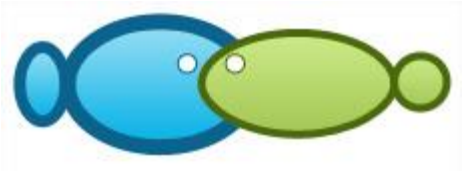

The therapeutic effect of black mangrove (*Rhizophora mucronata* Lam.) fruit extracts on estrogen levels in female white rats (*Rattus norvegicus*) post-ovariectomy *

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Abstract. The potential use of phytoestrogens in the treatment of estrogen deficiency continues to be investigated following reports of side effects of synthetic estrogen therapy. The study aims to evaluate the therapeutic effects of phytoestrogens from ethanol extract of black mangrove (*Rhizophora mucronata* Lam.) fruit on estrogen serum levels, body weight, and liver health in post-ovariectomy rats. Estrogen levels were measured using the ELISA method, while analysis of side effects of therapy was based on observations of body weight and liver enzyme activity. The results show that phytoestrogens in black mangrove fruit can increase estrogen levels in post-ovariectomy rats. Estrogenic effects began to manifest at 400 mg/kg BW/day. Estrogen levels in the Ovx+D2 and Ovx+D3 groups were not significantly different compared to the negative control. All groups of animals tested showed an increase in body weight, except for the group Ovx+D3. Enzyme activity (SGPT) in all treatment groups did not differ significantly compared to negative controls. The therapeutic effect of black mangrove fruit extract also showed a lower increase in enzyme activity (SGOT and ALP) compared to the positive control. Ethanol extract at a dose of 400 mg/kg BW/day was shown to provide the best therapeutic effect, because it significantly increased estrogen levels, but did not cause significant side effects on liver function (based on SGPT, SGOT, and ALP values). Further research will be conducted to determine the mechanism of action and long-term therapeutic effects of phytoestrogens from black mangrove fruit before they are recommended for clinical use in humans.
Key Words: therapeutic effect, ethanol extracts, *Rhizophora mucronata* Lam, estrogen level, ovariectomy.

Introduction. Estrogen plays a vital role in many body functions, including regulating bone metabolism, cardiovascular health, and liver function (Ernawati et al 2024). Decreased estrogen levels can lead to a variety of health problems, including osteoporosis, cardiovascular disorders, and several other symptoms of menopause (Khanjani & Panay 2019; Khosla & Pacifici 2021; Sagili & Rajan 2021; Cho et al 2022). Estrogen deficiency can occur in women who experience menopause or ovariectomy (Lobo 2017). In recent years, synthetic estrogen hormone therapy has often been used to treat menopausal symptoms (Fait 2019), but several studies have shown that this therapy also has risks and side effects that need to be considered (Lobo 2017; Tjoe et al 2021; Chang et al 2022; David et al 2023; Eliyahu et al 2023; Loizzi et al 2023; Kielb et al 2024). Alternatively, the

use of phytoestrogens from plant sources may be a safer option in treatment (Rietjens et al 2017; Wyse et al 2022).

Phytoestrogens are secondary metabolites found in plants that can bind to estrogen receptors, producing estrogenic effects in the body (Ernawati et al 2021 2024). A characteristic of phytoestrogens is the presence of a phenolic ring which is a requirement for the formation of bonds with estrogen receptors (Nikolić et al 2017). Phytoestrogens are divided into 4 groups: chalcones, flavonoids (flavones, flavonols, flavanones, isoflavonoids), lignans, and stilbenoids (Ström et al 2012). One of the natural sources of phytoestrogens is found in black mangroves (*Rhizophora mucronata* Lam.). In previous studies, we successfully identified ethylestrenol (phytoestrogen) from ethanol extract of black mangrove *R. mucronate* Lam. fruit which has similar molecular structure to 17β-estradiol and ethinyl estradiol (Figure 1). Both compounds are known as estrogen receptors α and β in mammals.

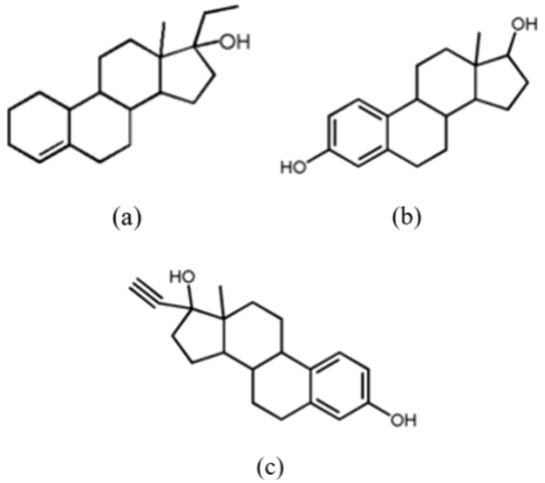

Figure 1. Molecular structure of (a) ethylestrenol, (b) 17β-estradiol, (c) ethinyl estradiol.

Material and Methods. The animal models were divided into 5 groups; namely the negative control group (NK), the positive control group/ovariectomy model (Ovx), the treatment group with a dose of 200 mg/kg BW/day (Ovx+D1), 400 mg/kg BW/day (Ovx+D2), 800 mg/kg BW/day (Ovx+D3). The amount of extract in each treatment was calculated and given orally using a sonde. The research variables consist of; independent variables (dose of *R. mucronata* Lam. extract), dependent variables (histological features of the uterus, liver, kidney, blood, estrogen in the blood), and control variables (ovariectomy, sex, age, body weight, feed). Each treatment group was repeated four times (4 rats per group). This number was obtained based on calculations using Equation 1.

$$t(n-1) \geq 15 \qquad (1)$$

t in Equation (1) is the number of treatment groups, while n is the number of repetitions. By entering a t value of 5, the n value is known to be 4.

First, the rats were adapted to the laboratory environment for 7 days with basal feeding and *ad libitum*. Rats were placed in a plastic tub cage measuring 17.5 x 23.75 x 17.5 cm. The bottom of the cage was given a rice husk base to absorb urine and feces, while the top was given a wire gauze cover. Rats were kept at room temperature (± 27ºC), with a relative humidity of around 60%.

Preparation of rats ovariectomy model. Female white rats (*Rattus norvegicus*), Wistar strain (20 rats), aged 10 weeks with an average initial weight of 203.5±9.76 g were adapted for 1 week in laboratory conditions. One week after adaptation, ovariectomy (removal of the ovaries) was performed on 16 rats with an incision in the left and right pelvic areas (Ström et al 2012; Kruger & Morel 2016). The rats were anesthetized using

ketamine at a dose of 10 mg kg^{-1} BW (100 mg mL^{-1}) and xylazine at a dose of 2 mg kg^{-1} BW (20 mg mL^{-1}). The intramuscular administration volume was 0.02 mL kg^{-1} BW for ketamine and 0.02 mL kg^{-1} BW for xylazine (Luengo-Mateos et al 2024).

After the rats were anesthetized, the flank hair was shaved and cleaned using alcohol. The skin was incised 1–1.5 cm long, followed by an incision of the subcutaneous tissue. The abdominal wall and fat tissue were retracted, so that the ovaries were removed from the abdominal cavity, and then suturing was done on the muscle with 3-0 cut gut chromic thread. While the skin was sutured with silk thread. After the muscle and skin were closed, iodine was given as an antiseptic. After the ovariectomy process was completed, the rats were adapted for 2 weeks for recovery.

Determination of dosage of R. mucronata fruit extracts*.* The dose of black mangrove fruit extract was determined by converting the dose commonly consumed by humans (200 mL) with a conversion factor for rats of 0.018. Administration of black mangrove extract was carried out 2 weeks after the ovariectomy wound had healed. Each rat was weighed to determine the volume of extract given. Rats weight was measured every week (in the 2nd to 4th week) using an analytical scale with an accuracy of 0.01 g. Administration of black mangrove extract was carried out for 28 days with doses of 200 (Ovx+D1), 400 (Ovx+D2), and 800 mg/kg BW/day (Ovx+D3), respectively. Oral administration of the extract was carried out once a day with an interval of 24 hours by the gavage method using a sonde. The extract was given in the afternoon as much as 0.5 mL/rat/day. At the end of the treatment, the rats were sacrificed by injecting ketamine intramuscularly into the back thigh to take blood and organ samples.

Analysis of estrogen levels and liver enzyme activity (SGPT, SGOT, and ALP*).* After anesthesia, rat blood was taken from the heart using a 1 mL syringe and transferred into a tube containing heparin to prevent clotting. The blood was collected in a tube and then centrifuged at 2000 rpm for 15 minutes to obtain serum. Serum was inserted into the Eppendorf tube, The estrogen hormone levels were analyzed using an Elisa Kit, followed by measuring SGOT, SGPT, and ALP. While the analysis of 17β-estradiol hormone levels used Elisa Kit (Bt Laboratory, Cat. No. E1393Ra) (Ningrum et al 2021).

Data analysis*.* Data on mouse weight, estrogen levels, and enzyme activity were analyzed using IBM SPSS Statistics 29.0.10 for Windows. ANOVA analysis or t-test was performed to determine the significance levels of observations in each group ($P<0.05$).

Results

Rats weight*.* Table 1 presents the weight of rats in the 2nd to 4th week after the post-ovariectomy recovery period. During the observation period, the rats were in healthy condition and could adapt to the surrounding environment, as shown by their ability to move actively.

Table 1 shows the average body weight of rats in each group varied in the range of 194.92-210.83 g. Significant increase in body weight of rats occurred in the negative control/NK group (210.83±2.37 g) and positive control/OVX (208.56±5.51 g). All treatment groups (with the administration of extracts) showed a decrease in body weight in the 4th week, where the highest weight loss occurred in the Ovx+D3 group. The average body weight of rats in each treatment group was 201.56±1.85 g (Ovx+D1), 200.08±3.62 g (Ovx+D2), and 194.92±9.66 g (Ovx+D3).

Table 1

Weight of rats in each group in the 2nd to 4th week

Groups	*Rats*	*Rats weight (g)*			*Average weight of rats per group (g)*
		2nd week	*3rd week*	*4th week*	
NK (negative control)	1	228	228	232	210.83±2.37
	2	198	198	208	
	3	186	186	186	
	4	225	225	230	
Ovx (positive control)	1	228	232	228	208.56±5.51
	2	191	202	222	
	3	183	192	199	
	4	201	209	216	
Ovx+D1	1	218	231	217	201.56±1.85
	2	202	201	195	
	3	180	188	182	
	4	200	207	198	
Ovx+D2	1	205	204	195	200.08±3.62
	2	219	217	202	
	3	198	196	184	
	4	205	198	178	
Ovx+D3	1	217	216	194	194.92±9.66
	2	222	219	211	
	3	164	170	178	
	4	152	202	194	

Estrogen levels. This was measured after 4 weeks of observation. The estrogen levels of rats in each group are presented in Figure 2.

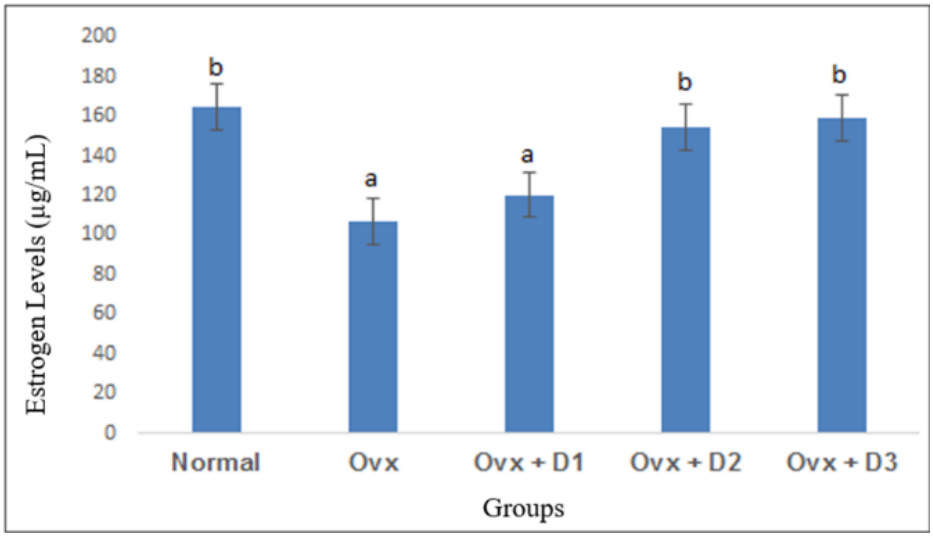

Figure 2. The estrogen levels of rats in each group.

Figure 2 shows that there was a significant decrease in estrogen levels in the positive control (Ovx) compared to Table 1 shows the average body weight of rats in each group varied in the range of 194.92-210.83 g. Significant increase in body weight of rats occurred in the negative control/NK group (210.83±2.37 g) and positive control/OVX (208.56±5.51 g). All treatment groups (with the administration of extracts) showed a decrease in body weight in the 4th week, where the highest weight loss occurred in the Ovx+D3 group. The average body weight of rats in each treatment group was 201.56±1.85 g (Ovx+D1), 200.08±3.62 g (Ovx+D2), and 194.92±9.66 g (Ovx+D3).

Liver enzyme activity. Analysis of enzyme activity (SGPT, SGOT, and ALP) was conducted to determine the effect of administering ethanol extract of *R. mucronata* fruit on liver function.

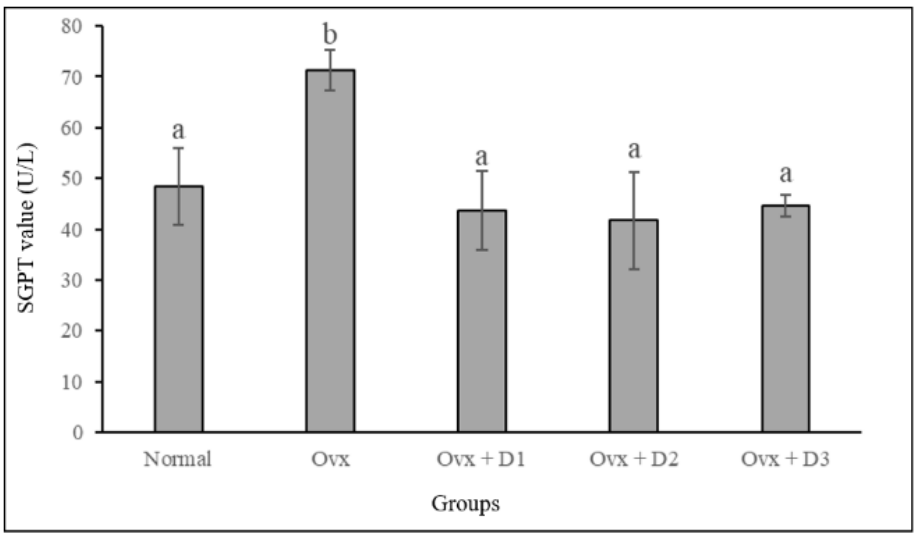

Figure 3. SGPT values for each treatment group.

Figure 3 shows the SGPT values of the extract dose treatment groups of 43.67±7.77 U L^{-1} (Ovx+D1), 41.67±9.50 U L^{-1} (Ovx+D2), and 44.67±2.08 U L^{-1} (Ovx+D3). The SGPT values in the three groups were not significantly different when compared to the SGPT values of the negative control group/normal rats (48.33±7.57 U L^{-1}). However, they were significantly different when compared to the positive control group/ ovariectomy rat model (71.33±4.04 U L^{-1}). This shows that the administration of ethanol extract of *R. mucronata* fruit (all doses) does not cause liver function disorder. Thus, based on the results of observations of SGPT values, it is believed that *R. mucronata* fruit extract is safe to be given for 4 weeks of therapy.

Analysis of liver function in the tested animals was also carried out by measuring for analysis of liver function. The results of the SGOT analysis in each group are presented in Figure 4.

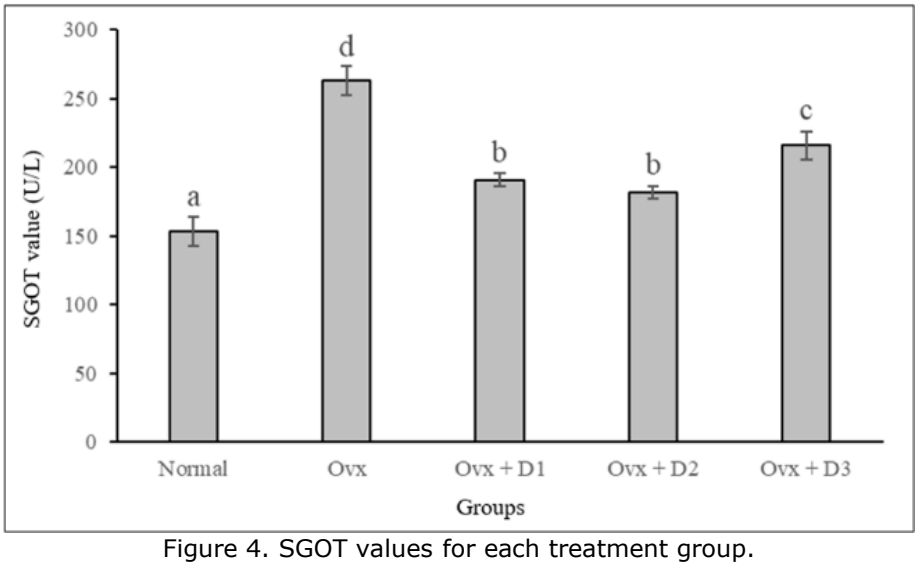

Figure 4. SGOT values for each treatment group.

Figure 4 shows an increase in SGOT enzyme activity in all treatment groups when compared with the negative control group/normal rats (153.00±10.58 U L^{-1}). The SGOT values of each treatment group were 190.67±5.03 U L^{-1} (Ovx+D1); 181.67±4.51 U L^{-1} (Ovx+D2) and 216±10.15 U L^{-1} (Ovx+D3). The highest increase in SGOT values occurred in the Ovx+D3 group which was significantly different from the other two dose groups. However, the increase in SGOT values in the Ovx+D3 group was lower and significantly different from the positive control group/ovariectomy rat model (263.33±10.60 U L^{-1}).

Liver function analysis was then continued with ALP enzyme activity to determine the health of the bile ducts in the animals being tested are presented in Figure 5.

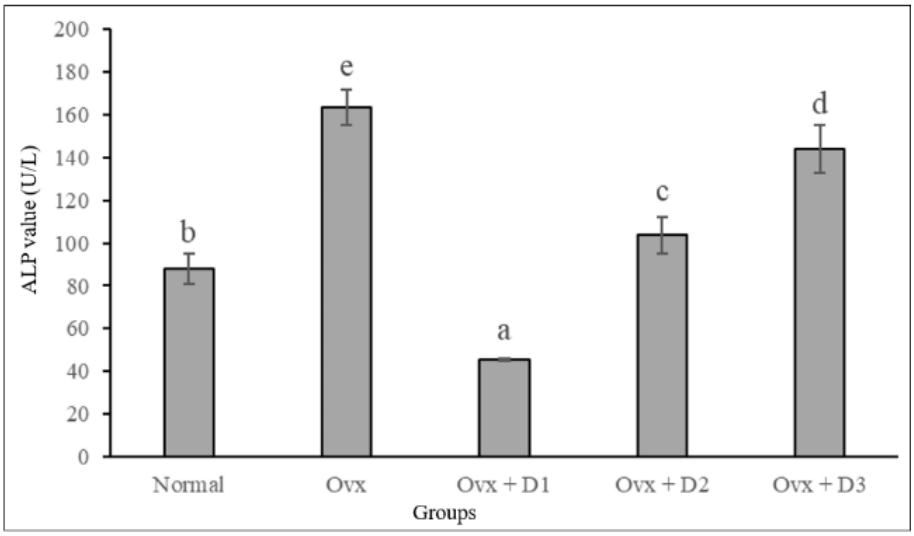

Figure 5. ALP values of each treatment group.

Figure 5 shows the ALP values that were significantly different in each group. The ALP value in the Ovx+D1 group (45.67±0.58 U L^{-1}) was lower than the negative control group/normal rats (88±7 U L^{-1}, Ovx+D2). Treatment of extract with higher doses (Ovx+D2 and Ovx+D3 groups) caused a significant increase in ALP values, exceeding the ALP values in the negative control group. The ALP values in the Ovx+D2 and Ovx+D3 groups were 103.67±8.39 and 144±11.36 U L^{-1}, respectively. Meanwhile, the highest ALP value based on the measurement results was in the positive control group/ovariectomy rat model, which was 163.33±8.33 U L^{-1}. ALP is sensitive to diseases related to the *biliary tract*.

Discussion. In this study, rats in the treatment group (all doses) and both control groups (positive and negative) had an average weight in the range of 194.92-208.56 g. Almost all groups showed an increase in body weight (except the Ovx+D3) compared to the initial weight (203.5±9.76 g). A decrease in mouse weight (not significant) only occurred in the Ovx+D3 group (dose 800 mg/kg BW/day) in the 4th week, although there was no decrease in activity and health condition. The increase in body weight of rats in the Ovx+D1 and Ovx+D2 groups showed that during 4 weeks of observation, the rats had a regular diet, normal activity, and were in healthy condition. This shows that therapy with ethanol extract of *R. mucronata* fruit is believed to be safe for the health of test animals.

Estrogen concentration in serum is measured by ELISA (Enzyme-Linked Immunosorbent Assay) method using a kit with Horse Radish Peroxidase (HRP) conjugate. The principle of this method is the interaction between antigen (in standard or sample) with specific antibodies that are passively adsorbed on the surface of the solid phase using antibody or antigen (enzyme) conjugates. The results showed that the positive control group (ovariectomy rat model) experienced a significant decrease in estrogen levels compared to the negative control group (normal rats). This indicates that post-ovariectomy, rats no longer have the main organs that produce steroid sex hormones, such

as estradiol, thus causing a hypoestrogenic condition (Medina-Contreras et al 2020). This condition also occurs in women who experience menopause or post-ovariectomy. In this condition, estrogen hormone production suddenly drops from 300-1000 pg/24 hours to 50-200 pg/24 hours (Esqueda et al 2007). Low estrogen levels during menopause cause symptoms such as hot flashes, pain during sexual intercourse, and decreased bone density.

This study shows that therapy with ethanol extract of *R. mucronata* fruit can increase estrogen levels in test animals. Significant increases in estrogen levels occurred in the 400 and 800 mg/kg BW/day treatment groups. Estrogen levels in both groups of test animals were equivalent to estrogen levels in normal rats. This shows that the phytoestrogen content in the ethanol extract of *R. mucronata* fruit can overcome the decrease in estrogen levels in test animals after ovariectomy.

Phytoestrogens work as estrogen agonists by filling estrogen receptor sites when estrogen is naturally no longer available in the body (Lecomte et al 2017). Estrogen is the main sex hormone in women that is synthesized in the ovaries with the help of follicle-stimulating hormone (FLH) and luteinizing hormone (LH). When women experience menopause, there will be changes in the structure and function of the ovaries. The number of ovarian follicles in the ovaries will decrease and affect the secretion of the hormone estrogen.

Generally, phytoestrogens have an important role in helping to restore the availability of estrogen in the body. There are two mechanisms of phytoestrogen action in the body, namely hormonal and non-hormonal. In terms of hormonal mechanisms, phytoestrogens that have a structure similar to estradiol will bind to ER (estrogen receptors) and produce estrogenic effects. While non-hormonally, the mechanism of action is not through binding to ER, but by producing several impacts such as antiproliferation activity, inhibition of tyrosine kinase, protein kinase C, DNA topoisomerase II, antioxidant activity, inhibition of angiogenesis, and inhibition of prostaglandin synthesis (Alexander 2014; Desmawati & Sulastri 2019; Sreepriya et al 2022; Cimmino et al 2023).

The side effects of ethanol extract therapy of *R. mucronata* fruit were studied based on analysis of liver function in each group. Disruption of liver function can be identified through examination of clinical parameters of *Serum Glutamate Pyruvate Transaminase* (SGPT)/*Alanine Transaminase* (ALT), *Serum Glutamate Oxalacetate Transaminase* (SGOT)/*Aspartate Transaminase* (AST), and *Alkaline Phosphatase* (ALP). SGOT and SGPT are related to liver cell parenchyma; the difference is that SGPT is mostly found in the liver, while SGOT is not only found in the liver but also the heart muscle, skeletal muscle, and red blood cells. Therefore, SGPT is a specific indicator of liver inflammation when compared to SGOT (Schneeberger et al 2012). ALP is generally found in the biliary tract. The biliary tract is related to the ducts and organs that lead to the secretion of the liver, gallbladder, and pancreas (Behar 2013; Keilson et al 2023).

The results showed that administration of *R. mucronata* fruit extract did not cause an increase in SGPT values. SGPT values in all treatment groups (all doses) were not significantly different from the negative control group (normal rats). An increase in SGPT values only occurred in the positive control group (ovariectomy rat model) which indicated inflammation or liver function disorder. SGPT is a liver enzyme indicating inflammation or cell death (Nebhinani et al 2019). Figure 4 shows an increase in SGOT values in each treatment group (all doses) compared to the negative control group (normal rats). The highest increase in SGOT values occurred in the treatment group with a dose of 800 mg/kg BW/day (216±10.15 U L^{-1}) but was still lower than the SGOT value in the positive control (ovariectomy rat model). Necrosis in the cell structure of the liver occurs when there is an increase in SGOT levels of more than 300 U L^{-1} (Aisah et al 2023).

The results showed a significant difference in ALP values in each group of test animals. The treatment group with a dose of 200 mg/kg BW/day (Ovx+D1) had a lower ALP value than the negative control (normal rats). Increasing the extract dose caused the ALP value to increase, but lower than the positive control group (ovariectomy rat model). This indicates that the plasma membrane in the liver organs of the rats in the treatment

group is healthier than the positive control group.

ALP is a marker enzyme for the plasma membrane and endoplasmic reticulum (Liu & Xu 2020). Increased ALP is most likely caused by membrane damage because ALP is an enzyme bound to the plasma membrane (Shu et al 2022). High activity of ALP serum is usually seen in liver damage, cancer, heart infection, obstructive jaundice, bone, kidney, leukocyte, placental, and intestinal diseases (Alvarez & Mukherjee 2011; Xanthopoulos et al 2019; Shu et al 2022). In addition, ALP is involved in bone growth and is excreted in bile. Its activity will increase if bile excretion is inhibited due to liver damage.

Based on this study, the phytoestrogen content in *R. mucronata* fruit has been proven to increase estrogen levels in ovariectomy rat models. The estrogenic effect began to appear at 400 mg/kg BW/day. Administration of *R. mucronata* fruit extract at a higher dose caused an increase in enzyme activity (SGOT and ALP) and a decrease in body weight after 4 weeks of observation.

Conclusion. This study supports the use of *R. mucronata* fruit extract as a safe estrogenic therapy to overcome the problem of estrogen deficiency. Ethanol extract of *R. mucronata* fruit at a dose of 400 mg/kg BW/day provides the best therapeutic effect because it significantly increases estrogen levels, but does not cause significant side effects on liver function. Further studies are needed to understand the mechanism of action of phytoestrogen compounds from *R. mucronata* fruit, as well as the effects of long-term use in humans before it is recommended for clinical use.

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Conflict of interest. The authors declare no conflict of interest in this research and publication.

Ethical approval. All animal welfare and research procedures were approved by the Ethics Committee of Brawijaya University, Malang, Indonesia (No. 955-KEP-UB).

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