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Original Article

The acute toxicity of ethyl acetate extract from soursop leaf endophytic fungi in rats

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ABSTRACT

Soursop leaf endophytic fungi is an endophytic fungus with a symbiotic relationship inside the soursop leaf. It possesses higher anticancer activity compared to the leaves themselves. This research aims to determine the acute toxicity of ethyl acetate extract from soursop leaf endophytic fungi (*Phomopsis* sp) so that it can be referred for future research. The research used Sprague-Dawley rats divided into 6 groups. Each group was administered with 0, 5, 50, 300, 2000, and 5000 mg/kg body weight (BW) of extract in water, respectively. A lethal dose of 50% was obtained at 4198 mg/kg BW. Blood creatinine level, SGPT, and SGOT values were all within normal concentration in rats, those were 0.443-0.408, 59.4-83.4, and 161.0-222.8 mg/dL, respectively. The results of acute toxicity testing on SD rats obtained a lethal dose value that caused 50% death or LD50 from Phomopsis sp. is 4198,564 mg/kg BW. After 14 days, creatinine levels ranged from 0.443-0.408, SGPT ranged from 59.4-83.4, and SGOT values were in the range of 161.0-222.8. All values were within normal levels in rats. It is recommended to use ethyl acetate extract with a dose below 300 mg/kg BW extract.

Keywords: LD50, Soursop leaf endophytic fungus, Phomopsis sp., Acute toxicity

Introduction

Cancer is the number one mortality caused worldwide with 14 million new cases and 8.2 million lethal cases in 2012 alone (WHO, 2014). More than 60% of new cases have occurred in Africa, Asia, Central, and South America. Indonesian Research and Development Agency, Ministry of Health stated that 347,792 persons, or roughly 1.4% of Indonesian are developing cancer in their bodies [1].

Abnormal cell growth in body tissues will transform to make cancer cells that have a genotypically and phenotypically diverse population [2]. The available therapy for cancer treatments is chemotherapy, surgery, radiotherapy, immunotherapy, and photodynamic therapy [3]. Chemotherapy is a therapy to kill cancer cells using certain chemicals. These chemicals will give side effects from limpness, nausea, hair loss, dermatitis, and significant loss of body weight, and kills surrounding normal cells in the body. Thus, we need safer cancer medication for patients. Herbal medicine can be made from anticancer plants with fewer side effects compared to chemical treatments [4].

Soursop (*Annona muricata*) is a natural ingredient with anticancer activity. Previous research includes antibacterial [5], antioxidant [6], and anticancer activities in soursop leaf extracts [7]. Research by [8] showed that soursop leaves were able to prevent and inhibit cancer cell growth. However, exploitation of soursop leaves will reduce soursop plant productivity in making fruits. A high number of leaves was also needed to isolate the bioactive constituents. Thus, we developed the production of anticancer extract using soursop leaf endophytic fungus.

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A researcher Wilson (1995) defined endophytic microbes are "fungi that are present in host plant tissues, during at least part of their life cycle, without causing visible symptoms. A researcher [9] reported that endophytic fungus from ethyl acetate extract expressed a cytotoxic effect on breast cancer cells by inhibiting the proliferation of the cancer cells. The active constituents in the extract are also safe because they showed no toxicity against normal cells. Endophytic microbes in [9] research were proven to be fungi with close kinship to *Phomopsis* sp. The ethyl acetate of soursop leaf endophytic fungus efficacy research in vivo can be used as the background for the use of the extract as an anticancer treatment.

The coordination meeting between 9 ministries and 5 national bodies in Jakarta on March 14th, 2019, about "Advances of Phytopharmaca Development and Usage" explained that cancer adjuvant is the priority for national drugs ingredients production priorities. But Indonesian Drugs and Food Administration Bodies (BPOM) stated that there are no phytopharmaca and traditional herbal medicine candidates in Indonesia as anticancer adjuvants until today. To be approved as a drug candidate, one formula needs to be tested for its Lethal Dose 50% (LD_{50}) toxicity test. Toxicity tests with animal models are required to know the toxicity risks posed by the substances [10]. Supporting data were obtained by observing the liver and kidneys of the laboratory animals. This research aims to obtain the LD50 value and determine the safe dose of ethyl acetate extract of soursop leaf endophytic fungus. We hope that the extract will be safe for consumption and do not give a toxic effect on the human body.

Materials and Methods

Chemicals and reagents

Materials were soursop leaf endophytic fungus isolate, glucose, yeast extract, malt extract, peptone, chloramphenicol, ethyl acetate, ethanol, Na-CMC 1% in water, female Sprague Dawley rats, Buffer Neutral Formalin (BNF) 10%, ethanol, xylol, hematoxylin solutions, eosin solutions, DPX adhesive liquid, and albumin. Materials in the blood analysis were creatinine kit reagents from ReiGed Diagnostic, blood urea nitrogen reagents from Reiged Diagnostic, deionized water, and optical tissues.

Soursop leaf endophytic fungus rejuvenation [11]

Inoculation of endophytic fungus isolate was done in a laminar airflow cabinet aseptically. The cabinet was sterilized by UV light for 1 hour, then 70% ethanol was used to clean throughout inside and outside of the cabinet. Next, the stock isolate was to be inoculated into a YMA medium and was incubated for 1 week at 27 °C temperatures.

Isolate cultivation [9]

Endophytic fungus isolate was taken using an inoculum needle after 5 days and was inoculated into a 200 mL yeast Malt Broth (YMB) medium. The isolate was then reincubated at the same time and condition. Thereafter, fungus in the YMB medium was transferred using an inoculum needle into a 2 L YMB medium in a 5 L Erlenmeyer flask. The transfer was carried out successively until reached 300 L volume and was collected in a 500 L bioreactor. The fermentation process in the bioreactor was done for 18 days.

Soursop leaf endophytic fungus extraction [12]

Soursop leaf endophytic fungus isolate was extracted after 18 days to obtain the main chemical constituents. Endophytic fungus isolate in YMB medium was added with ethyl acetate in the same volume and was incubated for 1 day. Bottom layer solutions were separated, and the top layer was collected as ethyl acetate extract. The extract was evaporated in a rotary vacuum evaporator at 57 °C temperature. The extract was then transferred into a dark bottle and was kept in cold storage before being used.

Acute toxicity test [13]

Sprague Dawley rats of 2-3 months old (95-140 g body weight) were used in the research. The adaptation period was carried out for 1 week with ad libitum feed and water. The animals were fasting for 8-12 hours before the treatment on day 0 with ad libitum access to water. The extract was given orally in various doses of 0, 5, 50, 300, 2000, and 5000 mg/kg body weight (BW). All solutions were prepared in 1% Na-CMC. The maximum administration volume was 0.5 mL. The lethality of the animals was evaluated after 1 x 24 hours and the data were analyzed by toxicity Probit methods. Toxic symptoms were also assessed after 14 days, including breathing, behavior, movement, feces, eyes, bodyweight changes, and mortality.

Creatinine (reiged diagnostic kit) [14]

Creatinine level measurements were done using Reiged Diagnostic Kit. The kit included R1, R2, and creatinine 2 mg/dL standard solutions. Five hundred microliters of R1 and R2 solutions were mixed with 100 µL of rat blood serum. The mixture was homogenized and incubated for 60 seconds at 37 °C. Then, the absorption was measured on a 510 nm spectrophotometer (A1). Second incubation was carried out for 120 seconds and the absorption was taken (A2). Standard solution absorption was also taken in the same manner. Creatinine level was calculated as follows:

Aspartic aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activities determination [15]

The assay was carried out using AST and ALT kit spectrophotometrically. AST kit contained tris buffer, L-aspartate, α-ketoglutarate, malate dehydrogenase, and NADH.

ALT kit included tris buffer, L-alanine, α-ketoglutarate, lactate dehydrogenase, and NADH. The fundamental measurement was to determine the reduction speed of NADH to NAD^+ in enzymatic reaction at 340 nm. Blood serum was taken using a pipette and transferred to an Eppendorf tube. One hundred microliters of blood were mixed with a 1000 µL reagent. The mixture was homogenized and was incubated for 1 minute at 30°C. The absorption was quantified on a 340 nm wavelength.

Results and Discussion

Acute toxicity of soursop endophytic fungus extract (Phomopsis sp.)

Acute toxicity extract of *Phomopsis* sp. was done in 6 groups of Sprague Dawley rats. There was no mortality after 24 hours of extract administration. The mortalities were found on day 2 (1 rat), day 3 (1 rat), day 4 (3 rats), and day 5 (1 rat). The mortalities were observed in the dose of 300, 2000, and 5000 mg/kg BW groups with a total of 1, 2, and 3 rats in each group, respectively. The number of lethal animals was calculated using Probit graph analysis.

Linear regression from the log dose in Probit analysis is $Y=0.2958\ X^2 - 0.6317\ X + 3.6374$, $R2=0.9973$ **(Figure 1)**. So, the calculated LD50 is 4198.564 mg/kg BW. Thus, the extract is a mild toxic material. **Table 1** shows *Phomopsis* sp. LD_{50} calculation based on Probit analysis.

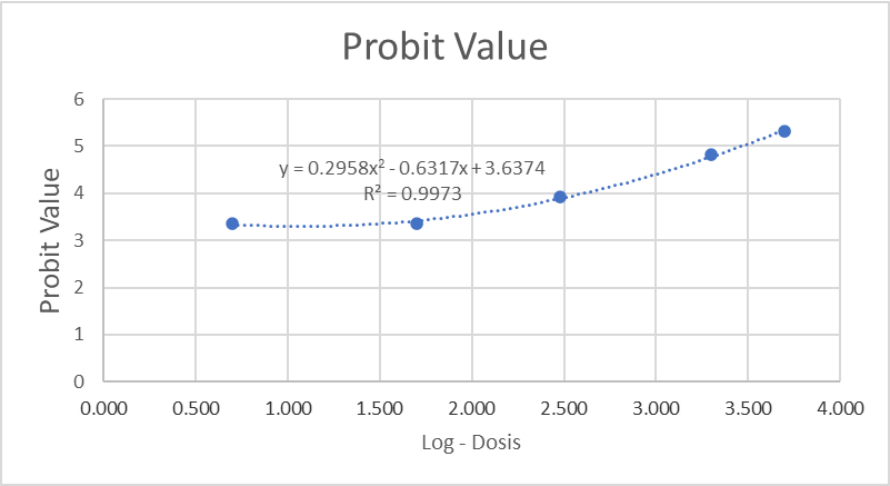

Figure 1. LD_{50} value of soursop leaf endophytic extract (*Phomopsis* sp.)

Table 1. LD_{50} value of soursop leaf endophytic extract (*Phomopsis* sp.)

Groups	Dose (mg/kg BW)	Log dose	Rat mortality	Normal rats	%Mortality	Probit
1	5	0.69897	0	5	0	3.36
2	50	1.69897	0	5	0	3.36
3	300	2.47712	1	4	20	3.92
4	2000	3.30103	2	3	40	4.75
5	5000	3.69897	3	2	60	5.31

Table 2. Toxic symptoms from the administration of Phomopsis sp. fungi extract on the first day

Extract Dose (mg/kg BW)	Toxic Symptoms	Notes
Control	-	-
5	-	-
50	-	-
300	-	-
2000	-	There was 1 rat experiencing lethargy on the 2nd day
5000	-	There was lethargy (2 rats) and salivation (1 rat) on the 2nd day

Extract administration with toxic activity can influence rat behavior. Rats did not show toxic behavior in the first hour. The toxic symptom was observed on the first, second, and third days of treatment. There was a rat volume increase observed but without the growth of bodyweight. The symptom was seen in the 2000 mg/kg BW group with 1 rat on the first day and 1 rat on the second day. The other toxic symptom was choking breath in

one of the rats in the 5000 mg/kg BW group on the second and third days. The symptom was lost after the fourth day **(Table 2)**.

Creatinine level

Creatinine is an endogenous product of muscle metabolism that is excreted by the body through the kidney [16]. Blood creatinine level is used as kidney function and glomerulus filtration indicators [17]. Creatinine measurements in this research were carried out using the Jaffe reaction. The reaction is based on a creatinine reaction with picric acid in base conditions to make an orange picric-creatinine complex that can be measured in 510 nm wavelength [18]. Ishak *et al.* [19] showed that normal blood creatinine value in rats is in the 0.2-0.8 mg/dL range.

Table 3. Blood creatinine, SGOT, and SGPT level

No	Extract dose (mg/kg BW)	Parameter		
		Creatinine (mg/dL)	AST (mg/dL)	ALT (mg/dL)
1	5000	0.42±0.99	162±2.83	52.5±3.54
2	2000	0.443±0.07	268.667±77.84	63.333±18.58
3	300	0.41±0.05	195.5±46.36	64.75±11.95
4	50	0.432±0.05	205.6±107.98	59.6±17.95
5	5	0.408±0.07	161±33.70	59.4±9.48
6	Control	0.436±0.06	222.8±57.14	83.4±18.81

Blood creatinine levels on day 14 showed that female creatinine rats were in the range of 0.408-0.443 mg/dL and do not significantly different between all the treatment groups. The creatinine level was considered normal **(Table 3)**.

The elevation of creatinine level by-products is an early indicator of acute kidney injury [20]. Hence, it will occur if the creatinine level is over 0.8 mg/dL [21]. Creatinine is a product of creatine catabolism or a by-product of nitrogen compounds catabolism in the muscle. The level in the blood is always constant or linear with the number of muscle cells [22]. So that the increase of creatinine can be an indicator of impaired kidney function or kidney disease [23].

Laboratory animals blood serum transaminase enzyme activities

The liver is a very important organ involved in coordinating several key metabolic activities [24]. There are several methods to evaluate liver condition, such as biopsy, radiological, and liver function tests (LFTs) [25]. Hepatic intracellular enzyme activity are highly affected during hepatocyte injury. One sensitive indicator is the release of liver intracellular enzymes such as transaminase [26]. The enzymes include aspartic aminotransferase (AST) or serum glutamate oxaloacetate transaminase (SGOT) and alanine aminotransferase (ALT) or serum glutamate pyruvate transaminase (SGPT) [27]. The increase of this enzyme activity indicate the leak and the loss of liver cells' membrane integrity and leads to liver disease [26].

An acute toxicity test was carried out in 5 doses of ethyl acetate extract of soursop leaf endophytic. Rats were divided into 6 treatment groups. Group 1 was a normal group without extract administration. Groups 2, 3, 4, 5, and 6 were treatment groups with various doses of 5, 50, 300, 2000, and 5000 mg/kg BW, respectively. Blood was collected on day 14.

All 5 groups administered with extract experienced a decrease in blood serum AST on day 14 compared to the normal group. Serum AST levels on day 14 varied between 161.00 to 205.6 U/L, unless those in group 5 give a slightly higher AST level of 268.67 U/L competes to the normal group. AST levels of the count groups was was slightly higher than previously reported with an ASTan level of 177.7-203.51 U/L [28]. However, some researchers showed a lower AST value of 147.25 U/L in the rats without treatment [29]. Suckow *et al.* [30] also state that normal AST enzyme activities are in the range of 77-157 U/L. Liver damage was known based on common biochemical parameters such as the increase of GPT or ALT and GOT or AST levels in the blood serum. Pratt [31] explained enzyme level increase up to 300 U/L are a sign of an unspecific defect. If the enzyme activity significantly increases over 500 U/L, then it was a sign of liver disease due to viral infection, heart failure, and hepatocyte disruption because of toxic substances. The decrease of AST enzyme activity in this research is in the range of normal so that the liver did not experience heavy disruption [32].

The decrease of AST enzyme activity in groups receiving 5, 50, and 300 mg/kg BW fungus ethyl acetate extract on day 14 is considered normal. AST activity was decreased because the extract has hepatoprotective ability. The researcher [9] explained that chemical constituents in soursop leaf extract have the hepatoprotective ability that can prevent hepatic disease. Hepatoprotectors are chemical compounds that can give liver cells protection against poisons, drugs, and other xenobiotics. The liver is a unique organ with its hepatocyte does not have self-repair ability.

The highest serum ALT or SGPT on day 14 was shown by the group administered with 50 mg/kg BW extract (64.75 U/L) and varied until 52.5 2 U/L in a group with 5000 mg/kg BW extract administration. The other groups also showed higher results compared to those of the control group. However, all AST levels are still in normal range concentration. Suckow *et al.* [30] stated that ALT enzyme activity in normal Sprague Dawley rats is 24-53 U/L. While according to [28], the normal ALT level is about 136.42 U/L. Thus, our result showed lower ALT levels in all treatment groups. Endophytic fungus ethyl acetate extract did not affect the ALT level significantly. The results are consistent [9] reports that endophytic fungus extract has hepatoprotective

abilities. Therefore, it can protect liver cells from damage by poisons, drugs, or other external conditions.

Nevertheless, the extract is not recommended in 5000 mg/kg BW dosage. Considering that liver has unique functionality and cannot self-repair itself in the case of cell damage. Hence, we observed mortality in the group during treatment.

AST/ALT ratio is one of screening test that can indicate liber abnormalities [33]. AST/ALT ratios on the rat at day 14 showed a value of more than 0.8 or more than 2.67 in control up to 4.25 in the 2000 mg/kg BW treatment group. The early hepatocellular inflammation and damage will cause damages to cell membrane leading to the leakage enzymes into the blood [26]. The process will cause the ALT level to rise more than ALT and the AST/ALT ratios <0.8 and indicate minor liver damage [34]. Acceptable extract administration based on AST/ALT ratios is up to 4198.564 mg/kg BW. Yet, the safe dose is below 300 mg/kg BW due to no clinical symptoms observed in the 300 mg/kg BW administration group.

Conclusion

The results of acute toxicity testing on SD rats obtained a lethal dose value that caused 50% death or LD50 from Phomopsis sp. is 4198,564 mg/kg BW. After 14 days, creatinine levels ranged from 0.443-0.408, SGPT ranged from 59.4-83.4, and SGOT values were in the range of 161.0-222.8. All values were within normal levels in the rat. It is recommended to use ethyl acetate extract with a dose below 300 mg/kg BW extract.

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