PROTECTIVE POTENTIAL ETHYL ACETATE OF SRIKAYA STEAM SKIN (*Annonasquamosa* L.) ON RAT LIVER HISTOLOGY (*Rattus norvegicus* B.) INDUCED BY PARACETAMOL

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ABSTRACT

This research aims to determine the hepatoprotective potential of ethyl acetate extract from soursop bark (*Annona squamosa* L.) (EEASB) on the histology of rat liver (*Rattus norvegicus* B.) induced by paracetamol. The method used in this study is a Completely Randomized Design (CRD) consisting of 6 treatments and 4 replications. A single dose of paracetamol 1,350mg/kgBW was administered on day 7, accompanied by the administration of distilled water (K-), HEPA-Q 11.34 mg/kgBB (K+), EEASB 150 mg/kg BB (P1), EEASB 300 mg/kg BB (P2), and EEASB 600 mg/kg BB (P3). Liver histology preparations were made using the paraffin method and Hematoxylin and Eosin (HE) staining. Parameters measured included the proportion of hepatocyte cells undergoing degeneration and necrosis, as well as the presence of hyperemia, hemorrhage, and inflammation. Mean data for necrosis and degeneration were analyzed using ANOVA with a significance level of 5% and followed by Duncan's multiple range test. Hyperemia, hemorrhage, and inflammation parameters were analyzed using Kruskal-Wallis and further tested with Mann-Whitney. The results showed that the administration of ethyl acetate extract from soursop bark (*Annona squamosa* L.) with doses up to 600 mg/kg BW did not have hepatoprotective potential against hepatocyte cells undergoing necrosis. However, the administration of ethyl acetate extract from soursop bark (Annona squamosa L.) at a dose of 600 mg/kg BW showed potential as a hepatoprotector against cells experiencing hyperemia and hemorrhage.

Keywords: *Annona squamosa* L., paracetamol, hepatoprotector without paracetamol, hepatocytes, necrosis, degeneration, hyperemia, hemorrhage, inflammation

A. INTRODUCTION

The soursop plant is one of the plants from the Annonaceae family that has medicinal properties as an herbal remedy. Soursop leaves have benefits as antioxidants, antidiabetic, hepatoprotective, and antitumor agents (Purwita et al., 2013). Soursop fruit contains vitamin C, which functions to combat free radicals in the body. The vitamin A found in soursop fruit is beneficial for maintaining skin, hair health, and improving eye function. Soursop fruit also contains potassium and magnesium, which are effective in preventing heart disease (Setiono et al., 2013). According to previous research conducted by Masykur et al. (2022), ethanol extract of soursop bark (Annona squamosa L.) has potential as a hepatoprotective agent based on macroscopic observations of the liver in paracetamol-induced rats.

Nurfisa (2023) found that ethanol extract of the bark of this plant has potential as a

hepatoprotective agent in Rattus norvegicus liver at a dose of 600 mg/kg BW. Ethanol is a type of solvent commonly used to extract phenolic compounds in herbal plants. The difference in previous studies (Nurfisa (2023); Masykur et al. (2023); Sari et al. (2023)) and this study lies in the type of solvent, which is ethyl acetate for maceration. Putri et al. (2013) stated that compounds in plant organs can also be extracted with ethyl acetate. Ethyl acetate is a semi-polar solvent that can attract both polar and nonpolar compounds. Ethyl acetate also has low toxicity and is highly volatile, making it suitable for extraction. The rate of extraction is influenced by the type of solvent, the solvent ratio, time, temperature, particle size, and the amount of solvent. Active ingredients will dissolve in the solvent during the extraction process, and these active ingredients will dissolve if the solvent matches their polarity (Rifai et al., 2018). Ethyl acetate is believed to be able to dissolve several herbs with hepatoprotective potential. According to Hayati et al. (2014), the ethyl acetate fraction of sangitan leaves (Sambucus canadensis L.) at a dose of 1.93 mg/kg BW can act as a hepatoprotective agent based on the measurement of SGPT and SGOT in Sprague-Dawley rats. The ethyl acetate fraction of sangitan leaves at a dose of 1.93 mg/kg BW has already shown hepatoprotective effects based on SGPT measurement.

Liver disease is an endemic disease that is a problem in Indonesia. According to the 2013 Basic Health Research (Riskesdas), the prevalence of hepatitis B virus is around 7.1%, or about 18 million, and hepatitis C virus is around 1.10%, or about 2.5 million. Chronic liver diseases include Hepatocellular Carcinoma (HCC), Hepatitis B, Hepatitis C, and Liver Cirrhosis. Liver diseases are characterized by inflammation and necrosis in the liver. Inflammation and necrosis in the liver usually last for six months before being diagnosed as chronic liver disease. Chronic liver disease is often caused by viral infections, alcohol addiction, fatty liver, and the influence of certain medications (Yasin et al., 2015).

Paracetamol is an analgesic and antipyretic drug that is commonly used among the public. The use of paracetamol as a fever and pain reliever has been known for a century. Paracetamol is very easy to find in pharmacies and stores, and many people consume paracetamol without a doctor's prescription. The recommended dose of paracetamol is 2000 mg per day (Zulizar, 2013). A toxic dose of paracetamol exceeding 2000 mg can cause hepatotoxicity. According to Darsono (2012), the toxic dose of paracetamol in adults is 6000 mg. Based on the above description, the potential protection of ethyl acetate extract from soursop bark (Annona squamosa L.) against the histology of paracetamol-induced rat liver (R. norvegicus) needs to be studied. This research aims to determine the hepatoprotective potential of ethyl acetate extract from soursop (Annona squamosa L.) as

a hepatoprotective agent against the histology of paracetamol-induced rat liver (Rattus norvegicus B.) by counting hepatocyte cells undergoing degeneration and necrosis, as well as the occurrence of hemorrhage, inflammation, and hyperemia in liver tissue.

B. RESEARCH METHOD

1. TIME AND PLACE

This research was conducted from October to December 2023. The preparation of ethyl acetate extract from the bark of Annona squamosa L. (EEAKBAS) was carried out at the Animal Structure and Development Laboratory, Faculty of Mathematics and Natural Sciences, USK. The provision and treatment of test animals were conducted at the Experimental Animal Unit Laboratory, Faculty of Veterinary Medicine, USK University. The preparation and observation of histological preparations were carried out at the Animal Structure and Development Laboratory, Faculty of Mathematics and Natural Sciences, USK.

2. TOOLS AND MATERIALS

The tools used in this research included rat cages measuring 50 cm x 40 cm x 30 cm with wire mesh covers, rat drinking bottles, Zeiss Primo star microscope, sample bottles, mortar and pestle, tweezers, staining jars, paraffin molds, oven, refrigerator, water bath and stretching table, blender, computer, microtome, a set of surgical instruments, 1 mL syringes, gavage needles, Erlenmeyer flasks, evaporating flasks, measuring glasses, analytical balance, petri dishes, beakers, rotary vacuum evaporator, funnel strainers, dropper pipettes, volumetric flasks, and writing instruments. The materials used in this research included 1 kg of soursop bark (Annona squamosa L.), ethyl acetate, cuvettes, DPPH crystals, Whatman filter paper, physiological NaCl solution, 24 male rats (Rattus norvegicus L.) aged 3 months with a body weight of 250 g, surgical gloves, surgical masks, cotton, feed, aluminum foil, 70%, 80%, and 90% alcohol, absolute alcohol, Bouin's solution, xylene, absolute ethanol, paraffin, hematoxylin dye, eosin dye, microtome blades, embedding
cassettes, Entellan, cover slips, glass slides, HEPA-Q, and label paper.

3. WORK PROCEDURE

The dried bark samples (1 kg) were pounded using a mortar and pestle and placed in a container for maceration. Annona squamosa L. extraction was performed by maceration using ethyl acetate for 3 x 24 hours. The filtrate was then filtered and concentrated using a rotary evaporator at 35°C. The rats were acclimated for 7 days in cages measuring 50 cm x

40 cm x 30 cm with wire mesh covers. The cages were lined with 3 cm of husk and cleaned every 3 days. The test animals were given doses of ethyl acetate extract from soursop bark (EEAKBS) at doses of 0 mg/kg BW (P0),
150 mg/kg BW (P1), 300 mg/kg BW (P2), and 600 mg/kg BW (P3) for 14 days after acclimatization. The volume of soursop extract and paracetamol given was 1 mL orally. Rats were given paracetamol on the 7th day at a toxic dose of 1350 mg for each group except K0, with a volume of 1 mL. The hepatoprotective testing procedure included the period and timing of paracetamol administration to the test animals based on Rafita et al. (2015).

After 14 days of treatment, the rats were euthanized, and their liver organs were harvested. The preparation of histological slides of the rat liver was performed using Hematoxylin Eosin staining. The liver organs were fixed in BNF solution for 24 hours. After 24 hours, the liver samples were washed with 70% alcohol until clear. The organs were then dehydrated in 70%, 80%, 90%, and absolute alcohol solutions for 2 hours each, with two repetitions. The tissues were then cleared with xylene solution for 1 hour, with two repetitions. The tissues were infiltrated with a xylene-paraffin solution (1:1) for 1 hour and with paraffin solution for 1 hour, with two repetitions. Next, the embedding stage was performed, where the infiltrated tissues were embedded in liquid paraffin in block molds and left to harden. The procedure for histological preparation is detailed in Appendix 4 (Kurnia et al., 2015).

The hardened blocks were removed from the molds and sectioned using a microtome. Paraffin sections were cut to a thickness of 6 µm to form paraffin ribbons, with 4 sections per sample at 10- section intervals. The sections were placed in a water bath at 56-58°C until they expanded. The expanded sections were then mounted on glass slides, placed on a stretching table, and left to dry.

The samples were stained using the Hematoxylin-Eosin staining method. The dried samples were immersed in xylene I and xylene II solutions for 2-3 minutes each. The samples were then washed with absolute alcohol I, absolute alcohol II, 90% alcohol II, 80% alcohol I, 80% alcohol II, 70% alcohol I, and 70% alcohol II for 2 minutes each. The samples were washed with distilled water for 1 minute. The samples were then stained with hematoxylin solution for 3-5 minutes, washed again with distilled water for 10 minutes, stained with eosin solution for 1-2 minutes, and then re-dehydrated by dipping in 70% alcohol I, 70% alcohol II, 80% alcohol I, 80% alcohol II, 90% alcohol I, 90% alcohol II, absolute alcohol I, and absolute alcohol II for 2-3 minutes each. The samples were cleared in xylene I and xylene II for 1 minute each. Finally, the samples were mounted with

Entellan, covered with cover slips, and left overnight to dry (Kurnia et al., 2015).

The histological structure of the rat liver was observed using a microscope at 400x magnification (Kurnia et al., 2015). Observations of hepatocytes undergoing degeneration and necrosis were made in 8 microscopic fields. The histological preparations were placed under a Zeiss microscope with a 400x objective lens. The microscopic fields were observed from left to right.

C. RESULT AND DISCUSSION

The histological structure of the liver of Rattus norvegicus Berkenhout treated with ethyl acetate extract from soursop bark (Annona squamosa L.) and induced with paracetamol can be seen in Figure 4.1. Liver tissue damage caused by toxicity in this treatment includes necrosis and degeneration. Necrosis of the hepatocytes in the test rats is characterized by changes in the cell nucleus structure, displaying karyopyknosis, karyorrhexis, and karyolysis.

Based on ANOVA analysis of the average data of hepatocytes experiencing necrosis and degeneration, there is a significant difference between the treatment groups ($P<0.05$). Therefore, the analysis was continued with Duncan's multiple range test. The results can be seen in Table 4.1.

Table 4.1. Average Proportion of Hepatocytes Experiencing Degeneration and Necrosis in Each Treatment

Treatment	Rata rata sel (Rerata ± SD)	
	Nekrosis	Degenerasi
K0	$0{,}31972^{a} \pm 0{,}1988$	$0{,}08360^{ab} \pm 0{,}328912$
K-	$0{,}55611^{b} \pm 0{,}49106$	$0{,}32062^{bc} \pm 0{,}11684$
K+	$0{,}46408^{ab} \pm 0{,}041924$	$0{,}04876^{a} \pm 0{,}20294$
P1	$1{,}71127^{d} \pm 0{,}228070$	$1{,}05408^{d} \pm 0{,}231720$
P2	$1{,}15046^{c} \pm 0{,}043837$	$0{,}93288^{\mathbf{d}} \pm 0{,}328726$
P3	$0{,}42650^{ab} \pm 0{,}064153$	$0{,}37975^{c} \pm 0{,}021891$

Note:Numbers followed by different superscript letters (a, b, c, and d) indicate significant differences at the 5% test level (P<0.05). Treatments K0:Administration of aquades K-: Administration of a single dose of 1350 mg/kg BW paracetamol on the 7th day accompanied by aquadest K+: Administration of HEPA-Q 11.34 mg/kg BW P1: Administration of EEAKBS 150 mg/kg BW P2: Administration of EEAKBS 300 mg/kg BW P3: Administration of EEAKBS 600 mg/kg BW for 14 days

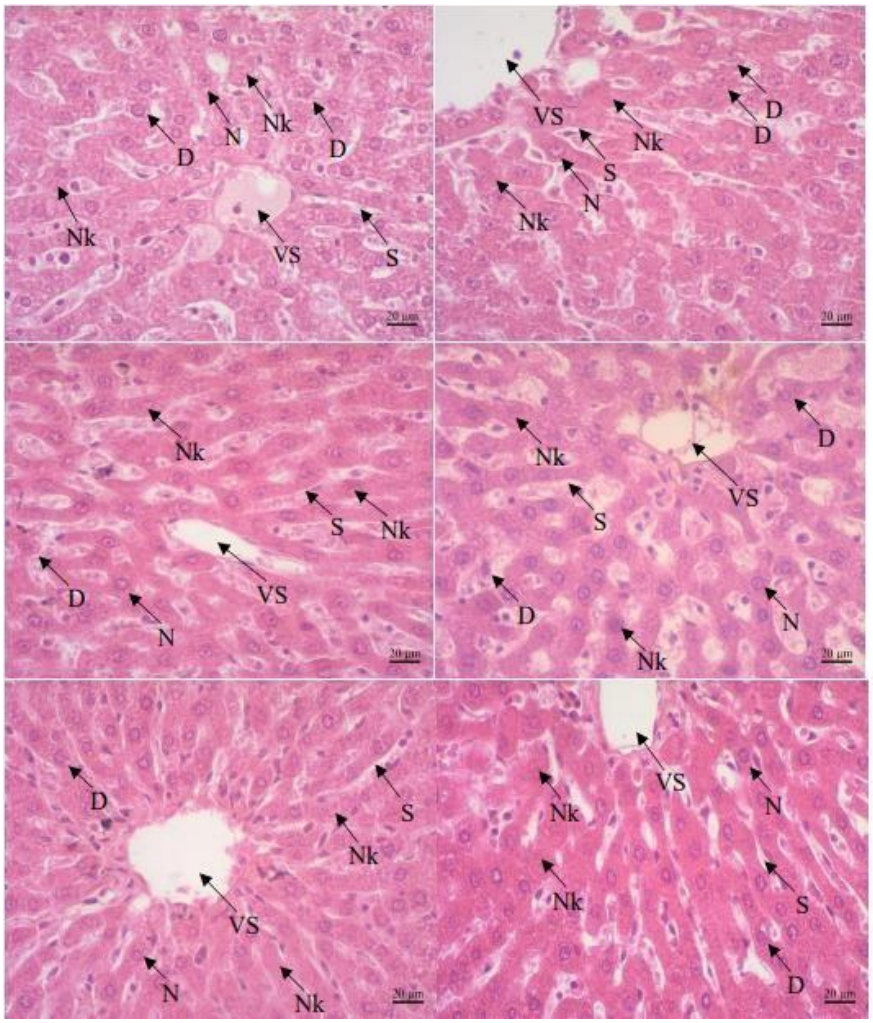

Figure 4.1. Histological Structure of Rat Liver (Rattus norvegicus Barkenhout) After Treatment Description:(A) Administration of aquadest (K0) (B) Administration of a single dose of 1350 mg/kg BW paracetamol on the 7th day accompanied by aquadest (K-) (C) Administration of HEPA-Q 11.34 mg/kg BW (K+) (D) Administration of EEAKBS 150 mg/kg BW (P1) (E) Administration of EEAKBS 300 mg/kg BW (P2) (F) Administration of EEAKBS 600 mg/kg BW (P3) for 14 days Abbreviations: VS: Central vein N: Normal hepatocytes D: Degeneration Nk: Necrosis S: Sinusoids He: Hemorrhage Hi: Hyperemia in: Inflammation

Based on Table 4.1 and Appendix 6.4, it is shown that there is an effect of treatment on rat liver cells. The administration of paracetamol (K-) can cause a significant increase in

necrosis and degeneration. According to Eric et al. (2016), hepatocyte cell damage can be caused by the consumption of high doses of paracetamol. Sidabuptar et al. (2016) state that paracetamol contains N-acetyl-p-benzoquinoneimine (NAPQI), which is a highly reactive metabolite capable of causing liver cell damage when consumed in high doses. This leads to the accumulation of NAPQI, resulting in necrosis and potentially progressing to liver failure. Sugiharto & Martini (2018) add that the administration of paracetamol causes the body's endogenous antioxidant GSH (glutathione) in the liver to be unable to control NAPQI. This condition causes free radicals formed to bind with the unsaturated fatty acids of the cell membrane, leading to lipid peroxidation and the formation of MDA (malondialdehyde). High levels of MDA in the plasma can be used as an indicator of free radicals attacking lipid membranes containing polyunsaturated fatty acids, forming MDA. MDA is one of the end products of lipid peroxidation. According to Rafita et al. (2010), toxic doses of paracetamol undergo biotransformation by cytochrome P450 (CYP2E1 isozyme), producing a reactive and unstable toxic metabolite, N-acetyl-para-benzoquinoneimine (NAPQI). Excessive formation of NAPQI will deplete glutathione, causing NAPQI to react with nucleophilic groups on liver cell macromolecules, such as proteins, leading to hepatotoxicity and resulting in liver necrosis.

Figure 4.1 also shows other liver tissue damage in rats, such as hyperemia, hemorrhage, and inflammation. Hyperemia is the initial stage of liver damage before hemorrhage occurs. Microscopically, hyperemia in liver tissue is characterized by redness in the blood vessels due to blood accumulation.

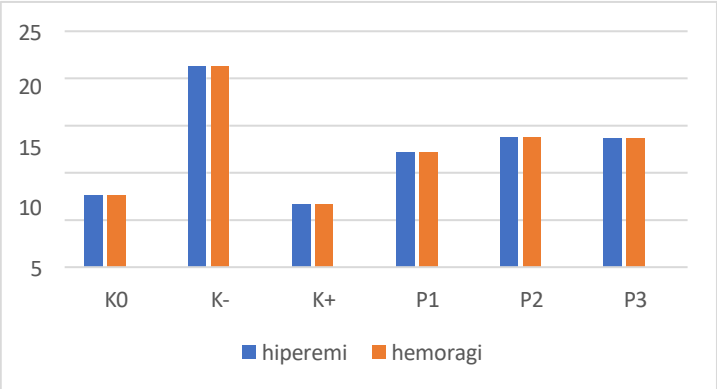

Figure 4.2. Graph of Mean Rank Proportion of Liver Tissue Damage Experiencing Hyperemia and Hemorrhage in Various Treatments - K0 Administration of aquadest. -K- Administration of a single dose of 1350 mg/kg BW paracetamol on the 7th day accompanied by aquadest.K+: Administration of HEPA-Q 11.34 mg/kg BW. P1: Administration of EEAKBS 150 mg/kg BW. P2Administration of EEAKBS 300 mg/kg BW.P3 Administration of EEAKBS 600 mg/kg BW for 14 days.

The conditions of hyperemia and hemorrhage in the liver tissue of rats at a dose of 600 mg/kg BW were significantly lower compared to the K- treatment (Table 4.2). However, there was no significant difference compared to K0, K+, P1, and P2. This indicates that the administration of EEAKBS at a dose of 600 mg/kg BW can reduce liver tissue damage such as hyperemia and hemorrhage caused by paracetamol toxicity. This is attributed to the saponin compounds contained in EEAKBS. According to Wiracakra et al. (2017), saponins have the ability to form hydroperoxides that act as secondary antioxidants, thereby inhibiting lipid peroxidation. The Kruskal-Wallis analysis of the inflammation data showed an asymp sig. value of 0.205 > 0.05, indicating no significant difference in the effect of treatments on inflammation.

D. CONCLUSION

Conclusion Based on the Research Findings:

1. The administration of ethyl acetate extract from the bark of soursop (Annona squamosa L.) at doses up to 600 mg/kg BW does not show hepatoprotective potential against hepatocyte cells experiencing necrosis.
2. The administration of ethyl acetate extract from the bark of soursop (Annona squamosa L.) at a dose of 600 mg/kg BW shows potential as a hepatoprotective agent against cells experiencing hyperemia and hemorrhage.

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