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ACUTE TOXICITY TEST OF SUNGKAI LEAF FRACTION (PERONEMA CANESCENS JACK.) ON BODY WEIGHT PERCENTAGE AND LIVER AND KIDNEY ORGANS RATIO

Elidahanum Husni, Alfathri Yunedi and Dwisari Dillasamola*

Faculty of Pharmacy, Universitas Andala Unand Padang Indonesia Indonesia.

***Corresponding Author: Dwisari Dillasamola**

Faculty of Pharmacy, Universitas Andala Unand Padang Indonesia Indonesia.

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ABSTRACT

Sungkai leaf (Peronema canescens jack.) has been widely used in traditional medicine because it has the potential to boost the immune system. To ensure safety in its use, it is necessary to carry out a toxicity test. The purpose of this study was to determine the range of LD50 values, safety status and the effect of the ethanol extract fraction of sungkai leaves on the percentage change in body weight, the ratio of liver and kidney weights and the behavior of white male mice. The test used 75 mice, which were divided into three groups per fraction. One fraction consists of 25 mice divided into 5 groups, consisting of 1 control and 4 treatment groups. The control group was given 0.5% Na-CMC suspension and the treatment group was given suspension of sungkai leaf fraction at doses of 1000, 2000, 4000, and 8000 mg/kgBB. The preparation was given once at the beginning of the research period. Observations were made on the symptoms of toxicity, the percentage change in body weight and the death of the test animal for 24 hours to 14 days. Oral administration of sungkai leaf fraction up to a dose of 8000 mg/kgBW did not indicate any death of the test animals, this indicated that the sungkai leaf fraction was in the non-toxic category (LD50>5000 mg/kgBW). Symptoms of toxicity that appear include weakness, salivation, tremors and walking with the stomach. The results of statistical tests using two-way ANOVA showed a significant difference between the type of fraction and the variation in dose on the percentage change in body weight in the water fraction with a dose of 4000 mg/kgBW ($P<0.05$). Then, for the results of the statistical test the percentage ratio of liver and kidney weights showed a significant difference in the ethyl acetate fraction ($P<0.05$), a significant difference could be seen in the ethyl acetate fraction with an average percentage ratio of kidney and liver organ weights obtained were 0.923% and 7.038%, respectively.

KEYWORDS: Sungkai (Peronema canescens Jack), Sungkai leaf fraction, Organ ratio, Body weight.

I. INTRODUCTION

The existence of herbal medicines from plants has been known for thousands of years, this is evident from the writings written on lontar leaves, on the walls of monuments and in old scriptures. Knowledge of herbal medicines as prescription drugs has been passed down as a legacy in the great communities of the world.[1] Many types of health related ailments can be cured by using herbal medicinal formulations. Therefore, knowledge of herbal recipes as medicine must be maintained and preserved as a national heritage.[1]

More than 248,000 species of higher plants have been identified and 12,000 plants are known to have medicinal properties. WHO data states that traditional medicine systems are still attached to the community, namely around 80% of the world's population. Long history shows that there are many traditional medicinal practices based on experience and then passed on from generation to generation, which have demonstrated the safety and efficacy of traditional medicines. However, scientific research is needed to prove the efficacy and safety of these traditional medicines.[2] WHO also recommends the use of traditional medicines including herbal medicines in the 2014–2023 strategy, with the aim of maintaining population health through providing access to effective and affordable alternative treatments.[3]

Indonesia is a country with very abundant natural resources, especially biological natural resources which have a total of around 40,000 types of medicinal plants, of which about 2.5% have been explored and used as traditional medicine.[4] There are no less than 30,000 plant species in our country, 9,600 of which are known to have medicinal properties, but not all of them are used optimally as medicine. Some of them have been equipped with safety and usefulness data. Meanwhile, there are also many studies to standardize ingredients, both pre-clinical trials to improve the status of jamu to become standardized herbal medicines, as well as

clinical trials as scientific evidence of the use of natural ingredients as phytopharmaca.[5]

At this time, the world's attention to the use of natural ingredients in medicine is increasing. Various countries have integrated natural materials in dealing with Covid-19. Traditional medicines from natural ingredients are believed to be able to restrain the rate of Covid-19 cases through their potential to increase the immune system because they are preventive and promotive through their secondary metabolite content.[5]

Sungkai as a traditional medicinal plant is a plant that is widely used by tribes in Sumatra and Kalimantan, with young leaves used as antiplasmodium, worm medicine, fever, cold medicine and can be used as a potion for women after giving birth and to increase body immunity.[1] Sungkai (Peronema canescens) is often referred to as jati sabrang, ki sabrang, skinny sungkai, or sekai. Sungkai leaves can be found in forests, gardens and yards, usually planted as a house border or as a living fence at the back of the house.[6,7] Sungkai leaves (Peronema canescens Jack) belong to the Verbenaceae family. Sungkai leaves are believed to have natural antioxidant activity and can also boost the immune system so that traditional medicine can be used in the health care system and according to the rules of formal health services.[4]

Some plants are reported not only contain toxic secondary metabolites, but are also polluted by air pollutants, especially heavy metals which can cause serious health problems. Thus, there is a need to assess the safety of sungkai leaf extract for human consumption before considering its therapeutic potential. One effective way that can be done is through in vivo acute oral toxicity tests.[8] Therefore, it is necessary to carry out a series of tests to ensure the safety of these natural ingredients. One of the traditional drug safety tests that is usually carried out is the toxicity test.[9]

Toxicity test is a test to detect the toxic effect of a substance on a biological system and to obtain typical dose-response data from the test preparation. The data obtained can be used to provide information regarding the degree of danger of the test preparation in the event of exposure to humans, so that the dosage can be determined for human safety.[10]

One type of toxicity test that is often used is the Oral Acute Toxicity Test. Oral acute toxicity test is a test to detect toxic effects that appear in a short time after administration of the test preparation given orally in a single dose, or repeated doses given within 24 hours.[10] Some acute toxicity tests (such as the “classical” LD50 test) are designed to determine the average lethal dose of the test substance.[11]

The LD50 (median lethal oral dose) is a single, statistically reduced dose of a substance that can cause death in 50 percent of animals when given by the oral route. The LD50 value is expressed in the weight of the test substance per unit weight of the test animal (mg/kg).[12] Parameters observed in this test were changes in the behavior of mice such as symptoms of toxicity (tremors, diarrhea, salivation, weakness, walking backwards, walking using the stomach), changes in body weight and the number of animals that died in each test group.[13]

Given the considerable potential of Sungkai leaves in boosting the immune system and assessing the LD50 in toxicity tests is one of the preclinical test requirements in the development of traditional medicines, researchers are encouraged to conduct research related to the LD50 of several graded fractions of the ethanol extract of Sungkai leaves (Peronema canescens Jack) against male white mice. The results of a previous study conducted by Melisa obtained an LD50 value > 5000 mg/kg, which means that it is in the non-toxic category.[14] These results are expected to provide information regarding the level of safety of the Sungkai leaf extract fraction and can be used as a basis for safety testing and continued with the isolation of pure compounds from the next Sungkai leaf.

II. RESEARCH METHODS

2.1 Tools and Materials

Rotary evaporator (Buchi R-210 Rotavapor), separating funnel, oven (Memmert), furnace, analytical balance (SF-400), desiccator, mortar and pestle, porcelain crucible, Moisture Analyzer , Hot plate, Uv-Visible lamp (Camag) , dark bottles, funnels, infusion bottles (500 ml and 100 ml), beaker glass (Pyrex), drip plate, rack and test tube, measuring cup (Pyrex), volumetric flask, spatula, stirring rod, dropping pipette, ointment pot, object glass, TLC chamber, capillary tube, syringe, sonde, animal cages, places to eat and drink animals. Sungkai leaf (I.), 70% ethanol, n-hexane, ethyl acetate, distilled water, 0.5% Na CMC, quercetin, phytochemical reagents, filter paper, dilute HCL, TLC mobile phase (methanol and ethyl acetate), whatman filter paper, aluminum foil, TLC Silica gel plate, standard mice food.

2.2 Test animals

The test animals used were male white mice aged ± 2-3 months with a body weight of 20-35 grams as many as 75 mice. Before the experiment, the mice were acclimatized to the animal house, Faculty of Pharmacy, Andalas University for 7 days, given enough food and drink.

2.3 Preparation of Simplicia and Extracts

4 kg of fresh sungkai leaves are sorted and washed thoroughly, then air-dried for 7 days or until the leaves can be crushed. After drying, do the sorting again to ensure the sample is free from impurities that are still left behind. The sorted dry samples were then chopped using a grinder and weighed to obtain 1 kg of simplicia powder. Furthermore, extraction using the maceration

method was carried out by soaking 1 kg of fine powdered simplicia added 10 parts of 70% ethanol solvent into a dark colored bottle as a maceration container. The mixture is stirred occasionally for the first 6 hours, allowed to stand for 18 hours, then filtered. Repeat the process twice with the same ratio of powder and solvent. All meserat was collected, then evaporated with a rotary evaporator to obtain a thick extract.[39]

2.4 Faction creation

Weigh the ethanol extract of Sungkai leaves as much as 150 g then dissolve it in 500 ml of warm distilled water, shake until homogeneous. The ratio between the volume of water and organic solvent that is put into the separatory funnel is 1:1, so that it is followed by the addition of 500 ml of n-hexane. Shake slowly, be sure to open the lid occasionally to prevent bumping. Then let stand for 30 minutes until two layers are formed, the top n-hexane layer and the bottom layer of water. Then separate the two layers and repeat this process three times.[40]

Then put the distilled water back into the separatory funnel, add 500 ml of ethyl acetate. With the same steps as n-hexane, two layers are formed, the ethyl fraction on top and the water fraction on the bottom. Then let stand for 30 minutes until two layers are formed, the top layer of ethyl acetate and the bottom layer of water. Then separate the two layers and repeat this process three times. The formed n- hexane, ethyl and water fractions were concentrated using a rotary evaporator.[40]

2.5 Determination of yield

Determination of yield is done by weighing the cleaned sungkai leaves (A), the viscous extract obtained is weighed again (B). The yield must reach the number at least as specified in each extract monograph. Determination of yield can be calculated using the following equation:[40]

$$\text{Rendemen} = \frac{B}{A} \text{ x } 100\%$$

Information :
A = Extract weight (grams)
B = Weight of the fraction obtained (grams)

2.6 Standardization of fractions

Consists of specific parameter tests (Organoleptic, Phytochemical, TLC) and non-specific tests (Drying Shrinkage and Ash Content)

2.7 Dosage planning and grouping of test animals

There were five dose variants of the ethanol extract of Sungkai leaves which were given to the test animals, namely 1000 mg/kg, 2,000 mg/kg, 4,000 mg/kg, 8,000 mg/kg. The test animals were grouped into 5 groups based on the variation in dose and control with the division as follows

a. Group I: only given 0.5% Na CMC
b. Group II: given extract dose of 1,000 mg/kgBB
c. Group III: given extract dose of 2,000 mg/kgBB
d. Group IV: given extract dose of 4,000 mg/kgBB
e. Group V: given extract dose of 8,000 mg/kgBB

The number of animals to be tested for each fraction was 25 male white mice, each group consisting of 5 mices.

2.8 Preparation of test preparations

The weighed fraction was suspended in 0.5% Na CMC. Weigh 50 mg of CMC Na into a mortar containing 1 mL of hot water. Grind until homogeneous then add distilled water to a volume of 10 mL. Suspend the extract with CMC Na solution according to the concentration to be made.[43]

2.9 Treatment of test animals

Healthy male white mice that had previously been acclimatized were divided randomly into 5 groups, each group consisting of 5 mice. The test preparation was given once on the first day based on the body weight of the mice. The test animals were observed for symptoms of toxicity that occurred until death within 24 hours. Observations were made intensively on the first day every 30 minutes for 6-8 hours, then for 4 hours periodically for 14 days. The body weight of the mice was monitored from before administration of the preparations until the animals were sacrificed. After 14 days, the animals were sacrificed by means of anesthesia.[44]

III. RESULTS AND DISCUSSION

This study used experimental research methods with in vivo techniques which were carried out to see the safety of the Sungkai leaf fraction in its use. The samples used were sungkai leaves (Peronema canescens jack.) taken from Jalan Bukit Ngalau, Indarung, Lubuk Kilangan District, Padang City, West Sumatra.

Plant identification was carried out at the Anda Herbarium, Department of Biology, Faculty of Mathematics and Natural Sciences, Unand. The purpose of identifying this plant is to find out the identity of the sample to be used. Based on the identification results, the samples used in this study were sungkai leaves (Peronema canescens jack.) from the Verbenaceae family.

The process of making Sungkai leaf extract begins with collecting 4 kg of fresh Sungkai leaf herb and then sorting it wet to separate it from impurities. The sorted plant parts are then washed with running water and air-dried. This drying process aims to get simplicia that is durable and can be stored for a longer time. The sample is said to have dried if the sample can be crushed. The dry sample was then chopped using a grinder and 1 kg of powdered simplicia was obtained. Slicing aims to expand the surface of the simplicia so that the solvent can penetrate easily so that the withdrawal of the active substance becomes more complete.

The extraction method used is maceration. Maceration was chosen because the process is simple, does not require special treatment, can use a large number of samples and does not go through a heating process. The solvent used is 70% ethanol.[50] The use of ethanol as a solvent is because it is a universal solvent so it can dissolve polar, semi-polar and non-polar compounds. The concentration of 70% is used because the sample used is a dry sample where the water content is small. The water content is needed to break down the plant cell walls so that the juice from the plant can come out.

The ethanol extract fraction was prepared by weighing 150 g of Sungkai leaf ethanol extract and then dissolved in 500 ml of warm distilled water, shaken until homogeneous. The ratio between the volume of water and organic solvent that is put into the separatory funnel is 1:1, so that it is followed by the addition of 500 ml of n-hexane. Shake slowly, be sure to open the lid occasionally to prevent bumping. Then let stand for 30 minutes until two layers are formed, the top n-hexane layer and the bottom layer of water. Then separate the two layers and repeat this process three times.[40]

Then put the distilled water back into the separatory funnel, add 500 ml of ethyl acetate. With the same steps as n-hexane, two layers are formed, the ethyl fraction on top and the water fraction on the bottom. Then let stand for 30 minutes until two layers are formed, the top layer of ethyl acetate and the bottom layer of water. Then separate the two layers and repeat this process three times. The formed n-hexane, ethyl and water fractions were concentrated using a rotary evaporator.[40]

3.1 Determination of yield

Sungkai leaf maceration process was carried out for 24 hours with 3 repetitions using the same solvent. Simplisia powder as much as 700 g is soaked for 3 days while occasionally stirring every day then allowed to stand. Filter using filter paper and the meserate obtained is collected and then evaporated with a rotary evaporator to obtain a thick extract. Rotary evaporator works by lowering the pressure on the surface so as to speed up the evaporation of the solvent. Condensed extract of Sungkai leaves was obtained as much as 120.24 g and the yield of the extract was obtained as much as 17.17%.

$$\%\text{Extract Yield} = \frac{\text{Extract weight obtained}}{\text{Simplisia Weight}} \text{ x } 100\%$$

$$= \frac{120{,}12 \text{ g}}{700 \text{ g}} \text{ x } 100\%$$

$$= 17{,}17\%$$

The n-hexane fraction obtained from 120.24 g of ethanol extract of Sungkai leaves after fractionation was 4.12 g.

$$\%\text{Yield of n} - \text{hexane fraction} = \frac{\text{Weight of Fraction obtained}}{\text{Weight of condensed extract}} \text{ x } 100\%$$

$$= \frac{4{,}12\ g}{120{,}12\ g}\ x\ 100\%$$

$$= 3{,}43\%$$

The remaining fraction obtained from the fractionation with n-hexane was followed by fractionation using ethyl acetate, then evaporated with a rotary evaporator to obtain a viscous fraction of 4.56 g of ethyl acetate.

$$\%\textit{Yield of ethyl acetate fraction} = \frac{\textit{Weight of Fraction obtained}}{\textit{Weight of condensed extract}} x\ 100\%$$

$$= \frac{4{,}56\ g}{120{,}12\ g}\ x\ 100\%$$

$$= 3{,}79\%$$

Furthermore, the remaining fraction or water fraction obtained after the fractionation using ethyl acetate was evaporated using a rotary evaporator, the weight of the viscous water fraction was obtained as much as 11.23 g, then the percent yield was calculated.

$$\%\textit{yield of water fraction} = \frac{\textit{Weight of Fraction obtained}}{\textit{Weight of condensed extract}} x\ 100\%$$

$$= \frac{11{,}23}{120{,}12} \text{x } 100\%$$

$$= 9{,}27\%$$

Based on the yield calculation results above, it can be seen that the highest yield value is the water fraction of Sungkai leaves with a weight fraction of 11.21 g with a percent yield of 9.27%, more than the n-hexane and ethyl acetate fractions. This indicates that Sungkai leaves contain more polar compounds.

The factors that affect the size of the yield value of the Sungkai leaf fraction are the ratio of the number of samples to the amount of solvent used, the type of solvent used, the amount of simplicia and the length of time needed in the extraction process.[45]

Determination of yield aims to determine the amount of bioactive compounds contained in the extracted sungkai leaf simplicia.[51] The extracts obtained were then standardized through non-specific and specific parameter testing. Standardization aims to obtain extracts that safe and guaranteed quality.

Non-specific parameters carried out in this study included drying shrinkage, total ash content and acid insoluble ash content. While the specific parameters carried out included organoleptic, phytochemical tests and TLC.

3.2 Non-Specific parameters

3.2.1 Drying shrink check

Drying shrinkage is one of the non-specific parameter extract characterizations. The drying shrinkage can be said to be a constant in determining the maximum limit for the amount of compound lost during the annealing process. In other words, the compound in question is not only water but chemical compounds other. Sungkai viscous extract as much as 2 g which has been placed in a crucible and then dried in an oven with a temperature of 105°C. Drying of the extract is carried out for approximately 30 minutes or until a constant weight is obtained.[46] Extract drying shrinkage was replicated three times to see the average.

The drying shrinkage value of the ethanol extract of Sungkai leaves obtained from an average of three repetitions was 10.89%. This value indicates that the ethanol extract of Sungkai leaves meets the requirements because it is below the maximum shrinkage limit for good drying, which is below 11%.

Table 1: Results of determination of drying shrinkage of sungkai leaf ethanol extract.

No.	Blank Exchange Rate (g)	Exchange rate + sample before heating (g)	Exchange rate + sample after heating (g)	Drying Shrinkage
1.	34.02	36.02	35,84	9.25 %
2.	21.76	23.77	23.54	11.45%
3.	31.54	33,56	33,32	11.98%
Mean ± SD				10.89%

3.2.2 Determination of total ash content

Determination of total ash content is also one of the non-specific parameter extract characterizations. Determination of total ash content aims to provide an overview of the internal and external mineral content from the initial process to the formation of the extract. The material is ignited at a temperature at which the compound organic matter and its derivatives can be destroyed and evaporate, so that only the mineral and inorganic elements are left.[21]

Table 2: Results of determination of dry simplisia ash content of sungkai leaves.

No.	Blank Exchange Rate (g)	Exchange rate + sample pre-ignition (g)	Exchange rate + sample after heating (g)	Ash Content
1.	31,13	33,13	31,24	5.64%
2.	33,23	35,24	33,36	6.63%
3.	29,51	31.52	29,61	5.32%
Average				5.86%

Table 3: Results of determination of ash content of sungkai leaf ethanol extract.

No.	Blank Exchange Rate (g)	Exchange rate + sample pre-ignition (g)	Exchange rate + sample after heating (g)	Ash Content (%)
1.	31,12	33,13	31,26	6.74%
2.	34.01	36.03	34,15	6.67%
3.	33,23	35,24	33,36	6.63%
Average				6.68%

3.3 Specific parameters

3.3.1 Organopleptic examination

Organoleptic examination is a specific parameter of the extract characterization test carried out on the viscous extract of Sungkai leaves obtained. Organoleptic testing aims as an initial introduction that is as simple and objective as possible because this test only uses the five senses for its examination.[21]

Table 4: Organoleptic Examination of the N-hexane Fraction.

No	Inspection	Results
1	Color	Black Green
2	Smell	Typical
3	Flavor	Bitter
4	Form	Thick

Table 5: Organoleptic examination of the ethyl acetate fraction.

No	Inspection	Results
1	Color	Black Green
2	Smell	Aromatic
3	Flavor	Bitter
4	Form	Thick

Table 6: Organoleptic examination of the water fraction.

No	Inspection	Results
1	Color	Blackish orange
2	Smell	typical
3	Flavor	Bitter
4	Form	Thick

3.3.2 Phytochemical screening ia

Phytochemical screening aims to determine the class of secondary metabolite compounds contained in the Sungkai leaf extract fraction by reacting the test extract with its specific reagent.[47] In this study several classes of secondary metabolites were tested, including alkaloids, phenolics, saponins, flavonoids and terpenoid steroids. Based on the tests that have been carried out using several specific reagents described in the table, the positive results of Sungkai leaf extract contain phenolic compounds, flavonoids, saponins and terpenoids.

Table 7: Phytochemical Test Results of the N-hexane Fraction of Sungkai Leaves.

No	Inspection	Reactor	Observation result	Results
1	Alkaloids	Mayer	No yellow or white precipitate formed	-
		Dragendorf	No orange precipitate formed	-
2	Flavonoids	Mg powder and concentrated HCl	Formed yellowish green color	+
3	Phenolic	FeCl3	Blackish green color formed	+
4	Saponins	Hot water and 2N HCl	Foam formed and did not disappear when 2 N HCL was dripped	+
5	Terpenoids & Steroids	Chloroform, anhydrous acetic acid and concentrated H2SO4	No blue green color formed	_

Table 8: Phytochemical test results of the ethyl acetate fraction of sungkai leaves.

No	Inspection	Reactor	Observation result	Results
1	Alkaloids	Mayer _	A yellow or white precipitate forms	+
		Dragendorf	An orange precipitate formed	+
2	Flavonoids	Mg powder and concentrated HCl	Formed purple red color	+
3	Phenolic	FeCl3	Blackish green color formed	+
4	Saponins	Hot water and 2N HCl	Foam formed and did not disappear when 2 N HCL was dripped	+
5	Terpenoids & Steroids	Chloroform, anhydrous acetic acid and concentrated H2SO4	No blue green color formed	-

Table 9: Phytochemical test results of the water fraction of sungkai leaves.

No	Inspection	Reactor	Observation result	Results
1	Alkaloids	Mayer	A yellow or white precipitate forms	+
		Dragendorf	An orange precipitate formed	+
2	Flavonoids	Mg powder and concentrated HCl	Formed purple red color	+
3	Phenolic	FeCl3	Blackish green color formed	+
4	Saponins	Hot water and 2N HCl	Foam formed and did not disappear when 2 N HCL was dripped	+
5	Terpenoids & Steroids	Chloroform, anhydrous acetic acid and concentrated H2SO4	No blue green color formed	-

Information:
(+) = Contains secondary metabolite compounds
(-) = Does not contain secondary metabolites

3.3.3 Examination of the KLT Profile

Thin layer chromatography is a technique for separating compounds based on their polarity. The TLC profile test aims to see the presence of chemical compounds contained in a sample by comparing the stain pattern and Rf value of the test sample with a comparison containing pure compound isolate.[48] On the TLC plate, the ethanol extract of Sungkai leaves and the comparator compound were dotted. The comparator compound used is quercetin which is a compound from the flavonoid group. As in the results of the phytochemical screening table, the ethanol extract of Sungkai leaf fractions positively contained flavonoids, therefore quercetin was used as a comparison. In addition, the reason for using quercetin as a comparison is because it belongs to the class of flavonoid glycosides and is the most widely distributed flavonoid compound in Sungkai leaf plants.

a. n-hexane fraction

In the TLC examination of the N-hexane fraction, ethyl acetate: n-hexane: methanol (2:4:4) was used as the eluent.

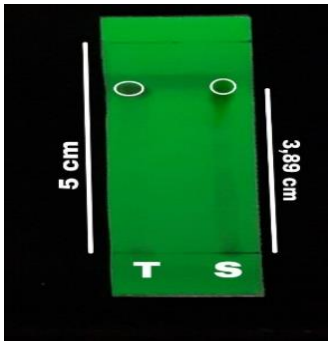

Figure 1: TLC results of the n-hexane fraction of sungkai leaves.

Information:
T = comparator (quercetin)
S = sample (n-hexane fraction of Sungkai leaves)

On the TLC plate above there are two spots, namely sample and comparison spots. Stains on the plate were observed under a UV lamp with a wavelength of 254 nm. From the results obtained, the distance traveled between the sample and comparison spots is the same, namely 3.89 cm. While the distance traveled by the eluent was 5 cm, the Rf n-hexane fraction of Sungkai leaves was obtained at 0.78 cm.

b. Ethyl acetate fraction

In the TLC examination of the Ethyl Acetate Fraction, ethyl acetate eluent was used: n-hexane (6:4)

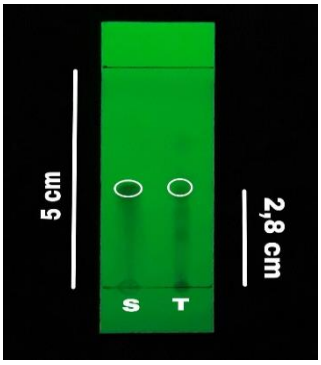

Figure 2: TLC results of the ethyl acetate fraction of sungkai leaves.

Information:
T = comparator (Quercetin)
S = sample (Ethyl acetate fraction of Sungkai leaves)

On the TLC plate above there are two spots, namely sample and comparison spots. Stains on the plate were observed under a UV lamp with a wavelength of 254 nm. From the results obtained, the distance traveled between the sample and comparison stains is the same, namely 2.8 cm. While the distance traveled by the eluent is 5 cm, the Rf n-hexane fraction of Sungkai leaves is 0.56 cm.

c. Water fraction

In the TLC examination of the Ethyl Acetate Fraction, methanol was used as eluent : n-hexane (8:2)

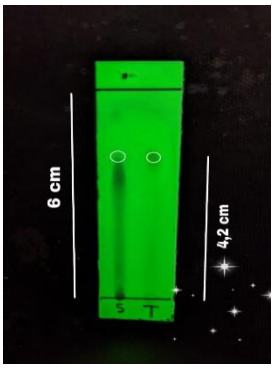

Figure 3: TLC results of the water fraction of sungkai leaves.

Information:
T = comparator (quercetin)
S = sample (water fraction of Sungkai leaves)

On the TLC plate above there are two spots, namely sample and comparison spots. Stains on the plate were observed under a UV lamp with a wavelength of 254 nm. From the results obtained, the distance traveled between the sample and comparison spots is the same, namely 4.2 cm. While the distance traveled by the eluent is 6 cm, the Rf n-hexane fraction of Sungkai leaves is 0.70 cm.

3.4 Acute toxicity test

Oral acute toxicity test is a test conducted to obtain information regarding toxic effects that appear in a short time after administration of the test preparation within 24 hours orally in single or repeated doses.[32] Acute toxicity tests are designed to determine the lethal dose (LD50) of a substance and provide guidance as to which dose should be used in longer trials.[49] The LD50 is a dose that is calculated statistically and is expected to cause death in 50% or half of the population receiving it. In general, the smaller the LD50 valuethe more toxic the compound is and the greater the LD50 value, the lower the toxicity.[49] Herbal medicines to be tested clinically require toxicity test data and at least LD50 data.[10] Observation of test animals was carried out during the first 24 hours with 4 intensive hours. If no death occurs, the observation is continued for 14 days. Parameters observed included symptoms of toxicity, percentage change in body weight, death of the test animals and the ratio of liver and kidney weight as the final parameters.

In this study, the animals used were swiss webster strain male white mice as many as 75 mice aged 2-3 months and weighing 20-35 g. White mice were chosen as experimental animals because they are physiologically similar to humans, easy to handle, maintain, easy to obtain and economical. White mice with male sex were chosen because they have more stable hormones than female mice. To reduce the results of deviations from the results of the study, mice of the same sex, age and body weight were relatively the same.[50] Before being given treatment, the mice were acclimatized for 7 days so that the mice were used to the new experimental environmental conditions and were not stressed. Mice selected for testing were healthy mice and did not show

significant changes in body weight, i.e. more than 10% during the acclimatization process.[42]

Mice were grouped into 5 groups for each fraction, each group consisting of 5 mice. Before being given the test preparation, the mice were fasted for 3-4 hours but still given drinking water.[30] Mice in group 1 were only given 0.5% Na CMC (control group) and mice in groups 2-5 were given test preparations suspended with 0.5% Na CMC. The use of CMC Na as a suspending agent is because it is easy to obtain, is inert, non-toxic, non-irritating and produces a stable suspension.[51] Sungkai leaf fraction is difficult to dissolve in water so it requires suspension. After being given the test preparation, feed may be given again after 1-2 hours of administration of the preparation.[30] The dose given is designed to cause the death of the test animals from 0% to 100%. Multiple doses were made according to the Thompson Weil method. Therefore, 4 levels of the test dosage were selected, namely 1000 mg/kg, 2,000 mg/kg, 4,000 mg/kg, and 8,000 mg/kg.

The test preparation was given once on day 0 and observed for the first 24 hours with 4 hours of intensive. If no death occurs, then the observation is continued to see the toxicity delayed for 14 days. Observations were made of the toxic symptoms that arose, body weight was measured every day and on the last day the animals were sacrificed and liver and kidney organs were taken to measure the ratio of each animal's organs.

3.4.1 Calculation of LD50 and determination of security status

This study tested several dose levels of the ethanol extract fraction of Sungkai leaves to test animals according to the dose limits that had been designed. Acute toxicity testing in this study used doses of 1000, 2000, 4000 and 8000 mg/kg BW, where up to a dose of 8000 mg/kg BW no animals died. toxic because it has an LD50 > 8000 mg/kgBB. In this acute toxicity evaluation, several other parameters were observed and examined: toxic symptoms, percentage change in body weight and organ weight ratio.

3.4.2 Symptoms of toxicity

The results of observations made for 14 days of toxic symptoms in the form of tremors, diarrhea, salivation, weakness, walking backwards, walking with the stomach, shortness of breath and pyrolection.[12]

a. n-hexane fraction

Table 10: Observation results of toxicity symptoms of the n-hexane fraction.

Toxicity Symptoms	Dose				
	K(-) NaCMC 0.5%	1000 mg/kgBB	2000 mg/kgBB	4000 mg/kgBB	8000 mg/kgBB
Breathing rate	-	-	-	+	++
Weak	-	-	-	+	++
stretched	-	-	-	-	-
Piloerection	-	-	-	-	-
Walk on the belly	-	-	-	+	+
Grooming	-	-	-	-	-
Tremors	-	-	-	-	-
Salivation	-	-	-	+	+
Urination	-	-	-	+	+
Death	-	-	-	-	-

Information:
(-) = Asymptomatic
(+) = Mild symptoms
(++) = Moderate symptoms
(+++) = Severe symptoms

In the above presented toxic symptoms from the administration of various doses of oral preparations of the n-hexane fraction of ethanol extract of Sungkai leaves. Based on the table it is known that after administration of the n-hexane fraction of the ethanol extract of Sungkai leaves, no toxic symptoms were found in the control group, treated at doses of 1000 and 2000 mg/kgbb. Mice were active and showed normal behavior after administration of the test preparation. Toxic symptoms began to be found at doses of 4,000 mg/kg and 8,000 mg/kg. At a dose of 4000 mg/kg BW, the mice moved slowly and there was a decrease in motor activity, weakness, little salivation and shortness of breath. However, these symptoms only lasted for the first 1 hour after administration of the test preparation, after which the mice returned to their active movements. There was a decrease in motor activity in mice marked by the movement of mice that slowed down even though they were touched. These symptoms of toxicity only lasted about the first hour after administration of the test preparation, after which the test animals returned to normal.

b. Ethyl acetate fraction

Table 11: Results of observation of toxicity symptoms of the ethyl acetate fraction.

Toxicity Symptoms	Dose				
	K(-) NaCMC 0.5%	1000 mg/kgBB	2000 mg/kgBB	4000 mg/kgBB	8000 mg/kgBB
Breathing rate	-	-	-	+	++
Weak	-	-	-	+	++
stretched	-	-	-	-	-
Piloerection	-	-	-	-	-
Walk on the belly	-	-	-	+	++
Grooming	-	-	-	-	-
Tremors	-	-	-	-	-
Salivation	-	-	-	+	+
Urination	-	-	-	+	+
Death	-	-	-	-	-

Information:
(-) = Asymptomatic
(+) = Mild symptoms
(++) = Moderate symptoms
(+++) = Severe symptoms

In the above presented toxic symptoms from the administration of various doses of oral preparations of the ethyl acetate fraction of the ethanol extract of Sungkai leaves. Based on the table it is known that after administration of the n-hexane fraction of the ethanol extract of Sungkai leaves, no toxic symptoms were found in the control group, treated at doses of 1000 and 2000 mg/kgBW. Mice were active and showed normal behavior after administration of the test preparation. Toxic symptoms began to be found at doses of 4,000 mg/kg and 8,000 mg/kg. At a dose of 4000 mg/kg BW, the mice moved slowly, there was a decrease in motor activity, weakness and a little shortness of breath. However, these symptoms only lasted for the first 1 hour after administration of the test preparation, after which the mice returned to active activity. Toxic symptoms increased at a dose of 8,000 mg/kg, namely weakness, rapid breathing, salivation, slight tremors and a slightly crouched walk. There was a decrease in motor activity in mice marked by the movement of mice that slowed down even though they were touched. These symptoms of toxicity only lasted about the first hour after administration of the test preparation, after which the test animals returned to normal.

c. Water fraction

Table 12: Observation results of water fraction toxicity symptoms.

Toxicity Symptoms	Dose				
	K(-) NaCMC 0.5%	1000 mg/kgBB	2000 mg/kgBB	4000 mg/kgBB	8000 mg/kgBB
Breathing rate	-	-	-	+	++
Weak	-	-	-	+	++
stretched	-	-	-	-	-
Piloerection	-	-	-	-	-
Walk on the belly	-	-	-	+	++
Grooming	-	-	-	-	-
Tremors	-	-	-	-	-
Salivation	-	-	-	+	+
Urination	-	-	-	+	+
Death	-	-	-	-	-

Information:
(-) = Asymptomatic
(+) = Mild symptoms
(++) = Moderate symptoms
(+++) = Severe symptoms

In the above presented toxic symptoms from the administration of various doses of oral preparations of the water fraction of the ethanol extract of Sungkai leaves. Based on the table it is known that after administration of the n-hexane fraction of the ethanol extract of Sungkai leaves, no toxic symptoms were found in the control group, treated at doses of 1000 and 2000

mg/kgBW. Mice were active and showed normal behavior after administration of the test preparation. Toxic symptoms began to be found at doses of 4,000 mg/kg and 8,000 mg/kg. At a dose of 4000 mg/kg BW, the mice moved slowly, there was a decrease in motor activity, a little weakness and shortness of breath. However, these symptoms only lasted for the first 1 hour after administration of the test preparation, after which the mice returned to active activity. Toxic symptoms increased at a dose of 8,000 mg/kg, namely weakness, rapid breathing, slight salivation, slight pyrolection and stooping. There was a decrease in motor activity in mice marked by the movement of mice that slowed down even though they were touched. These symptoms of toxicity only lasted about the first 1-2 hours after administration of the test preparation, after which the test animals returned to normal.

Table 13: Criteria for classifying test preparations according to OECD 420.

Dosage (mg/kgBB)	Death	Category
5	≥ 2 out of 5 dead 1	1
5	≥1 tail shows symptoms of toxicity and no death	2
50	≥ 2 out of 5 dead	
50	≥1 with symptoms of toxicity and no death	3
300	≥ 2 out of 5 dead	
300	≥1 head with symptoms of toxicity and or <1 off	4
2000	≥ 2 out of 5 dead	
2000	≥1 head with symptoms of toxicity and or no death	5
	There are no symptoms of toxicity	5/ unclassified

Evaluation of acute toxicity is not only about the LD50, but also on behavioral abnormalities, stimulation, motor activity to get an idea of the cause of death of the test animals. The data collected in the acute toxicity test is in the form of quantitative toxicity benchmarks, namely the range of lethal/toxic doses and benchmarks.[35]

Based on the above criteria for the classification of test animals according to the OECD, namely in category 5/ unclassified, which means that the oral preparations of the n-hexane fraction, ethyl acetate fraction and aqueous fraction of the ethanol extract of Sungkai leaves have relatively low acute toxicity.[12]

3.4.3 Percentage change in body weight

Body weight was weighed before being given the test preparation and after being given the test preparation for 14 days. Weighing was done every day by giving the same amount of feed to each group. Changes in body weight were obtained from the average difference in body weight in one group on days 1,2,3 and so on until day 14 minus the body weight before being given the test preparation (day 0). Data on the percentage of body weight change for 14 days were analyzed using the SPSS (Statistical Product and Service Solution) program with a one-way ANOVA test between dose and % body weight change and followed by Duncan's test.[52]

Table 14: Effect of fraction types and dose variations on body weight changes in male white mice.

Treatment Group	Change in BW (%) ± SE			Mean (%) ± SE
	n-hexane fraction	Ethyl acetate fraction	water fraction	
Control (-)	7.7135 ± 1.980	5.3754 ± 1.980	9.5844 ± 1.980	7.558 ± 1.143
1000 mg/kgBB	10.2873 ± 1.980	10.6476 ± 1.980	22.6125 ± 2.054	14.516 ± 1.157
2000 mg/kgBB	9.0399 ± 1.980	15.0194 ± 1.980	17.1688 ± 2.138	13,743 ± 1,174
4000 mg/kgBB	10.2178 ± 1.980	15.4813 ± 1.980	28.0733 ± 2.233	17,924 ± 1,194
8000 mg/kgBB	9.9551 ± 1.980	13.5214 ± 1.980	23.5162 ± 2.469	15.664 ± 1.224
Mean (%) ± SE	9.443 ± 0.885	12.009 ± 0.885	20.191 ± 0.976	

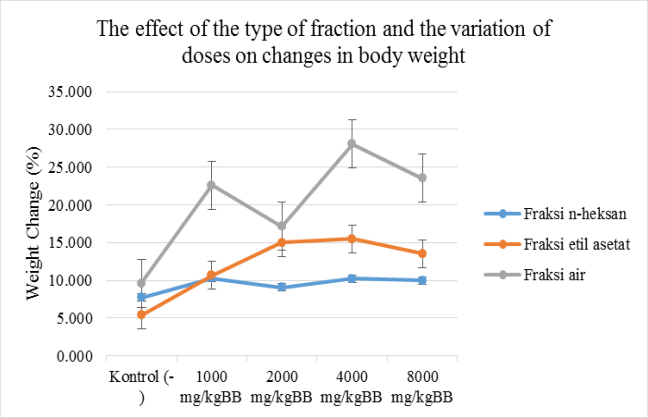

Figure 4: Graph of the effect of the types of Fractions and Variations in the doses of Sungkai leaves on changes in body weight of male white mice.

The results of the two-way ANOVA analysis showed that there was a significant effect of dose variation ($p<0.05$) on the percentage change in body weight of male white mice, then there was also a significant interaction between dose variation and type of fraction ($p<0.05$) percentage change in body weight of male white mice . The average value of the effect of the type of fraction and the variation in the dose of Sungkai leaves on the percentage change in body weight of male white mice can be seen in the table above.

The average percentage change in body weight of mice given the Sungkai leaf fraction was higher than the negative control group. Based on Duncan's follow-up test, it was seen that there was a significant difference in response to changes in body weight given the n-hexane, ethyl acetate and water fractions. The water fraction showed a significant increase in body weight compared to the other two fractions. Then it was also seen that there was a significant difference in response to changes in body weight given the various doses, the treatment group had a higher percentage of increase in body weight than the negative control group. The highest increase in body weight was seen at a dose of 4000 mg/kg BW.

Body weight is determined by the balance between caloric intake and energy release. In some way the body weight is maintained at a certain point. Appetite is regulated by the hypothalamus through an interaction between the "food center" and the "satiation center.[54]

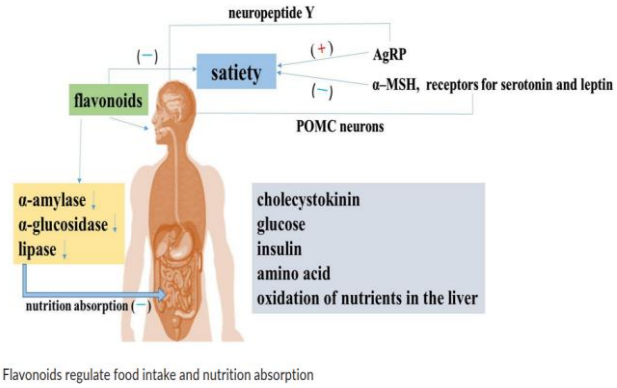

FIGURE 1 Flavonoids regulate food intake and nutrition absorption

Figure 5: Mechanism of flavonoids in regulating food Intake and Absorption of nutrients.

All phenolic acids and flavonoids tested had inhibitory effects on digestive enzymes including α-amylase, α-glucosidase, and lipase to varying degrees except salicylic acid which showed no inhibition on glucosidases. Flavanols and flavones have a higher inhibitory capacity for α-amylase and α-glucosidase.[55]

α-amylase secreted by saliva and the pancreas, plays an important role in the breakdown of complex carbohydrates into simple molecules that can be absorbed in the digestive tract. The inhibitory effect of flavonoids on α-amylase is related to the number of hydroxyl groups in ring B of the flavonoid skeleton, because the interaction occurs by the formation of hydrogen bonds between the hydroxyl groups on the flavonoid and the catalytic residue from where the α-amylase bond occurs.[55]

Reducing α-glucosidase activity can prevent the body from absorbing excess glucose, which can control blood

glucose levels, thereby reducing the absorption of carbohydrates and fats which causes blockage of digestive enzymes involved in carbohydrate and lipid metabolism can directly reduce digestion and absorption. This has been shown to be an additional means of preventing obesity and weight loss.[55]

Flavonoids contained in the sungkai leaf fraction are thought to have a function to modulate eating behavior. In vivo tests on mice showed an increase in the expression of the receptor gamma-aminobutyric acid B1 (GABAB1R) and a decrease in the expression of neuropeptide Y (NPY) in the hypothalamus, thereby modulating food intake/weight control behavior. Upregulation of GABABR1 is followed by decreases in active protein kinase A (PKA) and phosphorylated cAMP-response element binding protein (CREB), both located downstream of GABAR1. Similarly, administration of flavonoids to mice can decrease food intake.[56]

It is known that at a dose of 4000 mg/kgBB the ethyl acetate fraction and the water fraction of Sungkai leaves showed a significant increase in body weight. leptin, the secreted NPY will bind to its receptors on proopiomelanocortin (POMC) neurons (Y1 receptors) and cause inhibition of the activity of these POMC neurons. Activation of neurons expressing NPY and agouti related peptide (AgRP) during negative energy balance can stimulate eating in two ways, namely by releasing the appetite stimulant NPY and by reducing the action of the appetite suppressant melanocortin/POMC.[57]

3.4.4 Liver ratio

Table 15: The effect of the type of fraction and the dose variation on the liver organ ratio of male white mice.

Treatment Group	Liver Ratio (%) ± SE			Mean (%) ± SE
	n-hexane fraction	Ethyl acetate fraction	water fraction	
Control (-)	5.6000 ± 0.221	5.8480 ± 0.221	5.7042 ± 0.221	5.717 ± 0.128
1000 mg/kgBB	4.5800 ± 0.221	7.8540 ± 0.221	6.0052 ± 0.221	6.146 ± 0.128
2000 mg/kgBB	5.1220 ± 0.221	7.2900 ± 0.221	5.2048 ± 0.247	5.872 ± 0.133
4000 mg/kgBB	5.2500 ± 0.221	7.0800 ± 0.221	5.6042 ± 0.221	5.978 ± 0.128
8000 mg/kgBB	5.0840 ± 0.221	7.1200 ± 0.221	5.6347 ± 0.286	5.946 ± 0.141
Mean (%) ± SE	5.127 ± 0.99	7.038 ± 0.99	5.631 ± 0.108	

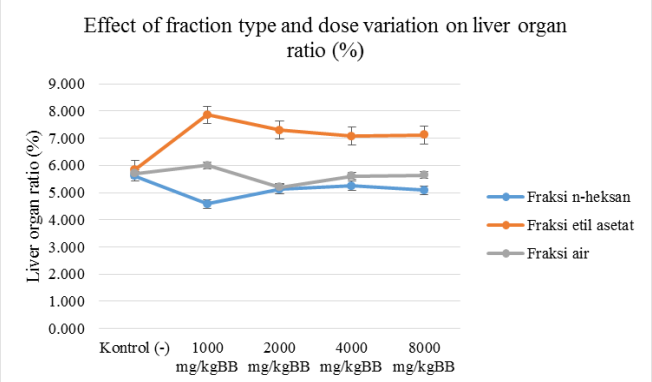

Figure 6: Graph of the effect of the types of fractions and variations in the doses of Sungkai leaves on the liver organ ratio of male white mice.

The results of the two-way ANOVA analysis showed that there was no significant effect of dose variation ($p>0.05$) on the percentage of liver organ ratio of male white mice, but there was a significant interaction between dose variation and type of fraction ($p<0.05$) on percentage of liver organ ratio of male white mice.

Based on the results of Duncan's test, the average percentage increase in liver organ ratio of male white mice in the ethyl acetate fraction was higher than the other two fractions. However, in this case, there was no significant difference in the response between the dose variations of each fraction and the percentage ratio of the liver organs of male white mice. The average value of the effect of the type of fraction and the variation in the dose of Sungkai leaves on the percentage of the liver organ ratio of male white mice can be seen in Table 18 above.

The liver is an organ that has the ability to repair enormous cell damage. The liver has cytochrome p-450 enzymes that can metabolize foreign substances in the body, by making some toxicants less toxic and more water soluble. Because we use the oral route, the test compound will undergo an enterohepatic cycle, after absorption occurs in the gastrointestinal tract, the compound will be carried by the portal vein to the liver. About 80% of the blood in the liver comes from the portal vein, so the liver is often the target organ for toxic compounds in the body.[53]

Rodent liver weight varies by species and strain but is usually in the range of 2–3 g (3–5% body weight, body weight) in mice.[58] It can be seen in this study that the percentage of liver weight ratio was 5-7% BW, so it can be concluded that the Sungkai leaf fraction with varying doses from 1000 – 8000 mg/kgBW does not significantly affect the percentage of liver weight ratio.

3.4.5 Organ kidney ratio

Table 16: Effect of fraction type and dose variation on liver organ ratio of male white mice.

Treatment Group	Liver Ratio (%) ± SE			Mean (%) ± SE
	n-hexane fraction	Ethyl acetate fraction	water fraction	
Control (-)	0.7160 ± 0.039	0.7460 ± 0.039	0.7980 ± 0.039	0.753 ± 0.22
1000 mg/kgBB	0.7600 ± 0.039	0.9480 ± 0.039	0.8100 ± 0.050	0.839 ± 0.25
2000 mg/kgBB	0.7580 ± 0.039	1.0020 ± 0.039	0.7433 ± 0.050	0.834 ± 0.25
4000 mg/kgBB	0.7720 ± 0.039	0.9440 ± 0.039	0.8310 ± 0.039	0.849 ± 0.22
8000 mg/kgBB	0.6220 ± 0.039	0.9760 ± 0.039	0.8454 ± 0.039	0.814 ± 0.22
Mean (%) ± SE	0.726 ± 0.17	0.923 ± 0.17	0.806 ± 0.20	

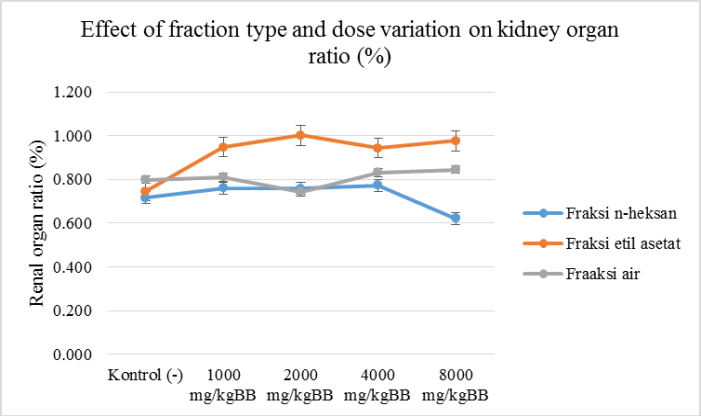

Figure 7: Graph of the effect of the types of fractions and variations in the doses of Sungkai leaves on the liver organ ratio of male white mice.

The results of the two-way ANOVA analysis showed that there was a significant effect of dose variation ($p<0.05$) on the percentage ratio of kidney organs of male white mice, then there was also a significant interaction between dose variation and type of fraction ($p<0.05$) on percentage of liver organ ratio of male white mice.

Based on the results of Duncan's test, the average percentage increase in kidney organ ratio of male white mice in the ethyl acetate fraction was higher than the other two fractions. Then in this case, there was a slightly significant difference in the response between the variation in the dose of each fraction on the percentage ratio of liver organs in male white mice, as seen in Duncan's test the effect of variations in the dose of each fraction on the ratio of kidney organs, where in the group given doses the value of the ratio of kidney organs slightly higher than the negative control group. The average value of the effect of the type of fraction and the variation in the dose of Sungkai leaves on the percentage of the liver organ ratio of male white mice can be seen in Table 19 above.

Large blood flow to the kidneys functions to maintain the body's homeostasis by regulating the body's electrolyte balance, regulating acid-base balance and regulating body osmolarity. The kidneys excrete solutes and dispose of the results of metabolism so that substances that are not useful to the body will be brought to the kidneys in large quantities. The kidney is a vital organ for the body, therefore it is often used as an observation parameter for testing the toxicity of a drug.[9]

Changes in organ weights are considered as sensitive indicators of toxic effects caused by new types of materials or compounds induced in experimental animals. To determine the effect of compounds induced on experimental animals and their effects on organs, absolute weights and relative weights are used as indicators.[59]

The average relative kidney weight (adjusted for 30 grams) of 21 inbred rat strains ranged from 0.6 g to 0.85% BW, in this study the kidney organ weight ranged from 0.7-0.9%. Abnormal organ weight ratios occurred in the ethyl acetate fraction, with a range of organ weight percentage ratios from 0.9 to 1.0% of the body weight of mice. Then for the n-hexane and water fractions from Sungkai leaves with varying doses from 1000 – 8000 mg/kgBW, it did not significantly affect the weight of

the kidney organ with an average percentage of organ ratio of 0.7-0.8% BW of mice.

CONCLUSION

1. The administration of each fraction of the ethanol extract of Sungkai leaves did not have a harmful toxic effect on mice, as evidenced by the OECD test preparation group criteria included in category 5/ unclassified and also the LD50 value> 8000 mg/kg BW
2. Giving fractions and varying doses of Sungkai leaves affected the percentage of body weight change in male white mice, a significant increase occurred in the water fraction at a dose of 4000 mg/kg BW
3. Giving fractions and varying doses of Sungkai leaves affected the weight percentage of the ratio of liver and kidney organs, a significant increase was found in the administration of the ethyl acetate fraction of Sungkai leaves. The weight ratio of the liver and kidneys in the ethyl acetate fraction of Sungkai leaves were 7.038% and 0.923%, respectively;

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