ILMU KELAUTAN: Indonesian Journal of Marine Sciences September 2024 Vol 29(3):414-424 e-ISSN 2406-7598

Exploring the Anti-Menopausal Potential of *Rhizophora mucronata* Lam. Ethanol Extract: A Comprehensive Study on Estrogen Receptor β Agonist Activity

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Abstract

Mangrove is a tropical forest that stores millions of benefits ecologically, biologically, and economically. Rhizophora fruit extract contained bioactive compounds components derived from natural ingredients and scientifically proven to have positive effects on health, among others, to prevent cancer, etc. This study aimed to investigate the potential of R. mucronata ethanol extract as an estrogen receptor β agonist for anti-menopausal purposes. Using a Completely Randomized Design (CRD), 25 mice were divided into five groups: a normal control group (NK), an ovariectomy control group (Ovx), and three ovariectomized groups (Ovx D1, D2, and D3) receiving different doses of the extract (200, 400, and 800 mg.kg^{-1} BW, respectively). The extract was administered orally, and various measurements were taken, including flavonoid content, using densitometry thin layer chromatography (TLC) and FTIR for functional group characterization. The study found a high rutin content (13.29%) in the fruit. Twelve compounds with potential estrogenic activity were identified, which were analyzed using SwissAdme software. Estradiol levels in serum increased with higher doses of the extract over four weeks. In silico and in vivo analysis showed 5 (five) selected compounds from the ethanol and ethyl acetate fractions with highest (most negative) to lowest binding affinity as candidates for anti-menopausal drugs. The administration of R. mucronata Lam. fruit extract in Ovx D1, Ovx D2 and Ovx D3 gave significantly different effects to each other on rat blood serum estradiol hormone levels. In this study, the dose 400 mg.L^{-1} BW rat gave P>5 increasing blood serum estradiol levels of ovariectomized rats.

Keywords: *anti-menopausal, bioactive, estrogen, phytoestrogen, Rhizophora*

Introduction

Mangrove forest is a tropical forest that stores millions of benefits ecologically, biologically, and economically (Syahidah and Subekti, 2019; Muhammad *et al*., 2022). The Rhizophora group of mangroves consists of three types, namely *R. apiculata* Blume, *R. stylosa* Griff, and *R. mucronata* Lam. This plant is widely used for various purposes, such as processed food, and explored as marine, pharmaceutical product (Zaky Zamani *et al*., 2019; Sultana *et al*., 2021; Wijaya and Indraningrat, 2021).

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https://ejournal.undip.ac.id/index.php/ijms
DOI: 10.14710/ik.ijms.29.3.414-424
Received : 21-06-2024
Accepted : 13-08-2024

Mangroves contain bioactive compounds such as alkaloids, carbohydrates, glycosides, tannins, proteins, amino acids, flavonoids, saponins, sterols, acid compounds, resins, peroxides, polyuronoids, etc. (Handayani *et al.,* 2020; Powar and Gaikwad, 2020; Cadamuro *et al.*, 2021). Bioactive compounds are components derived from natural ingredients and scientifically proven to have positive effects on health, among others, to prevent cancer (Chakraborty *et al.*, 2010; George *et al.*, 2020; Parthiban *et al.*, 2022), as antioxidants (Miranti *et al.*, 2018), anti-inflammatory (Alizadeh *et al.,* 2018; Muhammad *et al.*, 2022; Parthiban *et al.*, 2022), antibacterial (Wijaya and Indraningrat, 2021), antidiabetic (Hardoko *et al.,* 2020), and antimicrobial (Linh *et al.*, 2020; Qurrohman *et al.*, 2021; Hechaichi *et al.*, 2023),

One of the bioactive compounds in plants is phytoestrogens (Syahidah and Subekti, 2019). Phytoestrogens can be found in legumes such as soybeans. Phytoestrogens are plant substrates with estrogen-like activity (Błaszczuk *et al.*, 2022). Several studies on the estrogenic effect of mangroves are still limited to several species, including mangrove species of Avicennia (Rozirwan *et al.,* 2022), mangrove diterpenoid compounds *Exoeceria agallocha* (Handayani *et al.,* 2020), analysis of phytoestrogen content in various fruits and vegetables in both fresh and processed form (Kartika *et al.,* 2021). Rhizophora root extract contained flavonoids, alkaloids, coumarins, and polyphenols (Satria *et al.*, 2019; Pambudi and Haryoto, 2022; Udoji *et al.*, 2022), while the Rhizophora fruit extract contained flavonoids, hydroquinone, and triterpenoid saponins (Syahidah and Subekti, 2019 George *et al.*, 2020; Cadamuro *et al.*, 2021; Hechaichi *et al.*, 2023).

Natural ingredients containing phytoestrogens have now been widely used as an alternative to estrogen hormone therapy which is usually carried out to treat estrogen deficiency in the human body (Kar *et al.*, 2014; Illian *et al.,* 2019; Satria *et al.*, 2019). Aging is a decline in body functions and organ systems that occurs in all living things. The aging process that occurs in men is called andropause, while in women, it is called menopause (Chakraborty *et al.*, 2010; Rozirwan *et al.*, 2022; Sofia *et al.,* 2022). Decreased ovarian function in producing estrogen can result in various health complaints and physiological function disorders, including irregular menstrual cycles. The decrease in blood estrogen levels after menopause also causes uterine atrophy (Astutik *et al.,* 2019; Qurrohman *et al.,* 2021).

The uterine glands are in a state not secreting. Thus the uterus shrinks, its weight decreases, vaginal dryness and pain during intercourse intimacy (copulation), and osteoporosis disorders (Moulinié, 2014). Therapies commonly used to treat these complaints include estrogen hormone replacement therapy (Moulinié, 2014; Özgenç *et al.*, 2017; Veloz Martínez *et al.*, 2022). Alternative substances is conducted, the essence can be planted containing phytoestrogen compounds (Chakraborty *et al.,* 2010; Sihombing *et al.*, 2013). A study focused on determining and proving phytoestrogen's ability in *R. mucronata* Lam. fruit related to its role in overcoming the problem of physiological disorders in menopausal conditions is urgently.

Materials and Methods

This research was experimental using a Completely Randomized Design (CRD). The model animals were divided into 5 groups, namely the normal control group (NK), namely normal mice without ovariectomy and not given *R. mucronata* extract, the ovariectomy control group (Ovx), namely ovariectomized mice without *R. mucronata* extract, group (Ovx D1) ovariectomized mice and given *R. mucronata* extract at a dose of 200 $mg.kgBW^{-1}day^{-1}$, group (Ovx D2) ovariectomized mice and given *R. mucronata* extract at a dose of 400 $mg.kgBW^{-1}day^{-1}$, group (Ovx D3) ovariectomized mice and given *R. mucronata* extract with a dose of 800 $mg.kgBW^{-1}day^{-1}$. The volume of extract in each treatment was calculated and administered orally using a probe. The variables observed in this study consisted of the independent variable (therapeutic dose of *R. mucronata* extract, in ovariectomized mice at doses of 200 $mg.kgBW^{-1}$, 400 $mg.kgBW^{-1}$, and 800 $mg.kgBW^{-1}$. Dependent variables (histological features of the uterus, liver, kidneys). and blood, as well as estrogen in the blood) and control variables (ovariectomy, sex, age, body weight and feed).

Ethical approval

Animal experiments approved by the Research Ethics Committee, Brawijaya University, Indonesia (Ref. No. 955-Kep-UB).

Sample preparation and extraction

Extraction of samples was carried out by ultrasonication method using ethanol as solvent. Ultrasonic waves are formed from the local generation of ultrasonics from microcavitation around the material to be extracted, resulting in heating of the material, which will ultimately release the extract compound. The extraction results were filtered using a vacuum filter to obtain filtrate and residue. Followed by sonication with solvent, the same treatment was done in triplicates. The three filtrates were combined and concentrated using a rotary vacuum evaporator. The extract was collected in a dark bottle and stored at 4^{o}C. Phytochemical screening of the ethanolic section of fruit flour was carried out qualitatively, including groups of alkaloids, flavonoids, saponins, terpenoids, and tannins.

Characterization using Densitometry Thin Layer Chromatography (TLC)

Characterization using densitometry TLC modified (Jork *et al.*, 1990). The stationary phase used was silica gel 60F254, and chloroform, ethyl acetate, and formic acid were used as the mobile phase in a specific ratio. Before being used for elution, the chromatography tank was saturated with the mobile phase. The spots formed were observed with a UV lamp at 200 nm and 500 nm, and the Rf was calculated.

Functional group characterization of the FTIR method

The fraction results on the spot TLC were then identified the functional groups using an infrared (IR) spectrophotometer. The results of the FTIR spectrum were analyzed by an infrared spectroscopy correlation table modified (Fairbrother *et al.*, 1991).

Identification of phytoestrogen compounds

The extracted simplicia was screened for phytochemicals, characterized, and then fractionated. The extract was further fractionated into ethanol and ethyl acetate fractions. The fractionated results were then analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) (Thermo Scientific Dionex Ultimate 3000 RSLC nano). This method is chosen for its ability to physically separate from High-Performance Liquid Chromatography (HPLC) with mass analysis capabilities (mass spectrometry), which have high sensitivity and selectivity. The type of analysis column was hypersil gold aQ 50 x 1 mm x 1.9 particle size. While the mobile phase used was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). A total of 10 µl samples were injected into the LC-HRMS apparatus and eluted for 70 min using a flow rate of 40 $\mu l.min^{-1}$ at a column temperature of 30^{o}C. Furthermore, the data were analyzed using Discover Compound Software with mzCloud MS/MS Library.

Determination of doses and administration of R. mucronata extract

Determination of the dose of modified white rats with a conversion of 200 mL and a conversion factor of 0.018 (Moulinié, 2014). *R. mucronata* extract was administered after the ovariectomy wound had healed (±2 weeks). Rats were weighed as a basis for determining the volume of the extract.

R. mucronata extract was administered for 28 days. Ovx D1 group; the dose of 200 $mg.l^{-1}$ body weight, Ovx D2 group; the dose of 400 $mg.l^{-1}$ body weight, and the Ovx D3 group; a dose of 800 $mg.l^{-1}$ body weight. The extract was administered once a day at 24-h intervals. An oral method with the gavage used a probe. They were starting every afternoon, as much as 0.5 $ml.d.head^{-1}$ for 28 d. At the end of the treatment, female rats were sacrificed by intramuscular injection of ketamine in the hamstrings for blood sampling.

Analysis of estrogen hormone levels

Once anesthetized, the rat's blood was taken from the heart used a 1 ml syringe. Transferred to a tube containing heparin that does not happen freezing. Blood was collected in a holding tube. Then the blood was centrifuged at 2000 rpm for 15 min. Serum was obtained, then put into Eppendorf (Santen *et al.*, 2020). Serum was used to analyze the hormone estrogen levels in the blood using the Elisa Kit.

Result and Discussion

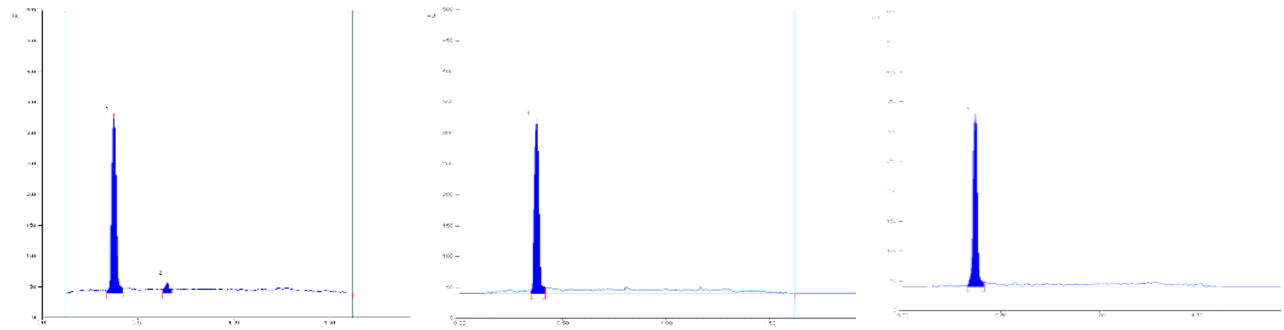
Flavonoid level

The chromatogram of *R. mucronata* Lam. fruit flour from 3 replications showed the presence of rutin compound. The chromatogram of rutin compound in *R. mucronata* Lam. fruit flour identified by TLC-densitometry is presented in Figure 1.

It can be seen in Figure 1 that the Rf (retention factor) values in 3 replications were 0.34-0.42, 0.35-0.42, and 0.33-0.42, in the Rf range of the chromatogram of rutin standard of 0.33-0.42 min.

Table 1. Rutin Compound in *R. mucronata* Lam. Fruit Flour

Category	I	II	III
Sample weight (g)	10.00	10.00	10.00
Spotting volume (µL)	3.00	3.00	3.00
Area (AUC)	3179.50	2874.90	3076.80
Rate (%)	13.67	12.81	13.38
Average (%)		13.29	



rf 0.34-0.42 (Replication I) rf 0.35-0.42 (Replication II) rf 0.33-0.42 (Replication III)

Figure 1. Chromatogram of routine compounds in *R. mucronata*Lam.fruit flour identified by TLC-Densitometry

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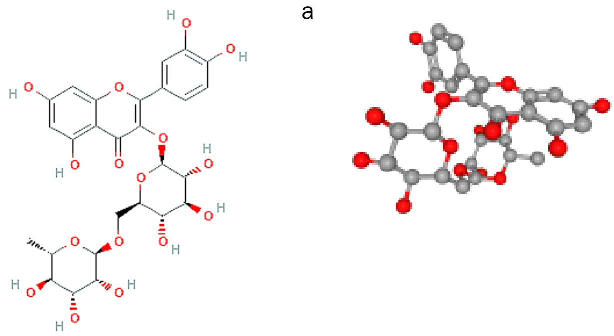

Figure 2. Molecular structure of Rutin compounds. a) 2-dimensional structure and b) 3-dimensional structure

The rutin compound in *R. mucronata* Lam. is relatively high at 13.29%. The rutin compound of *R. mucronata Lam.* fruit flour can be seen in Table 1, while the molecular structure of the rutin compound is presented in Figure 2.

The rutin compound has the chemical name Rutin (CAS 153-18-4) rutoside phytolin quercetin 3-rutinoside; belongs to the flavonoid group with a molecular weight of 610.5 g.mol^{-1} and the molecular formula C27H30O16, with canonical smiles CC1C(C(C(C(O1)OCC2C(C(C(C(O2)OC3=C(OC4=CC(=CC(=C4C3=O)O)O)C5=CC(=C(C=C5)O)O)O)O)O)O)O)O, PubChem CID 5280805. Rutin is an estrogen agonist and an estrogen antagonist.

Functional group characterization of FTIR

Figure 3 of the Infrared spectrum analysis shows the presence of bands in the absorption region. The X-axis is the frequency expressed in cm^{-1} units and indicates the presence of a functional group, while the Y-axis is % transmittance. Bands in the absorption region in the number region 3406, 2924, 2855, 1612, 1524, 1444, 1383, 1284, 1205, 1111, 1060, 862, 785, 637, 492 cm^{-1}. FT-IR is a high-resolution analytical technique to identify chemical elements and describe structural compounds (Toon *et al.,* 2018).

The absorption at a wavenumber of 3260 cm^{-1} is the OH absorption of phenol with hydrogen bonds (Adam *et al.*, 2013). The type of bond is shown in the absorption region of 1300-800 cm^{-1} (CC, CO, CN), 1900-1500 cm^{-1} (C═O, C═N, N═O), 2300-2000 cm^{-1} (C≡C, C≡N), and 3000-2200 (CH, OH, NH). The identification of functional groups through FTIR analysis can be seen in Table 2.

The isolated compound showed absorption at a wave number of 1612 cm^{-1}, indicating an absorption for the C=O carbonyl group, supported by a peak of 1111 cm^{-1}. The carbonyl compounds found are ester groups strengthened by firm peaks in the 1300 - 1000 cm^{-1} region. From the results of the FTIR analysis, the OH group (3260 cm^{-1}), the H group (3000 cm^{-1}), the C=O and C=C double bonds indicated the presence of a benzene ring, and the fingerprint area (600-1000 cm^{-1}). The main functional groups are around 4000-1500 cm^{-1}, while the absorption of the fingerprint region of molecules with very complex absorption is in the range of 1200-500 cm^{-1} (Fairbrother *et al.*, 1991; Toon *et al.*, 2018).

Identification of compounds using LC-HRMS

The results of the LC-HRMS chromatogram of the ethanol fraction of *R. mucronata* Lam. fruit flour is presented in Figure 4, while the Pa value of the compound is presented in Figure 5.

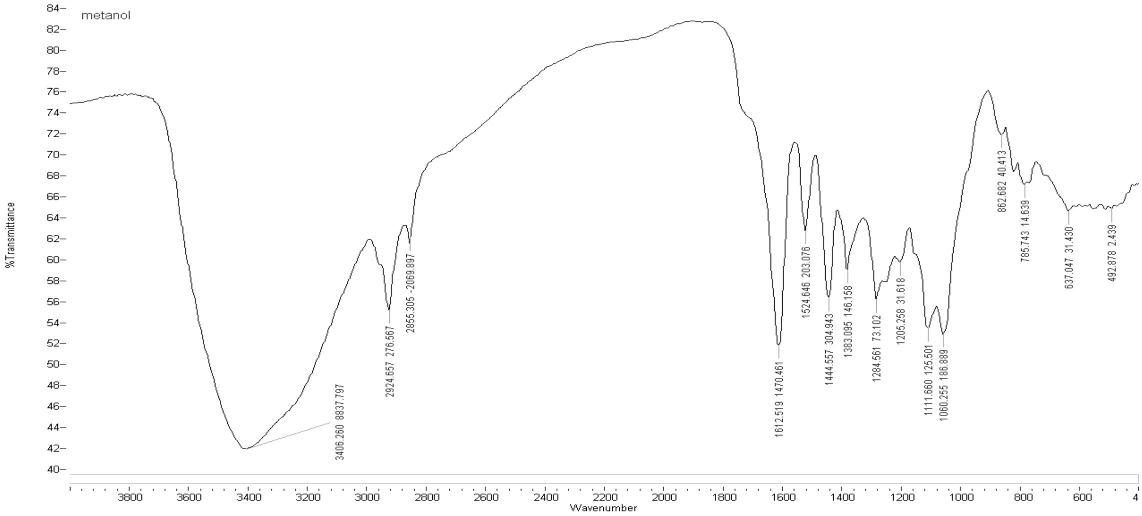

Figure 3. Infrared Spectrum of *R. mucronata*Lam. fruit flour

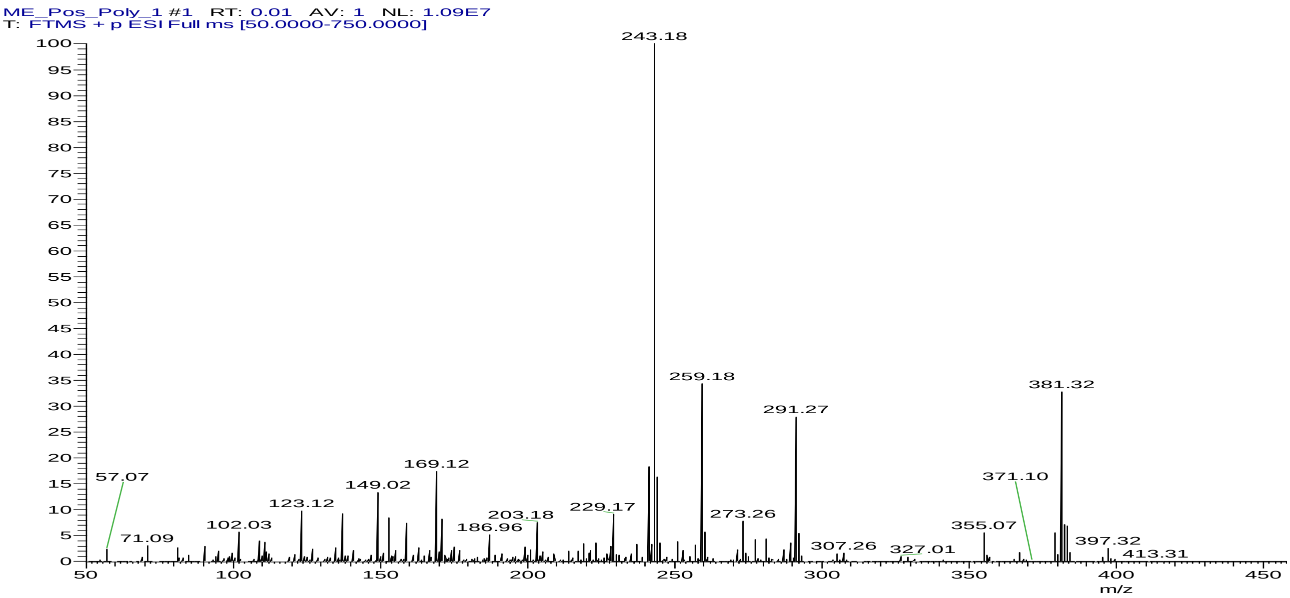

Figure 4. LC-HRMS chromatogram of the ethanol fraction of *R. mucronata* Lam. fruit flour

Table 2. Identification of Functional Groups through FTIR Analysis

Frequency (cm^{-1})	Bond	Functional Groups
3260 (strong, wide)	O-H dan N-H	Alcohols, Phenols, and Amines
1660(weak, narrow)	C=O	Alkenes and aromatic rings
1620 (weak, narrow)	C=C	Alkene
1520	C=C	Alkene
1440	C=C	Alkene
1175 (weak, narrow)	C-O	Esther and Ether
1125 (weak, narrow)	C-O	Alcohol, Esters, and Ether
780 (weak, narrow)	C-H, C-X	Substituted aromatics and organohalogens
745	C-H	Polyethylene and polypropylene

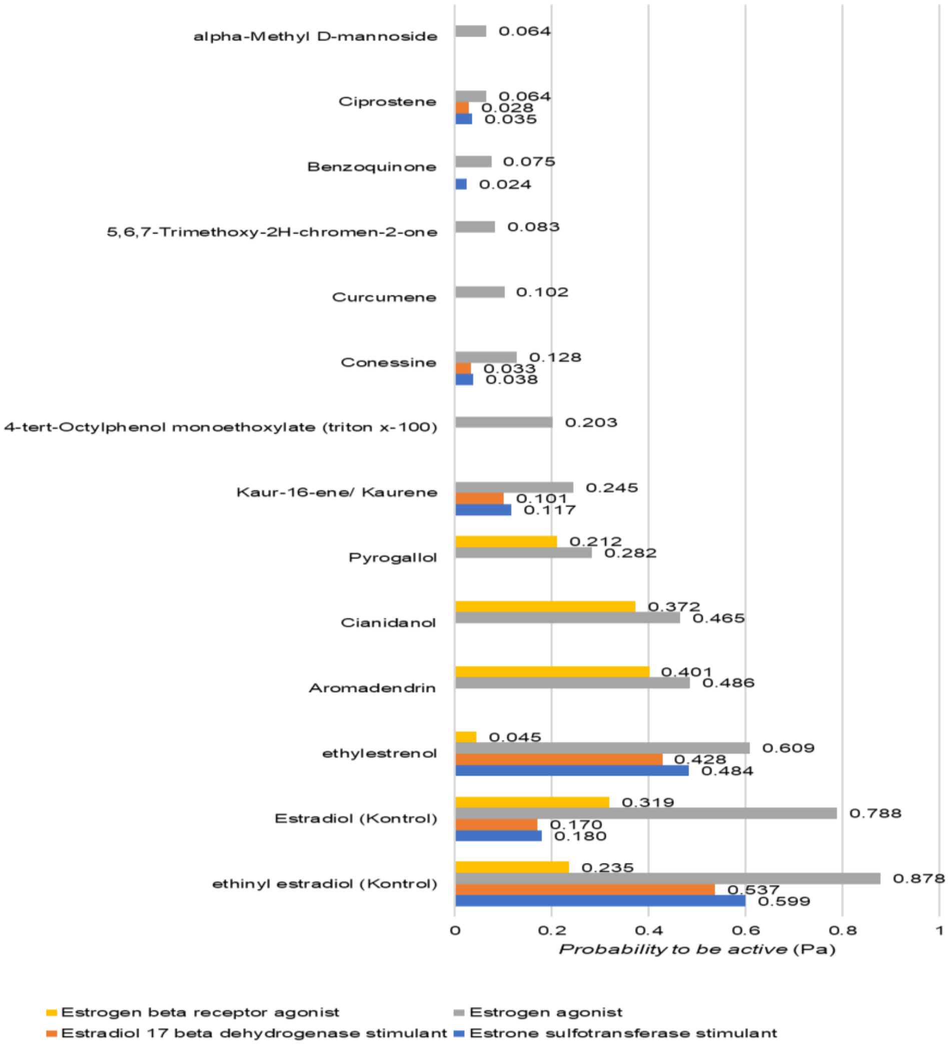

Figure 5. Pa Value of Ethanol Fraction Compound

The compound of *R. mucronata Lam.* fruit flour fractionated from ethanol and ethyl acetate was analyzed by LC-HRMS to analyze the probability of compounds having estrogenic potential. Identification of compounds was carried out by comparing them with a library search. The selected compounds have the highest SI (similarity index), ranging from 85-95%, the sample fractions, both the ethanol fraction and the ethyl acetate fraction.

Identification of ethyl acetate fraction compound

The results of the LC-HRMS chromatogram of the ethyl acetate fraction of *R. mucronata Lam.* fruit flour are presented in Figure 6, while the Pa value of the ethyl acetate fraction compound is presented in Figure 7.

Figure 7 shows that nine compounds have potential estrogen agonists. Of the nine compounds, three compounds with the highest potential of estrogen agonists were selected, namely pinoresinol, cyanidation, and zearalenone. The ethanol and ethyl acetate fractions of *R. mucronata Lam.* fruit flour were injected into a liquid chromatography column. The targeted compounds will be separated in the chromatographic column and recorded by mass spectrophotometry on the LC-HRMS equipment. The results of compounds identification of the two fractions were analyzed using in silico (Astutik *et al.*, 2019).

Rats weight

A weight measurement of rats was carried out after the post-operative recovery period. The size of the weight of rats during the treatment dose after ovariectomy can be seen in Table 6. The body weight (BW) of mice occurred in the negative control group (normal) and positive control group (Ovx), while the

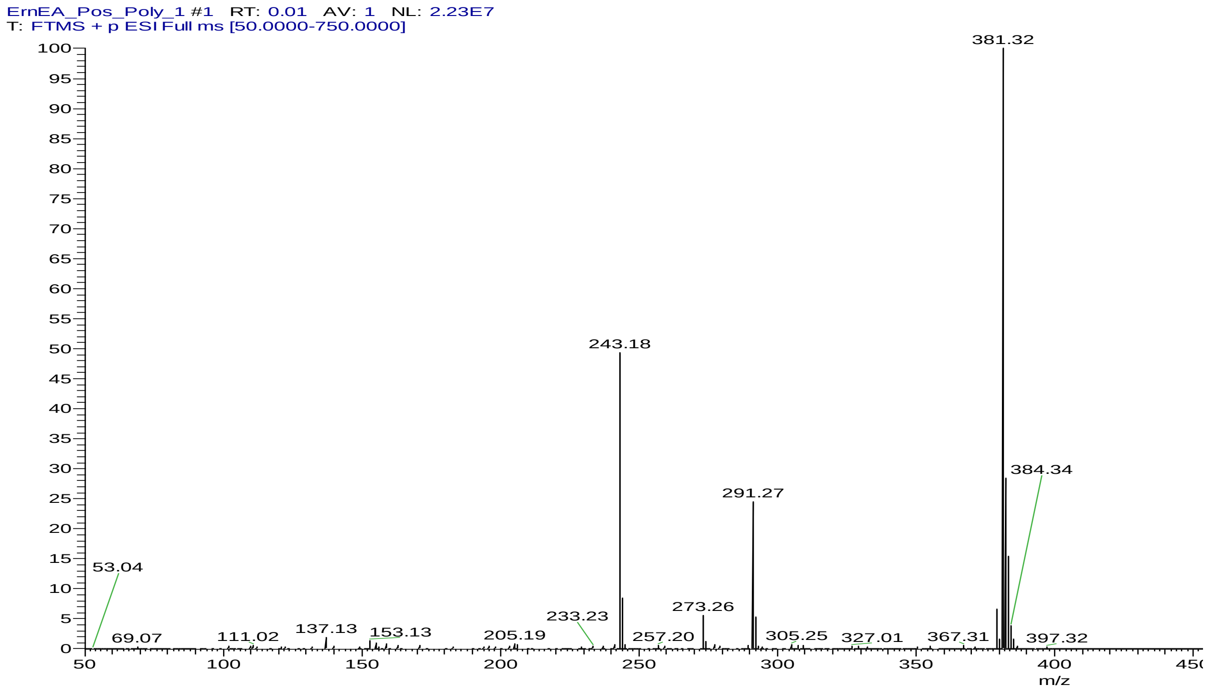

Figure 6. LC-HRMS chromatogram of the ethyl acetate fraction of *R. mucronata*Lam. fruit flour

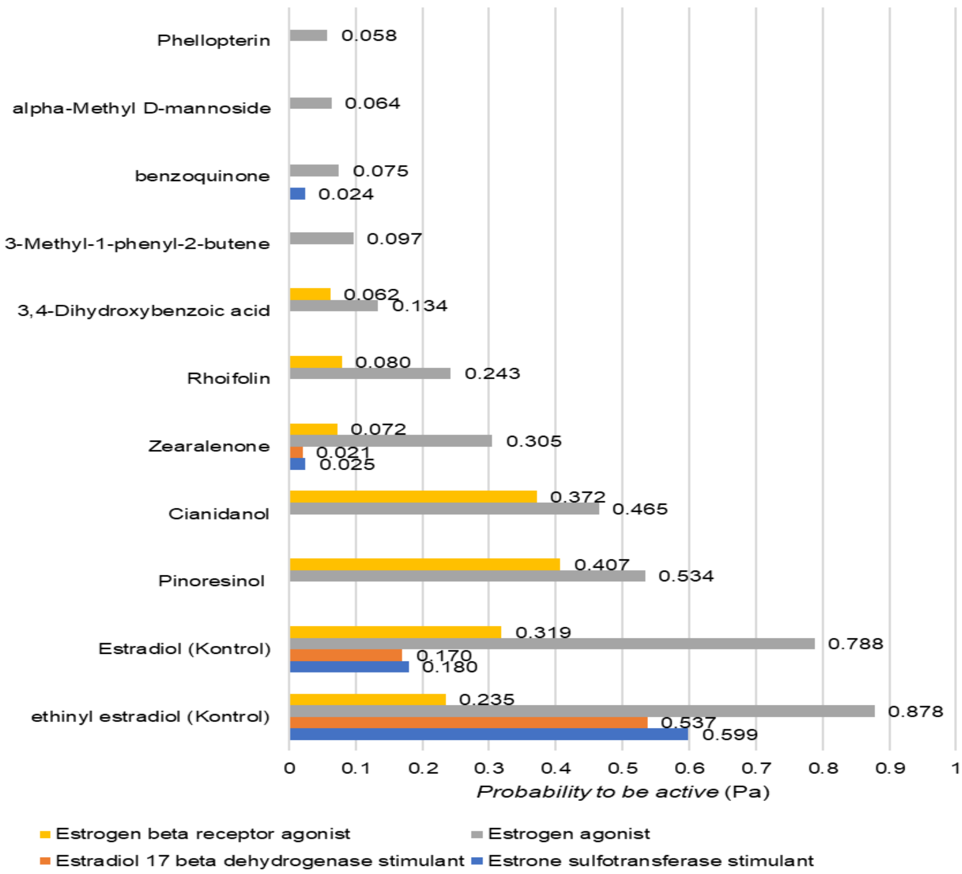

Figure 7. Pa Value of Ethyl Acetate Fraction Compound

Table 6. Size of Rats Weight During Treatment Doses After Ovariectomy

Kode	Rats Number	Weeks-2nd (g)	Weeks-3rd (g)	Weeks-4th (g)	Average
Normal	1	228	228	232	
	2	198	198	208	
	3	186	186	186	210.83 ± 2.37
	4	225	225	230	
Ovx	1	228	232	228	
	2	191	202	222	
	3	183	192	199	208.56 ± 5.51
	4	201	209	216	
Ovx +D1	1	218	231	217	
	2	202	201	195	
	3	180	188	182	201.56 ± 1.85
	4	200	207	198	
Ovx+D2	1	205	204	195	
	2	219	217	202	
	3	198	196	184	200.08 ± 3.62
	4	205	198	178	
Ovx+D3	1	217	216	194	
	2	222	219	211	
	3	164	170	178	194.92 ± 9.66
	4	152	202	194	

Noted: The average weight of rats in each group is obtained from the total average of the weight of rats in each observation.

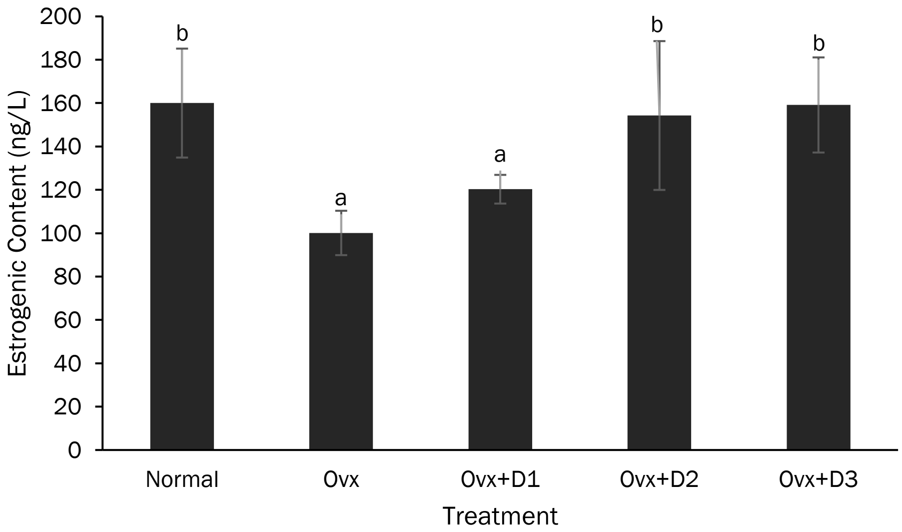

Figure 10. Graph of Estrogen Content (ng.l^{-1}) in Mouse Blood in All Test Groups

decrease in the 3rd week occurred for the group of mice with code Ovx+D1 (200 mg.l^{-1} BW crude ethanol extract of *R. mucronata* Lam. fruit), Ovx+ D2 (400 mg.l^{-1} BW crude ethanol extract of *R. mucronata* Lam. fruit), and Ovx+D3 (800 mg.l^{-1} BW crude ethanol extract of *R. mucronata* Lam. *fruit*).

The average body weight of rats varied in each normal treatment group, Ovx, ovx+D1, ovx+D2, ovx+D3 respectively 210.83 ± 2.37; 208.56 ± 5.51; 201.56 ± 1.85; 200.08 ± 3.62; 194.92 ± 9.66, and the rats were in normal condition, healthy and able to adapt to the surrounding environment as seen from the active rat activity.

Estrogen levels of rats

This study showed of the estrogen content in each test group was analyzed to determine whether the crude ethanol extract of *R. mucronata* Lam. mangrove fruit. have estrogenic abilities by knowing their levels by using Rat 17β-estradiol Elisa Kit (Bt-laboratory, Cat. No. E1393Ra). Estrogen Content Chart (ng.l^{-1}) in the blood of mouse in all groups test can be seen in Figure 10, while the results of statistical analysis of levels 17β estradiol.

Figure 10 showed that the estrogen content in each test group increased with increasing doses of the ethanol extract of *R. mucronata* Lam. mangrove fruit flour was given orally for fourth weeks. The highest estrogen content was found in the group Ovx+D3 with content of 159.15 ± 21.94 $ng.l^{-1}$. While in the group, Ovx+D2 and Ovx+D1 in the amount of 154.28 ± 34.32 $ng.l^{-1}$ and 120.29 ± 6.62 $ng.l^{-1}$. The mice undergoing ovarian removal in the group Ovx had a significantly. This condition represents menopausal condition in humans, the production of the hormone estrogen suddenly drops from 300-1000 $pg.h^{-1}$ to 50-200 $pg.h^{-1}$ (Sihombing *et al.,* 2013; Santen *et al.*, 2020).

Estrogen showed several advantages for the body and liver. In the liver, estrogen will be obstructed proliferation into cells (hepatic stellate cell) and fibrogenesis, protect mitochondrial structure and function, enhance the innate immune system (Adam *et al*., 2022), and elicit antioxidant effects (Kar *et al*., 2014; Hechaichi *et al.,* 2023). Phytoestrogens work as estrogen agonists by filling estrogen receptors when natural estrogen is no longer available in the body (Moulinié, 2014; Sultana *et al.,* 2021; Błaszczuk *et al*., 2022).

Conclusion

The administration of *R. mucronata Lam.* fruit flower extract in three dose variants gave significantly different effects to each other on rat blood serum estradiol hormone levels. The larger the dose given, the higher the serum estradiol level. In this study, the dose 400 $mg.l^{-1}$ BW rat gave the best effect on increasing blood serum estradiol levels of ovariectomized rats.

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