUSSION

This research used test material from purple leaf plants originating from Pasangkayu District, West Sulawesi, which has been identified as the true species *Graptophyllum pictum* (L.) Griff, from the *Acanthaceae* family at the Sulawesi Biological Resources UPT, Tadulako University. This is done with the aim of matching the morphological characteristics of the plants being studied and knowing the correctness of the plants used in order to avoid errors in taking test material. The extraction method used is maceration using 96% ethanol solvent. The thick of PLEE obtained was 188 grams with a yield of 7.8%.

The characterization results from checking the water content and total ash content in table 1 meet the requirements according to Indonesian Medical Materials (IMM). Qualitative analysis of secondary metabolite compounds (alkaloids, flavonoids, saponins and tannins) was carried out first on the thick extract of purple leaves in the form of a phytochemical screening test. This test aims to determine whether there are secondary metabolite compounds contained in the PLEE. Qualitative testing on purple leaf extract, obtained positive results containing flavonoids, alkaloids, tannins and saponins, as listed in table 2. This is in accordance with research by Fauzi 2016; Manoi 2010, which states that purple leaves contain alkaloids, flavonoids, saponins, tannins, steroids and glycosides (20).

Meanwhile, quantitative analysis aims to determine the total levels of secondary metabolite compounds contained in the PLEE using the UV-Vis Spectrophotometry method (21). The results of testing the total levels of secondary metabolites (quantitative) PLEE in table 3 show that PLEE contains more alkaloids (24,725.99 mg/g) than saponins (89.191 mg/g), flavonoids (6,332 mg/g) , and tannin (0.884 mg/g). Alkaloids are secondary metabolite compounds that are basic in nature, most of which are toxic and some can also be used as medicine (22).

The results of observations of clinical symptoms in Wistar rats did not show any clinical symptoms of illness. After administering a single dose of purple leaf extract, feed and drink consumption was stable for the rats and nothing remained. Wistar rats groom every day, which indicates that Wistar rats carry out normal activities for daily behavior. All mucosa such as the mouth, nose and eyes looked normal and there were no changes (observed within 24 hours until the 14th day for each dose), when observing signs of toxicity as in table 4, no changes were seen. The results of observing clinical symptoms showed that he did not experience tremors, convultion, salivation, urination, diarrhea, piloerection and normal eyes.

Acute toxicity is defined as the unwanted effect(s) that occurs either immediately or at a short time interval after a single or multiple administration of such substance within 24 hours (23). In this research, administration of purple leaves at a dose of 500 mg/kg to 5,000 mg/kg caused the death of test animals on days 2, 8, 11 and 14 as seen in table 5. Based on the mortality data obtained, the order of death in the acute toxicity test is 0,0,2,4, in the Weil table the r value of 0,0,2,4 has an f (factor) value of 0.10000. Then the analysis of mortality data was calculated using the Thomson and Weil method, the LD50 value was obtained at 3,890 mg/kg, the results of the data obtained were then analyzed based on the degree of toxicity. The degree of toxicity in PLEE at a dose of 3,890 mg/kg is included in the mild toxic category BPOM RI's acute toxicity potential at toxicity level 4 (500 – 5,000 mg/kg) (9). In accordance with previous research using different research subjects and low doses, namely 50, 150, 450 mg/kg carried out by Hilmarni in 2016, which stated that administration of PLEE was not toxic to the hematological profile of male white rats. So more research needs to be done more about usage PLEE on subchronic toxic test to look at long-term use.

CONCLUSION

The PLEE showed the highest total secondary metabolite content in alkaloids (24,725.99 mg/g), and the lowest in tannins (0.884 mg/g). The degree of toxicity in PLEE at a dose of 3,890 mg/kg is included in the BPOM RI mild toxic category at toxicity level 4 (500 – 5,000 mg/kg) and no significant clinical symptoms of acute toxicity occurred in all experimental animals.

AUTHOR'S CONTRIBUTION STATEMENT

The authors of this paper consist of six people, i.e N.P.D, M.,M., A.K.K. and I.D. This paper was finished way to the collaboration of the writing crew at each degree.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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