Exploring the Therapeutic Potential of Lumnitzera racemosa in an Accelerated Ovarian Failure model of Menopausal Syndrome

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KEYWORDS
Disregulation, Menopausal syndrome, Proinflammatory cytokines, Hormonal replacement therapy

ABSTRACT

Aim: This research aims to assess the therapeutic potential of *L. racemosa* in an Accelerated Ovarian Failure (AOF) rat model using 4-Vinyl cyclohexene diepoxide (VCD) to mimic menopausal syndrome.

Materials and Methods: Six groups of rats were used, with six rats in each group. All animals, except the control group, were administered VCD (160 mg/kg) intraperitoneally. Methanolic extract of *L. racemosa* leaves were given orally at doses of 100 mg/kg, 250 mg/kg, and 500 mg/kg and one group administered with standard estrogen 110µg/kg. Biochemical & Histopathological parameters were assessed at the end of the study.

Results: One-way ANOVA in Graph Pad Prism 10.3.0 was used to analyse results and presented as Mean ± SEM. LDL levels significantly decreased (***P<0.0001) in the 500 mg/kg treatment group compared to the AOF group. The AOF group showed a significant increase in proinflammatory cytokines, while the 500 mg/kg treatment group saw a significant decrease in IL-1β (***P<0.0001), IL-6 (****P<0.0001), and TNF (****P<0.0001). FSH (****P<0.0001) and LH (****P<0.0001) levels significantly increased, but progesterone levels did not (P<0.0001). By the end of the study, the 500 mg/kg treatment group showed a decline in progesterone and estrogen levels compared to all other groups. Serum phosphorus levels showed no significant change (****P<0.0001). The decline in calcium levels in the AOF group was rectified (P=0.7019) in the 250 mg/kg and 500 mg/kg treatment groups. Liver enzyme levels increased in the AOF group but decreased in ALP (***P<0.0001), ALT (***P<0.0001), and AST (P<0.0001) in the 250 mg/kg and 500 mg/kg treatment groups.

Conclusion:
L.racemosa is a mangrove plant which has wide range of secondary metabolites that are beneficial in reducing hormonal dysregulation. From all the findings we conclude that *L.racemosa* extract will be the good alternative for Hormonal replacement therapy. Receptor level studies are further required to establish the activity.

Article Highlights:

- Endocrine disruptive chemicals (EDC) have a direct impact on the female reproductive system, resulting in issues including accelerated ovarian aging and decreased follicle development, size, and activity. In this study we have used 4-VCD for the induction of menopause which is an EDC.
- *L.racemosa* extract reduced the hormonal imbalance, inflammatory cyckine levels, improved bone density.
- *L.racemosa* extract will be the best alternative for the present Hormonal replacement therapies.

Introduction

Menopause typically seen between the ages of 45 & 52, characterized by the cessation of menstruation [1]. Nearly 85% of postmenopausal women worldwide suffer from symptoms of menopause at a particular point in their lives. By 2060, 59.8% of women will have undergone menopause, an increase from the current percentage of nearly half in 2030 [2]. Among menopausal women, common symptoms reported include vasomotor, vulvo vaginal, and sexual dysfunction [3,4]. Hormone therapy (Estrogen) has traditionally been the first and foremost drug therapy for managing complications of menopause. However, due to health hazards & individual preferences, many women avoiding hormonal replacement therapy (HRT) [5]. As a result, there is a growing need to explore alternative approaches to alleviate menopausal complications. Extensive research has identified numerous phytochemical constituents present in mangroves, revealing their diverse pharmacological properties. Under highly challenging environmental stress conditions, these plants enhance the synthesis of secondary metabolites, which exhibit a wide range of therapeutic effects across various ailments. Previous studies explained valuable potential of these bioactive compounds in contributing to the medicinal properties of mangroves [6].

Qualitative investigations of ***Lumnitzera racemosa*** (*L. racemosa*) exhibited the occurrence of a rich array of bioactive substances in its methanolic & aqueous extracts. These compounds include secondary metabolites, sugars, tannins, glycosides [7,8]. Specifically, *L. racemosa* has been found to contain triterpenoids, such as friedeline, and phytosterols, including stigmasterol, campesterol, and β-sitosterol, as well as flavonoids like quercetin and myricetin, and Polyphenolic compounds like punicalagin [9].

In this present work induction of menopause is done by accelerated ovarian failure model(AOF) using 4-Vinylcyclohexene diepoxide (VCD) as a inducing agent with repeated daily dosing for 15 days [10]. AOF model serves as a naturally progressing menopause with intact ovaries [11]. This present research is for evaluation of the therapeutic potential of *L.racemosa* extract in an AOF model of menopause in rats.

2. Material and Methods:

2.1 Reagents

VCD was procured from Sigma Aldrich in India (Lot number 2022A102). The test kits used to measure lipid profile, liver function tests were purchased from Roche (Basel, Switzerland). Pfizer provided the standard oestrogen tablets (New York, NY, USA) (Lot number FF-716-30184). We procured Mayer's haematoxylin and eosin yellow stain from Thermo Scientific in Waltham, Massachusetts, USA. To analyze cytokines and reproductive hormones chemical were purchased from Millipore,USA.

2.2 Standard drug

Conjugated oestrogen (0.3mg/tablet) was used as standard drug, each animal was administered with 0.11mg/kg.

2.3 Plant material

Lumnitzera racemosa was collected from 16º-30’ to 17º-00’ N latitudes and 82º-14’ to 82º-23’ E longitudes of Koringa Magroove forest located in Kakinda, Andhra Pradesh, India. Leaves were washed thoroughly with water, shade dried and grounded into coarse powder.

2.4 Extract Preparation

Freshly collected, shade dried, coarsely powdered *L.racemosa* leaves were passed through sieve number 18 and was packed in a sturdy filter paper and extracted by using a Soxhlet apparatus by a continuous hot filtrate extraction process. This was then put in the middle chamber of the Soxhlet apparatus and extracted using solvents like distilled water, petroleum ether (60–80ºC), chloroform, ethyl acetate, and methanol [12]. The filtrate was dried in a desiccator, concentrated in a rotary vacuum evaporator, and the yield % was determined. Rotary Flash evaporator was used for concentration of the extracts obtained. The yield of the methanolic extract was 17.6% which is higher compared to other solvents. Methanolic extract so obtained was subjected for preliminary phytochemical analysis and used for animal study.

2.5 Experimental Animals

Sainath Agencies, Hyderabad, Telangana, India, provided wistar albino rats of both sexes weighing between 180 and 220 grams. These were kept in cages made of polypropylene and provided with distilled water and standard sterile pellet diet. To prevent animal anxiety, the animals were adjusted to the laboratory environment one week before starting of the study. Standard environmental conditions were maintained for all experimental animals, including 23º ± 2ºC temperature, 50 ± 5% humidity, and 12 hours of light and dark cycles. Every day, the husk in the cages was replaced with fresh husk to maintain hygienic conditions and optimal animal comfort (D’ Souza UJA 2003). All the experimental protocols were approved by Ethics committee of Vikas Institute

of Pharmaceutical Sciences on 15 March 2024 under the guidelines of CCSEA. The registration number is 2098/PO/RE/S/20/CPCSEA.

2.6 Study design

Acute toxicity testing was performed on the female albino mice in accordance with OECD guidelines 423. In this instance, Animals were divided into three in each group and administered with graduated dose of natural extracts (5–5000 mg/kg). Extracts were administered orally by adding 0.1% carboxymethyl cellulose. Over the course of the 14 days, some of the animals exhibited restlessness, alertness, fearfulness, and irritabilities, with specific attention provided during the first four hours [13].

All the selected rats were grouped into six in each group (n=6). Group I animals served as control group and were received dH_2o. Group II, III, IV, V and VI are given with 4-vinyl cyclohexene diepoxide (VCD) i.e 160mg/kg. Group II animals served as Accelerated Ovarian failure model induced menopause group which were administered with 4-vinyl cyclohexene diepoxide (VCD) i.e 160mg/kg, Group III served as standard which were administered with oestrogen (110µg/kg) by oral route of administration)., Group IV,V,VI served as test groups administered with *L.racemosa* MeOH extract 100 mg/kg, 250 mg/kg and 500 mg/kg respectively [14]. On Day 16 AOF induced animal were tested for Biochemical and clinical markers and this day served as the day 1 of experimental procedure [15]. The study design was depicted in Figure. 1. & Table 1.

Until the completion of the experiment, Weekly records were kept on body weight (BW), food and water consumption. Blood samples from the orbital plexus were withdrawn days 0,15, 24, 52, 73, and 129 [16] and analyzed for cytokine profile, reproductive hormone levels, calcium, and phosphorus levels.

Animals were euthanized by cervical dislocation for the histological examination because it severely damages the brainstem and results in instant unconsciousness and death. Diethyl ether (5%) was used to sedate experimental rats prior to dislocation.

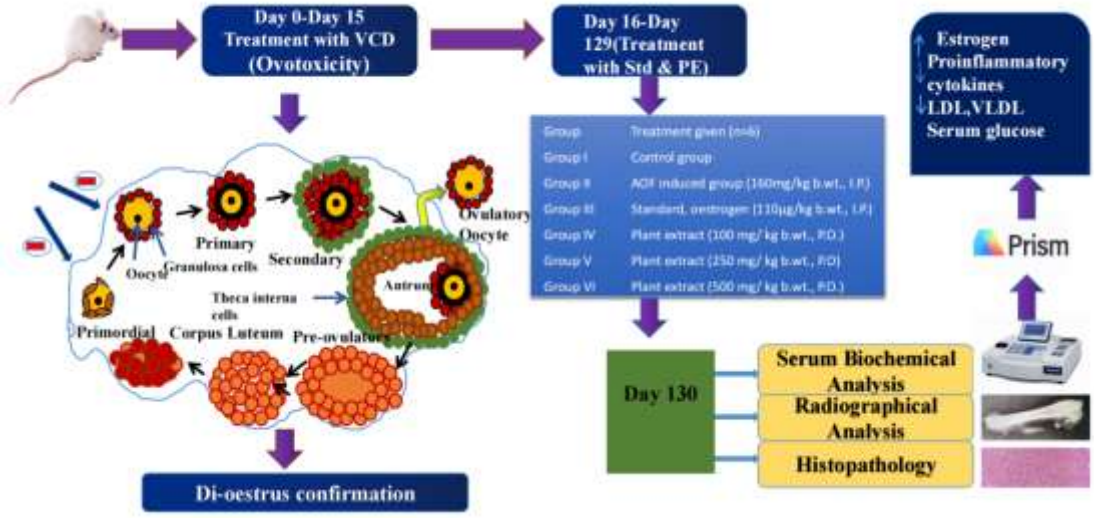

Figure 1: Study design

Table 1: Study design

Group	Treatment given (n=6)
Group I	Control group
Group II	AOF induced group (160mg/kg b.wt., I.P.)
Group III	Standard, oestrogen (110µg/kg b.wt., I.P.)
Group IV	Plant extract (100 mg/ kg b.wt., P.O.)
Group V	Plant extract (250 mg/ kg b.wt., P.O)
Group VI	Plant extract (500 mg/ kg b.wt., P.O.)

2.7 Biochemical Parameters

After collection of the blood from the retro orbital plexus, subjected for centrifugation at 2000 revolutions per min rpm to separate serum for five minutes. This serum was used for the analysis of serum glucose by GOD-

POD method, HDL and LDL by direct method, total cholesterol by CHOD-POD method, and triglycerides (TG) by glycerokinase peroxidase method, Proinflammatory cytokines (like Inter leukin-1β, Inter leukin-6, and Tumor necrosis factor-α) by Chemiluminescence immunoassay method, reproductive hormones by chemilumniscence immunoassay method), serum calcium and phosphorus are analysed by Arsenazo method. Parameters like Hemoglobin was analyzed by colorimetric method, RBC and Platelets were analysed by electrical impedance method,total WBC and Differential leukocyte count were analysed by Flowcytometry method. Liver functional indicators such as AST and ALT were analysed with UV assay whereas ALP with IFCC modified method and Bilirubin with Diazotized Sulfanilic Acid method.

2.8 Histopathological Parameters

After completion of the experimental procedures, animal's vital organs like liver, ovary were removed in accordance with the recommendations made by CPCSEA. Hematoxylin and eosin was used to stain a section of the organs that had been made from blocks fixed in paraffin. Various organs were examined using the accepted histopathological protocol [17].

2.9 Endometrial and Myometrial thickness

According to the OECD recommendations, the thickness of the endometrium and myometrium was measured under 40 x magnification under Magnus MX 21i binocular microscope. As directed by the Westwood Method, characteristics of the uterus lumen, lining of the uterus cavity, and uterine glands were noted [18].

2.10 Parameters of bone

During necropsy, the right femur bone was extracted from each rat. After the soft tissues were removed, the bones were weighed. The femur bone was preserved for one week in 10% buffered neutral formalin, followed by a dH2O wash and ten days in formic acid, formic acid was changed regularly until the bone softened. The bones were then bent or punctured with a sharp needle to check for decalcification. After cleaning with water and xylene, embedded longitudinally in paraffin wax after being dehydrated with graded alcohol. Three to five longitudinal serial sections with a thickness of 5 μm were cut, and stained with Mayer's hematoxylin and eosin [19]. Bone mineral density was assessed using X-Ray in three areas [20].

3. Results

3.1 Phytochemical constituents

Qualitative phytochemical analyses of *L racemosa* have revealed the presence triterpenoids like lupeol and betulin, phenolic acids like protocatechuic acid and gallic acid, and new glycosides, flavonoids, specifically myricetin and quercetin derivatives like myricitrin and Kaempferol have also been identified. Additionally, the plant contains sugars like acid-esterified saccharides, tannins such as 3-O-methylellagic acid-a hydrolysable tannin and Punicalagin, as well as glycopyrazone derivative, *L. racemosa* extracts have also been found to contain phytosterols like stigmasterols and β-sitosterol, lignans like Isoguaiacin, alhehydes like furfural, organochlorines coumarins, anthraquinones, macrolactone (racemolide), carotenoids like oxoisophorone and saponins in the leaves. Fatty acid derivatives were also identified in the stems and bark [21,22,23].

3.2 Acute toxicity studies

Group received high doses of the methanolic extract of L.racemosa exhibited some sort of symptoms and mortality compared to the groups administered with low doses of the extract.The results were dipicted in Table 2.

Table 2 The Acute toxicity studies results of methanolic extract

Experimental animal(n=3)	Dose	Mortality	Symptoms
Group 1	5 mg/kg	NIL	None
Group 2	50 mg/kg	NIL	None
Group 3	500 mg/kg	NIL	None
Group 4	1000 mg/kg	NIL	Restless,Jumping
Group 5	2000 mg/kg	NIL	No passage of stools,dyspnea
Group 6	5000 mg/kg	one	No passage of stools Difficulty in breathing,death

3.3 Regular observations (Body Weight, food and water intake)

Body weight was significantly decreased in VCD induced group and was increased with standard and test material treatment. There was no significant difference in the body weight of groups treated with standard estrogen and test. These observations were represented in Table 3 & Figure 2.

Table 3 Regular observations (Body Weight, food and water intake)

S.NO	Parameters	Control(n=6)	AOF(n=6)	AOF+Std(n=6)	AOF+100mg/kg Extract	AOF+250 mg/kg Extract	AOF+500mg/kg Extract
1	Body weight(g)	257.34±7.34	200.81±2.9	203.46±2.71	202.37±3.03	202.57±2.97	204.97±3.00
2	Food Intake(g)	16.52±1.16	16.63±1.02	18.94±0.91	18.21±1.01	19.84±0.67	19.57±1.04
3	Water Intake(mL)	18.42±0.64	18.94±0.42	21.26±0.89	21.36±0.70	21.84±0.70	22.36±0.64

Data is represented as Mean±SEM (n=6) Control,AOF treated,AOF with Estrogen treated,AOF with 100mg/Kg extract, AOF with 250mg/Kg extract,AOF with 500mg/Kg extract. For Body weight(g) ***P<0.0001,Food intake P=0.0666,Water intake ***P=0.0001. One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.1(509)

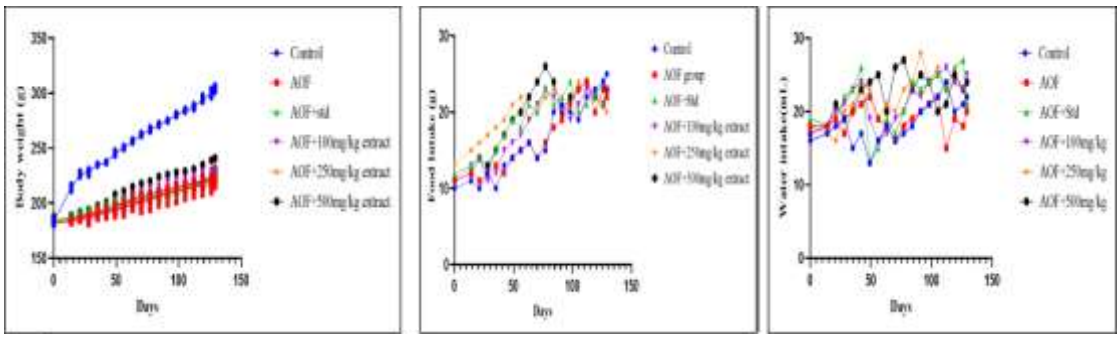

Fig 2: A. Change in the Body weight during treatment,monitored every week for 129 days, in all the groups there is a significant increase (***P<0.0001)in the Body weight was observed. B. Food intake in all the groups observed every weekly (P=0.0666). C. Water intake graphical representation n=6 (***P=0.0001). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.1(509)

3.4 Biochemical Parameters evaluation

Serum glucose, albumin, and total protein levels were within the normal range throughout the trial, despite considerable disparities between the groups. The LDL levels in the AOF group were significantly increased but returned to normal levels in the treatment group that received 500 mg/kg of the test substance, with a very strong statistical significance (P<0.0001). Significant increase in cytokines- Interleukin-1β, Intereukin-6, and TNF-α in the AOF group and were decreased to the normal level in group treated with 500 mg/kg of test. Group 6 received 500 mg/kg of test showed increase in the levels of progesterone and estrogen compared to all other groups. No significant change in the serum phosphorus levels. Decline in calcium levels in AOF group compared to normal and this decline was rectified in the groups treated with test 250mg/kg and 500mg/kg respectively. Liver enzymes levels were increased in AOF group and were declined in Group V and group VI received 250mg/kg and 500mg/kg respectively. No change were observed in ALP levels during study period in all groups. The treated groups did not exhibit any significant variations in haematological markers. The data was depicted in Table 4-8 & Figure 3-7.

Table 4: Biochemical Parameters

S.NO	Parameters	Control(n=6)	AOF(n=6)	AOF+Std(n=6)	AOF+100mg/kg Extract	AOF+250 mg/kg Extract	AOF+500mg/kg Extract

1	Serum glucose(mg/dL)	65.7±0.6	50.8±4.9	58.42±2.11	58.95±1.84	63.31±1.04	65.9±0.5
2	ALP(U/ L)	93.6±0.8 1	103.29±1 .25	85.82±0.63	101.92±1.35	88.51±1.11	85.26±0. 68
3	ALT(U/ L)	54.4±1.2 4	68.52±0. 52	57.4±0.39	68.39±0.59	63.12±0.66	61.04±0. 50
4	AST(U/ L)	167.47±3 .81	200±4.33	158.9±1.82	198.83±3.9	177.05±2.8	162.7±2. 09
5	Bilirubin (mg/dL)	0.17±0.0 6	0.10±0.0 2	0.03±0.006	0.08±0.02	0.06±0.02	0.05±0.0 1
6	Plasma Choleste rol(mg/d L)	74.28±0. 37	75.74±0. 97	74.21±1.11	75.63±0.90	73.48±1.10	72.95±0. 85
7	Plasma HDL(m g/dL)	62.25±0. 81	67.77±1. 21	62.56±1.68	62.5±1.68	61.22±1.63	64.86±0. 70
8	Plasma LDL(mg /dL)	52.8±0.5 3	64.01±0. 58	58.3±1.9	64.01±0.61	55.01±1.51	54.36±1. 41
9	Plasma VLDL	5.45±0.2 1	10.77±0. 14	10.41±0.42	13.42±2.90	9.62±0.35	9.07±0.3 1
10	Triglyce rides(mg /dL)	26.53±0. 42	40.06±0. 93	41.48±0.54	40.63±0.90	36.9±1.01	34.72±1. 83
11	Serum albumin(mg/dL	3.98±0.2 2	3.70±0.0 6	3.62±0.05	3.66±0.06	3.77±0.06	3.78±0.0 5
12	Total protein(mg/dL)	7.32±0.0 5	7.23±0.0 5	7.40±0.04	7.23±0.05	7.33±0.04	7.36±0.0 3

Table 4: Results were expressed in Mean±SEM (n=6) Control, AOF treated, AOF with Estrogen treated, AOF with 100mg/Kg extract, AOF with 250mg/Kg extract, AOF with 500mg/Kg extract. Serum glucose (***P<0.0006), ALP (***P<0.0001), ALT (***P<0.0001), AST (P<0.0001), Bilirubin (*0.0289), Plasma Cholesterol (P=0.192), Plasma HDL (*0.0167), Plasma LDL (***P<0.0001), Plasma VLDL (**P<0.0019), Triglycerides (***P<0.0001), Serum albumin (P=0.0753), Total Protein (**P=0.0021). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507)

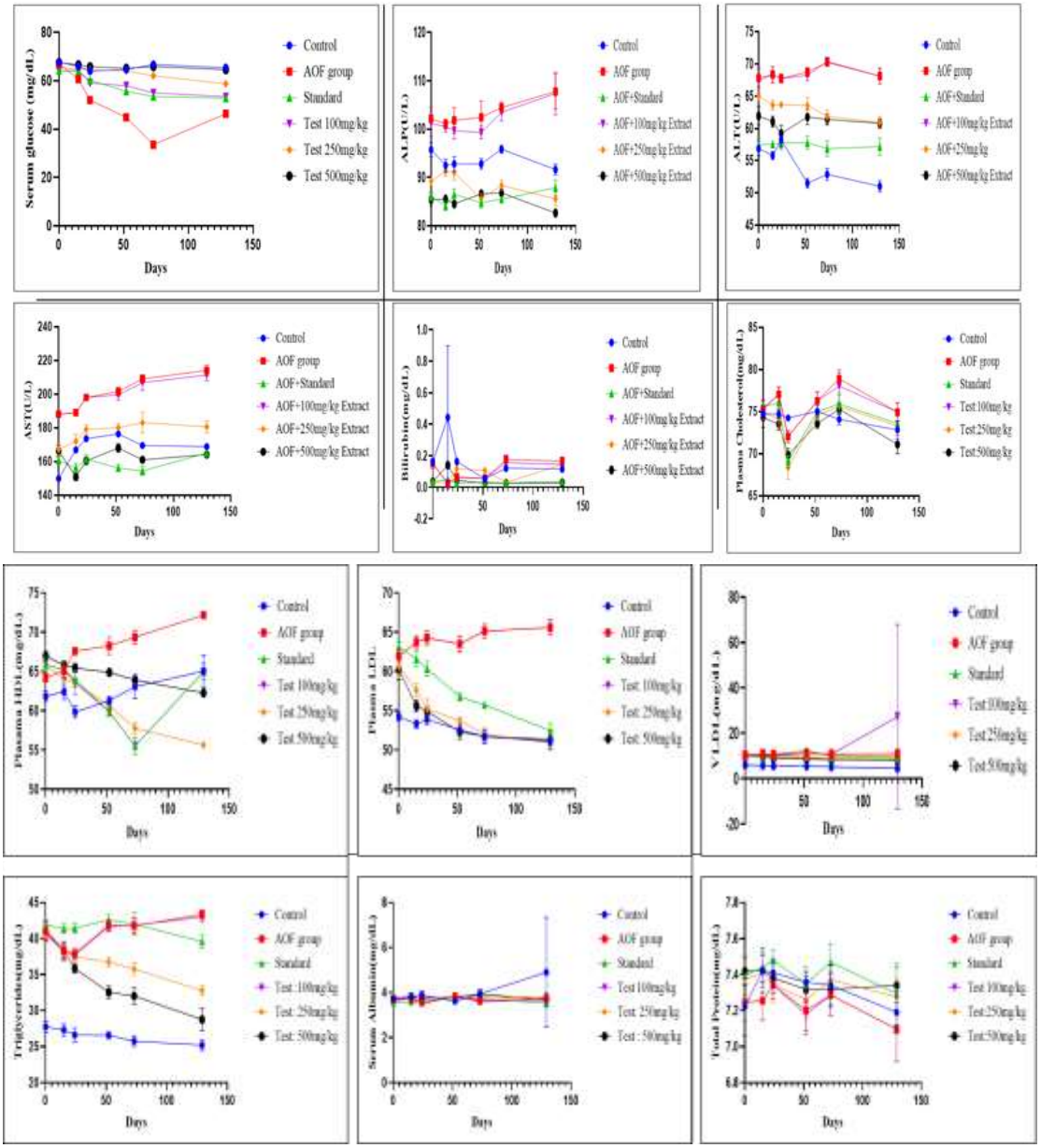

Fig 3: A. Change in the Serum glucose levels (***P<0.0001); B. Change in ALP levels (****P<0.0001); C. Change in ALT levels (****P<0.0001); D. Change in AST levels (****P<0.0001); E. Change in Bilirubin levels(*P=0.0289); F. Change in Plasma Cholesterol levels (P=0.1920); G. Change in Plasma HDL levels (*P=0.0167); H. Change in Plasma LDL levels (****P<0.0001); I. Change in Plasma VLDL levels(**P=0.0019); J. Change in Plasma Triglyceride levels(****P<0.0001). K. Change in Serum albumin levels(P=0.0753). L. Change in Total Protein levels (**P=0.0021) during treatment for 129 days in all the groups . One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507)

Table 5: Proinflammatory Cytokines

S.NO	Parameters	Control(n=6)	AOF(n=6)	AOF+Std(n=6)	AOF+100mg/kg Extract	AOF+250mg/kg Extract	AOF+500mg/kg Extract
1	IL-1β(pg/mL)	14.73±0.13	9.14±0.20	11.50±0.19	9.14±0.19	9.25±0.18	11.20±0.18
2	IL-6(pg/mL)	173.59±1.52	346.85±4.51	381.85±5.14	346.91±4.45	414.72±3.72	329.08±6.05
3	TNF(pg/mL)	1.79±0.09	3.31±0.12	3.74±0.16	3.61±0.12	3.68±0.07	2.30±0.20

Data is represented in Mean±SEM (n=6) Control, AOF treated, AOF with Estrogen treated, AOF with 100mg/Kg extract, AOF with 250mg/Kg extract, AOF with 500mg/Kg extract. IL-1β (***P<0.0001), IL-6(****P<0.0001), TNF (****P<0.0001). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507).

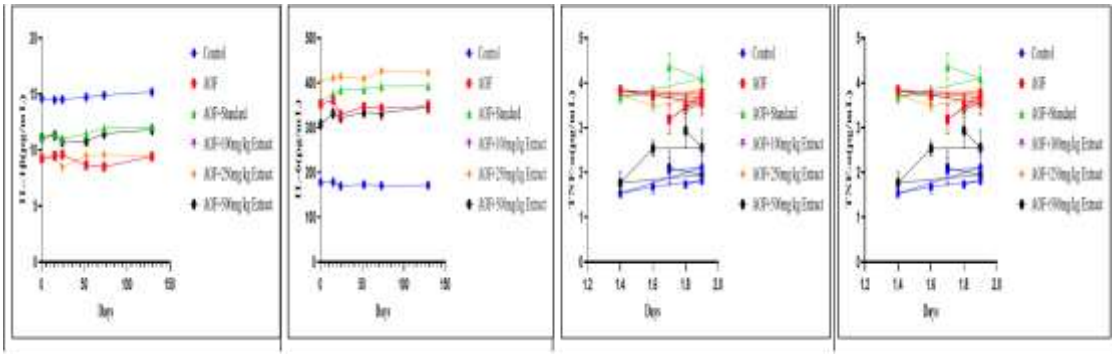

Fig 4: Proinflammatory Cytokine levels ; IL-1β-Interleukin 1β (****P<0.0001) , IL-6-Interleukin-6 (****P<0.0001), TNF-Tumor necrotic factor (****P<0.0001). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507)

Table 6: Reproductive Hormone Levels

S.NO	**Parameters**	**Control(n=6)**	**AOF(n=6)**	**AOF+Std(n=6)**	**AOF+100mg/kg Extract**	**AOF+250mg/kg Extract**	**AOF+500mg/kg Extract**
1	FSH(ng/mL)	2.43±0.10	3.87±0.07	3.05±0.15	3.87±0.07	3.08±0.12	2.73±0.08
2	LH(pg/mL)	6539.07±111.39	7306.23±52.97	6635.91±68.21	7306.23±52.92	7081.51±32.63	6922.65±39.98
3	Estradiol (pg/mL)	50.41±3.30	51.41±0.41	37.37±0.46	51.41±0.39	47.84±0.37	37.97±0.49
4	Progesterone(ng/mL)	0.40±0.04	5.08±0.31	7.53±0.34	5.08±0.31	8.19±0.41	9.48±0.26

Values are represented in Mean±SEM (n=6) Control,AOF treated,AOF with Estrogen treated,AOF with 100mg/Kg extract, AOF with 250mg/Kg extract,AOF with 500mg/Kg extract. FSH (****P<0.0001), LH (****P<0.0001), Estradiol (****P<0.0001), Progesterone (p<0.0001). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507)

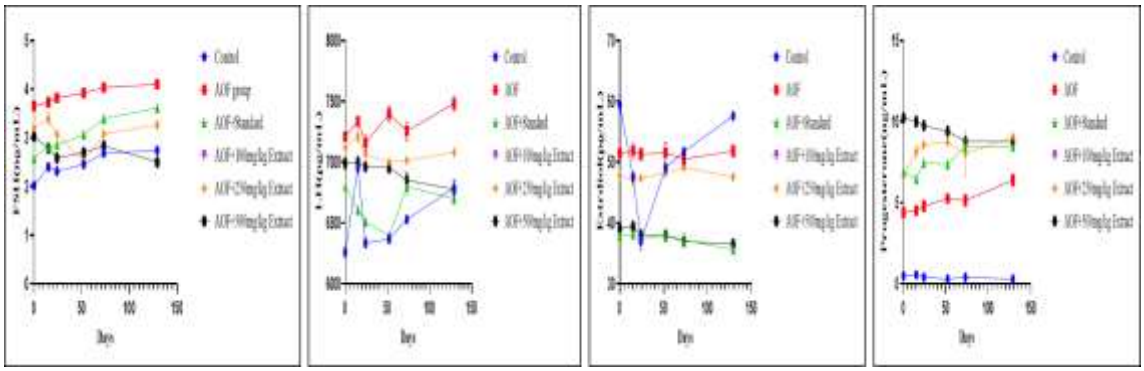

Fig 5: Reproductive Hormones FSH- Follicle stimulating hormone, LH-Leutinizing hormone, Estradiol and Progesterone.

Table 7: Serum Calcium and Phosphorus levels

S.NO	Parameters	Control(n=6)	AOF(n=6)	AOF+Std(n=6)	AOF+100mg/kg Extract	AOF+250 mg/kg Extract	AOF+500mg/kg Extract
1	Serum Calcium (mg/dL)	13.45±0.35	13.82±0.34	14.41±0.43	14.35±0.78	14.81±0.56	13.94±0.46
2	Serum Phosphorus(mg/dL)	7.35±0.19	8.34±0.22	8.52±0.11	8.27±0.13	8.0±0.15	8.43±0.13

Values are expressed in Mean±SEM (n=6) Control,AOF treated,AOF with Estrogen treated,AOF with 100mg/Kg extract, AOF with 250mg/Kg extract,AOF with 500mg/Kg extract. Serum calcium (P=0.7019), Serum Phosphorus (****P<0.0001). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.1(509)

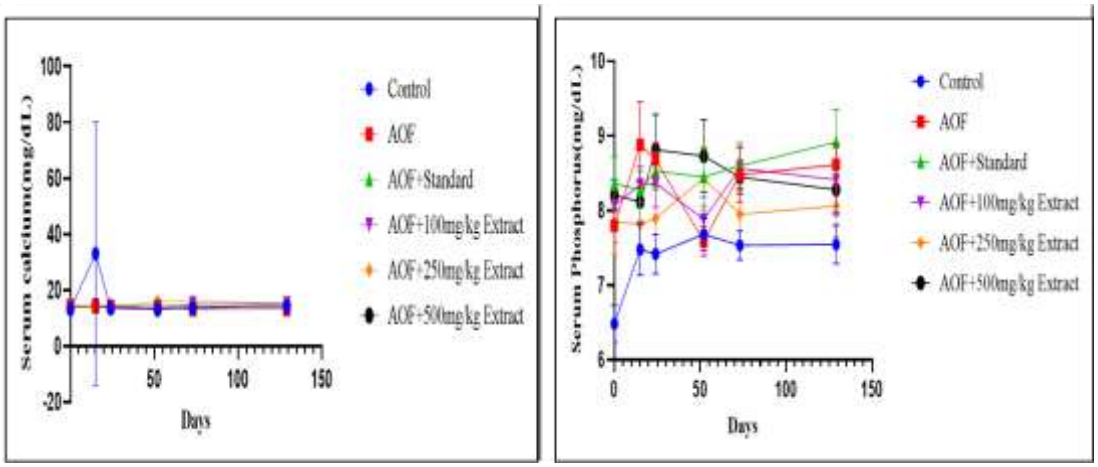

Fig 6: A. Serum calcium and B. Serum Phosphorus

Table 8: Endometrial and Myometrial thickness

1	Endometrial (µm)	99.28±1.32	49.06±0.70	61.68±1.05	51.71±1.28	81.06±0.74	51.98±1.00
2	Myometrial thickness(µm)	11.51±0.78	12.53±0.95	18.65±0.68	20.8±0.52	18±0.39	16.93±0.36

Values are expressed in Mean±SEM (n=6) Control,AOF treated,AOF with Estrogen treated,AOF with 100mg/Kg extract, AOF with 250mg/Kg extract,AOF with 500mg/Kg extract. Endometrial and Myometrial

thickness (P=0.9824). One way ANOVA method is used for analysis of the data using Graph pad Prism 10.3.0 (507).

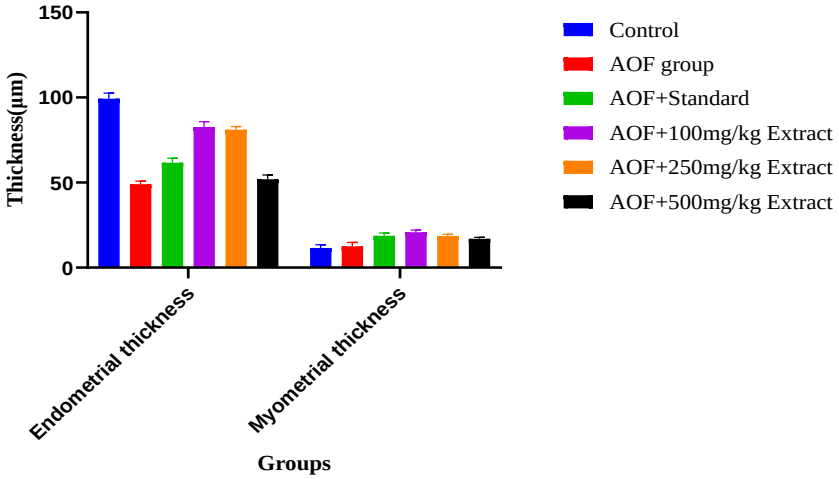

Fig 7: Endometrial and myometrial thickness in all groups

3.4 Histopathological Parameters

After careful removal of the vital organs (Liver and ovaries) and were observed for the change in weight and necropsy. There is no significant change was noted in all the study groups except ovary weight in the AOF group. Reduced weight of the ovary was seen in AOF group. Result depicted in Figure 8 & 9. Radigraphic analysis of Bone was depicted in Figure 10.

3.5 Uterotrophic bioassay

It was found that there was no significant changes in the uterus weight in AOF,standard drug treated and different concentrations of the test drug in comparison to the control group.
A decline in the endometrial and myometrial thickness was observed in AOF group compared to control and was almost similar in the standard and test drug treated groups.

3.6 Histopathology

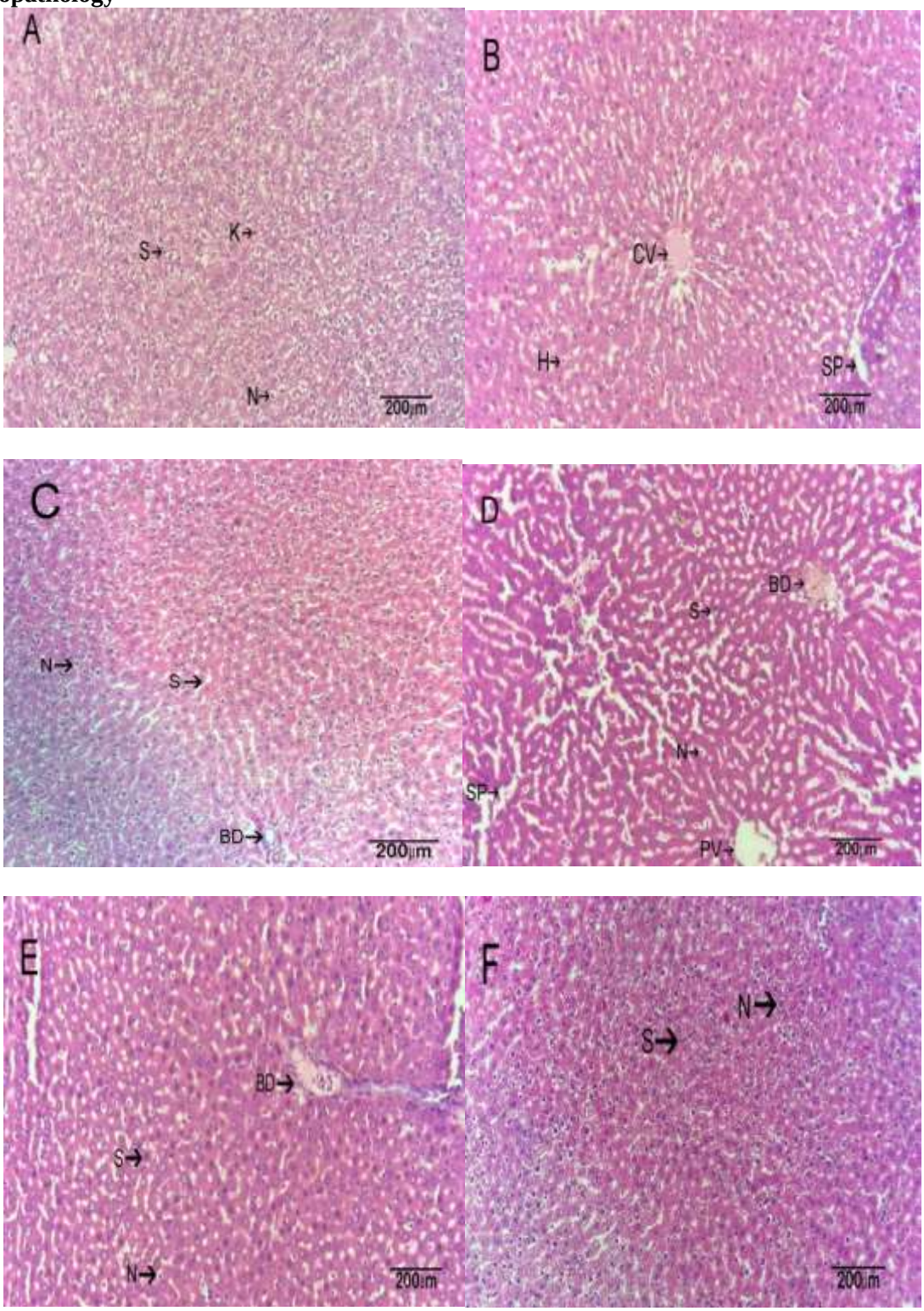

Fig 8: Histological representation of rat liver A. Control animal's liver showing nucleated hepatocytes (N), Sinusoids (S), Kupffer cells (K). B. Representing AOF group with partial changes with increased sinusoid spaces (SP). C. Standard drug treated group with minimal damage to hepatocytes. D. Animals treated with 100 mg/kg of test, dilated sinusoid spaces. E. Animals treated with 250 mg/kg of test with decreased sinusoid spaces. F. Animals treated with 500 mg/kg of test with healthy hepotocytes.

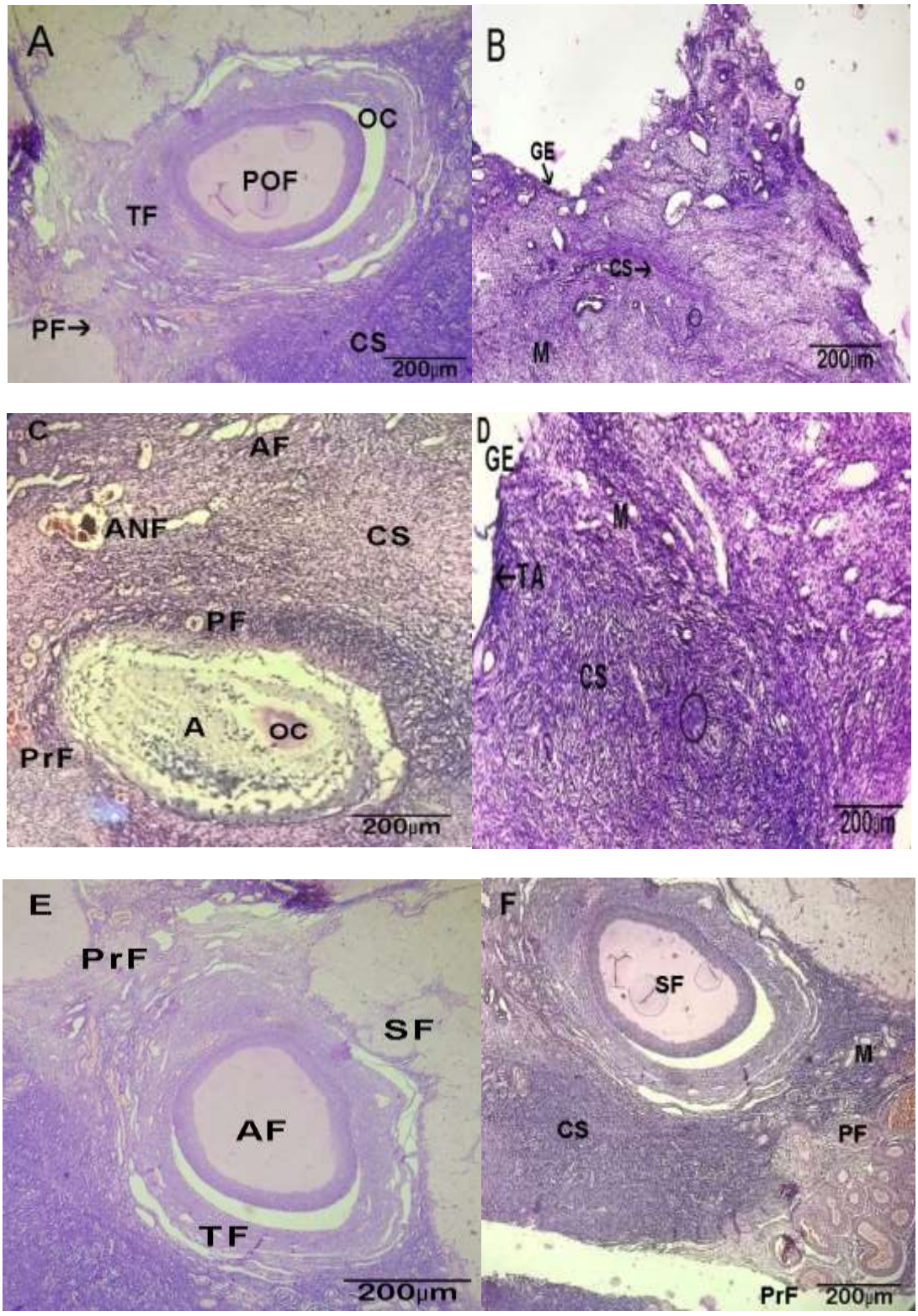

Fig 9: Histopathological representation of rats ovaries. A. Normal rat with matured follicles, POF-Preovulatory follicle, OC- Oocyte, TF- Theca follicle, PF- Primordial follicle, CS- Cortical stroma; B. AOF group with lack of Primordial and primary follicles, GE- Germinal epithelium, M-Medulla; C. Animals treated with standard estrogen showing follicles; ANF-Antral follicles, PF- Primordial follicles, A- Antrum, PrF- Primary follicle; D. Animal treated with 100 mg/kg of test showing no improvement in the ovary functioning; E. Animal treated with 250 mg/kg of the test showing well defined follicles with Primary follicles, secondary follicles; F. Animals treated with 500 mg/Kg test with SF-secondary follicle, PF-Primary follicle, M- medulla.

3.7 Radiographical analysis

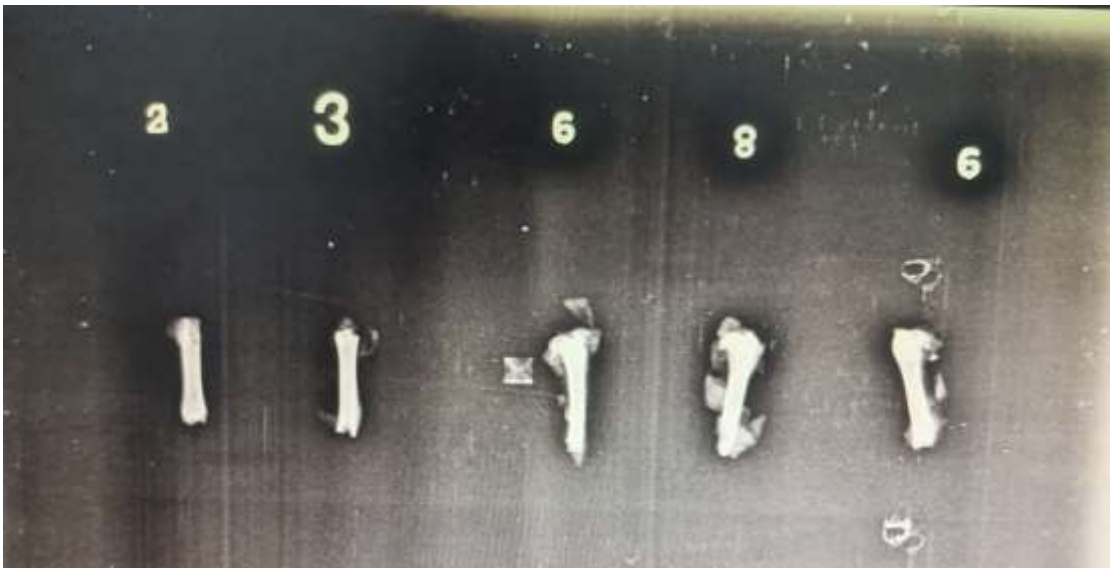

Fig 10: Radiographical analysis of Femur bone 2. Control, 3. AOF treated, 6. Standard estrogen treated, 8. Animal treated with 250 mg/kg test, 6(represented last). Animal treated with 500 mg/kg test.

Discussion

In recent decades, the prescribing practices for menopausal hormone therapy have evolved with growing awareness of its benefits and risks. Prior to the 2002 Women's Health Initiative findings, hormone therapy was commonly viewed as safe and effective for treating menopausal symptoms and preventing chronic illnesses like cardiovascular disease (CVD) [24,25,26]. The high risk of CVD and breast cancer has led to the preference for estrogen combinations over hormone therapy. WHI data showed women over 60 or more than a decade post-menopause starting HT had an increased risk of CHD. Due to health concerns, many women avoid HT, turning to complementary and alternative medicine (CAM) instead [27-29]. CAM treatments for menopause include natural products like herbs and supplements, and mind-body therapies like aromatherapy, massage, hypnosis, relaxation, acupuncture, reflexology, and meditation [30-32].

Flavonoids, such as Quercetine, reduce osteoporosis-related fracture risk in menopause [33]. Phytosterols offer chemoprotective, antioxidant, and anti-diabetic benefits, helping with conditions like atherosclerosis and cardiovascular disorders, including those associated with menopause [21,34,35]. Phenolic acids, like Protocathechuic acid found in L. racemosa extract, improve thermoregulation, mood, and sleep, benefiting menopausal symptoms [22].

In this study, 4-Vinyl cyclohexene diepoxide (VCD) was used at 160 mg/kg for fifteen days to induce accelerated ovarian failure (AOF) and menopause. Previous research indicates that VCD exposure in female rats and primates causes atretic degeneration of primordial and primary follicles [36,37]. Endocrine disruptors, like VCD, imbalance sex hormones by interfering with steroid-producing enzymes such as CYP17A1[38]. This enzyme is crucial for sex hormone synthesis, and its deficiencies can impair steroid production, leading to premature ovarian insufficiency (POI) and menopause [39,40,41].

Animal studies with VCD indicated changes in follicle count, estrous cycle, and hormone levels (FSH, LH, estrogen), pointing to POI and menopause. Traditional rodent models don't fully capture perimenopause. The new AOF model better mimics human perimenopause and postmenopause, differentiating hormonal effects from aging.

The results showed a highly significant difference in body weight ($P<0.0001$). Administering standard estrogen and increasing doses of L. racemosa extract restored body weight to normal levels compared to the control group. Food and water intake remained unchanged throughout the study. There was an evidence of elevated Total Cholesterol (TC) and Low density lipoprotein (LDL) values in obese menopausal women from previous research works [42]. AOF mice (VCD treated) had higher amounts of cholesterol and free fatty acids than regular cycling animals. The LDL levels were significantly reduced (***$P<0.0001$) in 500mg/kg treatment group compared to the AOF group. Previous research has shown that the onset of menopause is associated with low-level systemic inflammation, marked by increased levels of proinflammatory cytokines such as interleukin (IL-1, IL-6) and tumor necrosis factor α (TNF-α) in the serum [43,44]. A study conducted over a shorter duration has revealed an increase in both TNF-α and IL-6 [45]. In both natural and surgically induced menopause, women exhibit significantly higher serum levels of proinflammatory cytokines compared to fertile women. Similarly, in the AOF group, there was a significant increase in proinflammatory cytokines with AOF

and significant decrease of IL-1β (***P<0.0001), IL-6(****P<0.0001), TNF (****P<0.0001) with the 500 mg/kg treatment group.

In our study, groups treated with 500 mg/kg showed a significant reduction in inflammatory cytokines compared to the AOF group. While IL-6 decreased in the estrogen-treated groups, this reduction was not sustained throughout the investigation, consistent with previous findings [46,47]. VCD particularly targets ovarian follicles in its reproductive toxicity, and individuals exposure develop follicular atresia without even realizing it. This causes them to approach menopause early and not exhibit symptoms of interruption of the menstrual cycle. The structure and function of the ovaries may already altered even before occurrence of the symptoms of the menopause [48]. Significant increase in the FSH (****P<0.0001) and LH (****P<0.0001) levels but not progesterone levels (p<0.0001). At the end of the study in group 6 received 500mg/kg of test showed decline in the levels of progesterone and estrogen compared to all other groups. Bone architecture and quality, which are measured by bone mineral density (60–70%), together determine bone strength. Because of increased osteoclast activity, trabecular bone is the first to lose density in postmenopausal women, followed by the wrists and spine. In keeping with a prior study, BMD increased with oestrogen therapy, confirming amelioration of osteoporosis [49]. No significant change in the serum phosphorus levels (****P<0.0001). Percentage decrease in calcium levels in AOF group compared to normal and this decline was rectified (P=0.7019) in the groups treated with test 250mg/kg and 500mg/kg respectively. Liver enzymes levels were increased in AOF group and were decreased in ALP (***P<0.0001), ALT (***P<0.0001), AST (P<0.0001) Group V and group VI received 250mg/kg and 500mg/kg respectively. No significant change observed in ALP levels during study period in all groups. The treated groups did not exhibit any significant variations in haematological markers.

After careful removal of the vital organs (Liver and ovaries) were observed for the change in weight and necropsy. There is no significant change was noted in all the study groups except ovary weight in the AOF group. Decrease in the weight of the ovary was observed in AOF group.

Conclusion

L.racemosa is a mangrove plant which has wide range of secondary metabolites that are beneficial in reducing hormonal dysregulation. From all the findings we conclude that *L.racemosa* extract will be the good alternative for Hormonal replacement therapy. Receptor level studies are further required to establish the activity.

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CRediT autherhip :

Amala Masa: Design the study, and conducted experimental procedures along with manuscript writing. Dr.Samriti faujdar: Supervisor of the study facilitated the design, planning, experimental procedures of the study and manuscript writing. Dr.Saraswati Patel: Co-supervisor of the study and facilitated the design, experimental procedures of the study, checking of final manuscript. Dr.M.Suresh: Supported in Histopathology and Biochemical analysis. Dr.G.Sumalatha: Supported in manuscript writing, animal experimentation. Dr. Pratibha Sharma, Supported in Manuscript writing.

Declaration of Interest:

The authors declare that there is no conflict of competing interest.

Data availability:

All the data obtained during study was included in the published article.

Ethics and Consent to Participate: Not applicable

Consent to Publish: Not applicable

Appendix

List of Abbreviation

AOF	Accelerated Ovarian Failure
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
ALP	Alkaline phosphatase
BW	Body weight
CCSEA	Committee for control and supervision of Experiments on animals
CHOD-POD	Cholesterol oxidase- Peroxidase
CLIA	Chemilumniscence Immunoassay
dH_2O	Distilled water
FSH	Follicle Stimulating hormone
GOD-POD	Glucose Oxidase-Peroxidase coupled method
HDL	High-density lipoprotein
IFCC	The International Federation of Clinical Chemistry and Laboratory Medicine
IAEC	Institutional Animal ethical committee
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IP	Intra peritoneal
LDL	Low-density lipoprotein
LH	Leutinizing hormone
MRSA	Methicillin resistant staphylococcus aureus
MeOH	Methanolic extract
NIH	National Institutes of health
OECD	The organization for Economic Cooperation and Development
PO	Peroral
RBC	Red blood cells
SEM	Standard error mean
TNF-α	Tumor necotic factor-α
TC	Total cholesterol
TG	Triglyceride
TP	Total protein
UV	Ultra violet
VCD	4-Vinylcyclohexene diepoxide
VLDL	Very low density lipoproteins
WHI	Women's Health Initiative
WBC	White blood cells

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