Eremomastax speciosa and *Eremomastax polysperma* leaf fractions ameliorate the adverse effects of indomethacin in ovary and serum of treated rats

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Background and objective
Eremomastax speciosa and Eremomastax polysperma plants potentially contain bioactive principles against reproductive toxicants and oxidative stress. Thus, the ameliorative action of methanol and ethyl acetate fractions of *E. speciosa* and *E. polysperma* leaves on cyclooxygenase-2 (COX-2) and oxidative-stressed states in indomethacin-induced rat tissues have been performed.
Materials and methods
The dried-leaf extract of *E .speciosa* and *E. polysperma* was subjected to liquid–liquid fractionation to obtain the ethyl acetate and methanol fractions. The ethyl acetate and methanol fractions were respectively administered orally to rats (200 mg/kg), 30 min, and 10 h after subcutaneous injection with indomethacin (5 mg/kg) for 4 days. The postadministration of *E. speciosa* and *E. polysperma* fractions was used to determine their effect on ovarian and serum COX-2 concentration, ovarian malondialdehyde, and ovarian nitric oxide concentrations.
Results and conclusion
E. speciosa and *E. polysperma* fractions significantly ($P<0.05$) increased the concentration of COX-2 in ovaries and serum compared with the group treated with indomethacin only. Malondialdehyde and nitric oxide concentrations were significantly ($P<0.05$) decreased in all the animal groups posttreated with plant fractions compared with indomethacin only. Histological assessment of the ovary showed proliferating ovarian follicles and mature Graafian follicles in the groups treated with the plant fractions, while the indomethacin-only group showed scanty primary follicles.
These results showed that *E. speciosa* and *E. polysperma* leaf fractions mediated their protective effect on the ovaries and serum through the regulated COX-2 action and inhibited indomethacin-induced oxidative stress.

Keywords:
Eremomastax polysperma, *Eremomastax speciosa*, indomethacin, malondialdehyde, nitric oxide, ovary cyclooxygenase-2

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Introduction

Eremomastax speciosa (Hochst.) Cufod. and *Eremomastax polysperma* (Benth) Dandy are medicinal plants valued in West African traditional medicine by rural women in Nigeria and Cameroon. Their leaves are used to treat infertility in females of reproductive age [1]. Studies showed that *E. speciosa* and *E. polysperma* extracts increased fecundity in pubertal and prepubertal rats [2]. In another study, *E. speciosa* leaf extracts expressed reproductive hormone modulatory activities in females [3], and treated urinary-tract infections [4]. *E. speciosa* extract had expressed cytotoxic potential against DMBA-induced breast cancer in rats [5]. *E. speciosa* effectively ameliorated gastric ulcer, diarrhea, and nociception conditions [6,7]. Despite the success stories associated with the pharmacological activities of these medicinal plants, an effectual relationship between proper ovulatory function and *Eremomastax* species needs to be explored.

Ovulation is an indispensable regulated inflammatory process in the female reproductive cycle that precedes fertilization and implantation [8]. The mechanism of ovulation involves follicular development, oocyte maturation, and follicular rupture [9]. In some females, the ovulatory signal is usually accompanied by a painful discomfort on the lower side of the abdomen, a condition known as mittelschmerz [10]. Over-the-counter medications like NSAIDs are taken to relieve this pain by those women who are unable to

tolerate ovulatory discomfort. Indomethacin, a commonly used NSAID, effectively ameliorates pain, pyretic, and inflammatory conditions [11]. The side effect associated with indomethacin use is blockage of ovulation via inhibiting cyclooxygenase-2 (COX-2), an enzyme expressed in the ovarian granulosa and thecal cells [12]. COX-2 catalyzes the conversion of arachidonic acid to prostaglandin-2, a rate-limiting step in prostaglandin biosynthesis inhibited by indomethacin [13,14]. Indomethacin prevents prostaglandin-induced vasodilation in ovaries, consequently blocking ovulation in immature and adult rats [15,16]. Prostaglandins stimulate the production of proteolytic enzymes, these enzymes disrupt the follicular wall to release the matured egg during ovulation [17]. The report by Lim *et al.* [18] showed that ovulation was severely compromised in COX-2-deficient mice even in the presence of developed follicles. Indomethacin is therefore a reproductive toxicant due to the effect on the normal ovulatory process also generating free radicals.

Free radicals are generated following oxidative stress by reactive oxygen species (ROS) and reactive nitrogen species. Oxidative-stressed states in reproductive tissues impede normal reproductive processes that interfere with specific ovulatory processes [19,20]. Oxidative stress has been implicated in ovarian aging, reduction in ovarian function [21], and unsuccessful ovarian survival after ovarian transplantation [22]. Antioxidants play a protective role in the ovary by reducing the negative effect of generated oxidant species like malondialdehyde (MDA) and nitric oxide (NO). Increased MDA concentration is observed in ovarian dysfunctional conditions like polycystic ovarian syndrome and ovarian cancer [23,24]. NO is a free-radical compound that is expressed in the thecal cells of the ovary [25,26]. Unregulated NO leads to excessive concentrations, resulting in impaired ovarian function and eventual reduction in fertility [27–29]. Excessive tissue MDA and NO concentrations are markers for oxidative stress. Thus, antioxidants reduce excessive concentrations of MDA and NO in tissues. Antioxidants mediate their effect by maintaining a balance between ROS generation and clearance within the tissues [30].

Natural products sourced from medicinal plants are innovative alternative remedies to conventional drug-therapeutic systems having compounds hat regulate reproductive functions [31,32]. The pharmaceutical therapy mediated by *E. speciosa* and *E. polysperma* leaf fractions on ovarian function is not available. Therefore, this study was carried out to investigate the effect of methanol and ethyl acetate fractions of *E. speciosa* and *E. polysperma* leaf on COX-2 in indomethacin-treated ovaries and serum and in indomethacin-induced ovarian oxidative-stressed states.

Materials and methods

Reagents and chemicals

Indomethacin (Shanxi Guangsheng Pharmaceutical, Jinszhong, China, Registration No: 047650, batch No: 170302). Rat COX-2 ELISA KIT Cat #E0296Ra was provided by Bioassay Technology Laboratory, China. The following reagents were provided by Sigma-Aldrich: thiobarbituric acid (TBA), trichloroacetic acid 20%, EDTA powder, phosphate-buffered saline, sodium chloride, adrenaline powder, hydrogen peroxide, sodium nitrite, tris-HCL buffer, anhydrous sodium bicarbonate, potassium chloride, Griess reagent, xylazine, absolute ethanol, acetic acid (30%), formalin, Triton-X, potassium dichromate, glacial acetic acid, Ellman's reagent, and chromium potassium-sulfate powder.

Extract preparation and fractionation

E. speciosa (Herb/Bot/UCC/363) and *E. polysperma* (Herb/Bot/UCC/362) leaves were obtained from a farm at Akai Effa in Calabar municipality, Nigeria, and authenticated in the herbarium unit of the Department of Botany, Faculty of Sciences in the University of Calabar, Nigeria. The fresh leaves were washed and air-dried at room temperature. The dried leaves were blended into a fine powder using an electrical blender. 560.0 g of blended *E. speciosa* and 400.10 g of blended *E. polysperma* were extracted in 98% ethanol for 72 h at room temperature. The crude ethanol extract was filtered and concentrated in a vacuum at a low temperature of 37–40°C using a rotary evaporator. The concentrates yielded 53.2 and 46.0 g, respectively. The concentrated crude extracts were subjected to liquid–liquid fractionation using a separation funnel with solvents based on their increasing polarity [33]. The methanol and ethyl acetate fractions, each obtained from *E. speciosa* and *E. polysperma* plant extracts, were concentrated and administered to female rats at a dose of 200 mg/kg. The dose of 200 mg/kg was obtained based on information gathered from research on the crude extracts and acute-toxicity studies showing LD50 greater than 2000 mg/kg [34,35].

Animal protocol

The animal study was conducted in accordance with International Guidelines on the Care and Use of Animals and approved by the Animal Research Committee of the International Center for Chemical and Biological Sciences (ICCBS), Karachi, Pakistan (2017–0061).

Animals

Thirty-six female Wistar rats of normal breeding (10-week-old) weighing between 110 and 120 g were used in this study. The rats were housed under uniform husbandry conditions of light (12-h cycle) with water and the rat was fed *ad libitum*.

Vaginal smears were taken daily from the rats for 5 days and examined on a microscopic slide to determine the phase of the estrus cycle [36]. Animals in the proestrus phase of their cyclicity were replicated into six groups of six animals each. These animals were assigned as follows: control, indomethacin, ethyl acetate, and methanol fractions of *E. speciosa* and *E. polysperma*, respectively.

Experimental design

Indomethacin (50 mg) was dissolved in 300 µl of acetone and diluted with 5 ml of normal saline. Treatment of rats with the leaf fractions after a daily dose of indomethacin commenced at estrus. At proestrus, the preovulatory LH surge occurs with a concomitant increase in COX-2. The LH peaks before 8 a.m. the following morning, the estrus day of their cycle [37]. Indomethacin (12 mg/kg) was administered subcutaneously at 8 a.m. daily for 4 days (for the duration of the rat cycle). The ethyl acetate and methanol fractions of *E. speciosa* and *E. polysperma* leaves (200 mg/kg body weight) were respectively administered orally, twice daily. The first dose was administered 30 min after indomethacin injection (12 mg/kg) and the second dose after 10 h for 4 days [38,39].

On day 5, all the rats were anesthetized with ketamine 70 mg/kg and xylazine 7 mg/kg cocktail by intraperitoneal route. Blood was collected through a cardiac puncture, allowed to clot, and centrifuged at 3000 rpm/min for 15 min to obtain the serum for COX-2 assay. The ovaries were harvested and homogenized in normal saline (25 mg/ml) using a manual homogenizer, thereafter centrifuged at 10 000 rpm/min for 20 min. The supernatant was transferred into Eppendorf tubes and preserved at 4°C and later used for COX-2, MDA, and NO assays. Three ovarian samples collected from each group for histopathological assay were fixed in 10% formalin [40].

Cyclooxygenase-2 assay

The procedure followed all the steps outlined in the commercially Rat COX-2 ELISA KIT provided by Bioassay Technology Laboratory, Shanghai, China.

Nitric oxide assay

NO assay was carried out using the Griess reagent method [41]. Deproteinized serum samples were used for NO determination. Griess reagent (50 µl) was added to 50 µl of the homogenized sample and centrifuged. These were incubated in the dark for 10 min Thereafter, the absorbance was read at 540 nm. A standard curve of sodium nitrite ($NaNO_2$) with different concentration ranges (0–120 µM) was plotted to determine the concentration.

Malondialdehyde assay

MDA assay was evaluated according to the TBA test described by [42]. TBA assay is commonly used to assess lipid-peroxidation activity in cells. TBA forms a pink adduct with MDA in tissues, which is measured spectroscopically at the wavelength of 530 nm [43]. The results are expressed in mmol/g of the organ. Into each test tube was introduced 50 mg/2 ml (0.05 g) of homogenized ovary, 0.15 ml of trichloroacetic acid 20%, and 0.3 ml of TBA 0.67%. In the control tube, the homogenate was replaced with 0.3 ml of 50 mM Tris-HCL buffer, 50 mM potassium chloride. The tubes were then incubated in a water bath for 10 min at 95°C. These were then cooled in tap water and centrifuged at 5700 rpm for 10 min. The supernatant was removed and dispensed in plates where the absorbance was read at 530 nm. After reading the absorbance of the test and control, the MDA content was calculated using the molar extinction coefficient as follows:

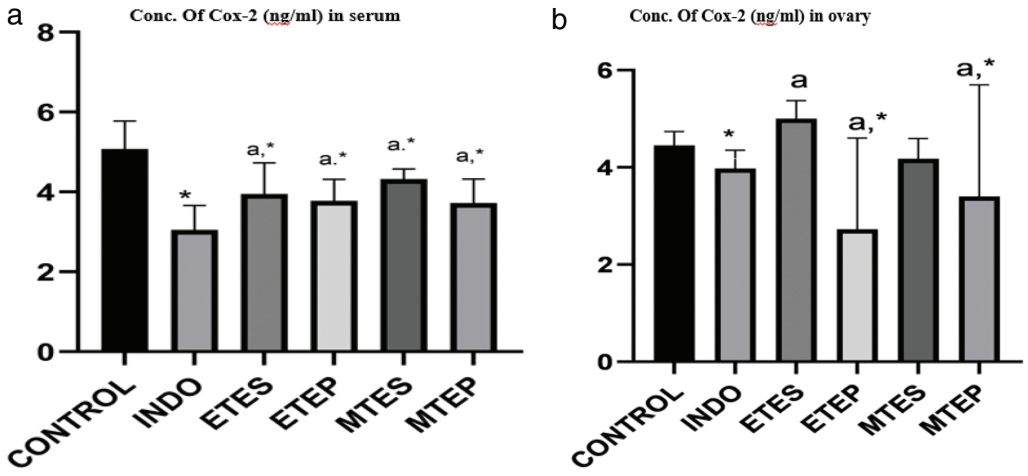
$$\mathrm{MDA}\left(\frac{\mathrm{mmol}}{\mathrm{gof}\varepsilon}\mathrm{organ}\right) = \mathrm{DO}.\frac{\mathrm{vt}}{}.\mathrm{L}.\mathrm{Morg}.\mathrm{Vi}\,.$$

DO=absorbance@530 nm (this was taken from the mean absorbance of each sample that was dispensed) Vt=total volume of homogenate 0.75 ml. ε=molar extinction coefficient=13 600 mol.cm. L=cuvette length 1 cm. Vi=volume used for dosage=0.2 ml. Morg=(organ mass used to prepare homogenate=0.05 g).

Statistical analysis

The data from biological assays were statistically analyzed and graphs were done using the Graph Pad Prism 8.2.1 (441) software, San Diego, California, USA. Analysis of variance was used to determine the significance of assays, which were presented as

Figure 1



(a) Concentration of COX-2 in serum. ETEP, ethyl acetate fraction of EP; ETES, ethyl acetate fraction of ES; INDO, indomethacin; MTEP, methanol fraction of EP; MTES, methanol fraction of ES. (b) Concentration of COX-2 in the ovary. COX-2, cyclooxygenase-2; ETEP, ethyl acetate fraction of EP; ETES, ethyl acetate fraction of ES; INDO, indomethacin; MTEP, methanol fraction of EP; MTES, methanol fraction of ES.

mean±SD. *P* value less than 0.05 was considered significant. Post-hoc multiple Dunnet's comparison test was carried out to establish the significant difference between treatments.

Results

Effect of *Eremomastax polysperma* and *Eremomastax speciosa* leaf fractions on cyclooxygenase-2 concentration in experimental rat serum

The results summarized in Fig. 1a showed that the concentration of COX-2 in groups treated with *E. speciosa* and *E. polysperma* fractions was significantly ($P<0.05$) increased compared with the indomethacin-only treated group. Also, the COX-2 concentration in *E. speciose*-treated groups recorded values higher than *E. polysperma*.

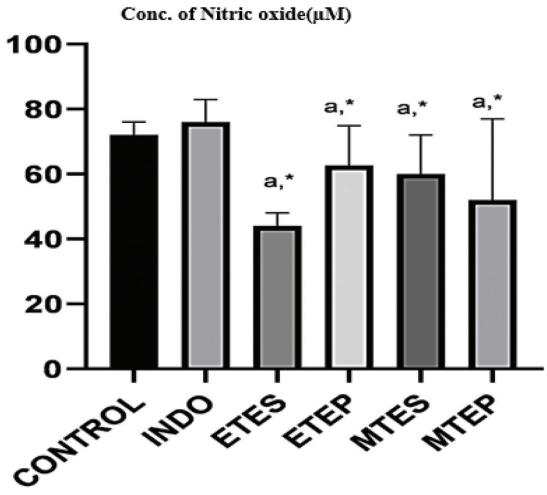
Effect of *Eremomastax polysperma* and *Eremomastax speciosa* leaf fractions on cyclooxygenase-2 concentration in experimental rat ovaries

Ovarian COX-2 concentration was significantly ($P<0.05$) increased in groups treated with *E. speciosa* plant fractions (ETES and MTES) compared with indomethacin-only treated group as shown in Fig. 1b. The *E. polysperma* ethyl acetate (ETEP) and methanol (MTEP) fraction treatment groups recorded values less than the indomethacin-only treated group.

Effect of *Eremomastax polysperma* and *Eremomastax speciosa* leaf fractions on nitric oxide concentration in experimental rat ovaries

The ovarian NO concentration was significantly ($P<0.05$) decreased in all the groups treated with plant fractions compared with indomethacin only and control. The ethyl acetate fraction of the *E. speciosa* (ETES) group recorded the least concentrations of NO as shown in Fig. 2.

Figure 2



Concentration of nitric oxide (NO) in the ovary. ETEP, ethyl acetate fraction of EP; ETES, ethyl acetate fraction of ES; INDO, indomethacin; MTEP, methanol fraction of EP; MTES, methanol fraction of ES.

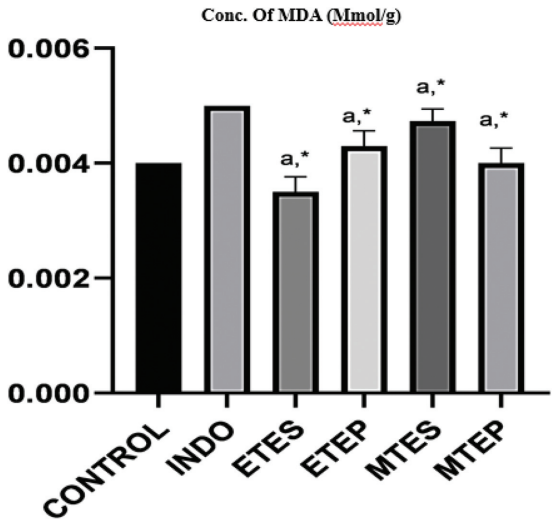
Effect of *Eremomastax polysperma* and *Eremomastax speciosa* leaf fractions on malondialdehyde concentration in experimental rat ovaries

The MDA assay on the ovaries showed a significant ($P<0.05$) decrease in all the experimental groups treated with plant fractions of *E. speciosa* and. *E. polysperma* after the administration of indomethacin. The ethyl acetate fraction of the *E. speciosa* (ETES) group recorded the least MDA concentration as shown in Figs 3 and 4.

Histopathology

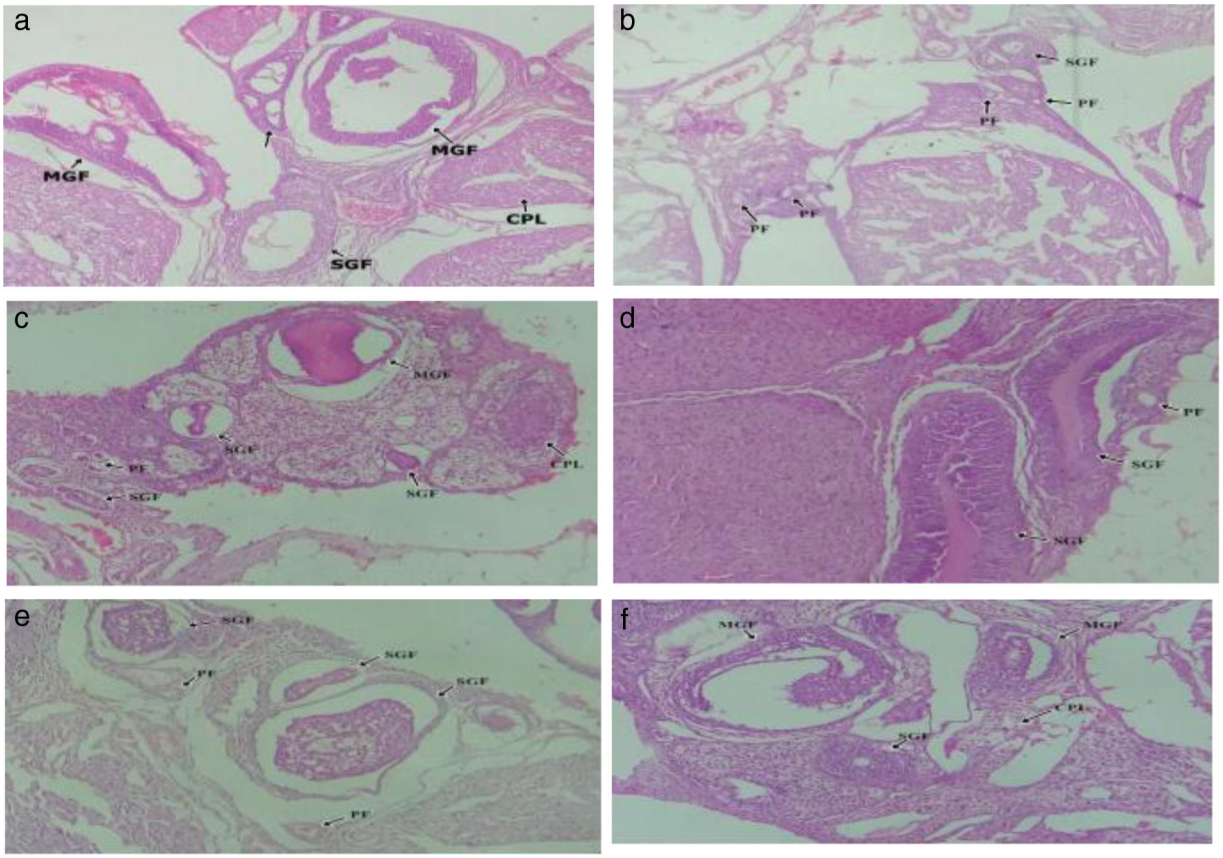
The histopathological investigation of the ovaries presented in Fig. 4a, 4c, 4d, 4e, and 4f revealed the effect of treatment with *E. speciosa* and *E. polysperma* fractions after administration of indomethacin on ovarian morphology. Figure 4a (control), shows the photomicrograph of the control group having abundant ovarian follicles at various levels of maturation. Figure 4c (MTES), photomicrograph of the ovary treated with methanol fractions of *E. speciosa* showed proliferating ovary follicles of the early series consisting of primary follicles and atrophy of the secondary follicles and Graafian follicles, also noted were granulosa cells with clear cytoplasm. Figure 4d (MTEP), photomicrograph of the ovary treated with methanol fraction of *E. polysperma*, showed a cellular stroma consisting of cortically located numerous primordial follicles and scanty secondary follicles. Figure 4e (ETES), photomicrograph of the ovary treated with ethyl acetate fractions of ES showed prominent regenerating ovarian follicles of the early series consisting of primary follicles and few secondary follicles. Figure 4f (ETEP), photomicrograph of the ovary treated with ethyl acetate fraction of *E. polysperma* showed proliferating ovarian follicles consisting of secondary and mature Graafian follicles with the corpus luteum. Histology of the ovary treated with indomethacin only in Fig. 5b showed degenerated and atrophic ovarian follicles with poorly outlined

Figure 3



Concentration of malondialdehyde (MDA) in ovaries. ETEP, ethyl acetate fraction of EP; ETES, ethyl acetate fraction of ES; INDO, indomethacin; MTEP, methanol fraction of EP; MTES, methanol fraction of ES.

Figure 4



Photomicrograph Showing The Histology of The Ovaries. (a) Control H&E 400. (b) Indomethacin H&E 400. (c) MTES methanol fraction of ES H&E 400. (d) MTEP , methanol fraction of EP H&E 400. (e) ETES ethyl acetate fraction of ES H&E 400. (f) ETEP ethyl acetate fraction of EP H&E 400.

primordial follicles. No mature Grafiaan follicle and corpus luteum was seen.

Discussion

This research showed a significant mechanism mediated by *E. speciosa* and *E. polysperma* leaf fractions to alleviate indomethacin-induced toxicity and oxidative stress during ovulation. COX-2 is a significant enzyme involved in the prostaglandin-signaling pathway associated with ovulation [20]. The results from this study showed that fractions of ETES-treated and MTES-treated ovaries and ETES-treated, ETEP MTES, and MTEP-treated serum resulted in increased COX-2 concentrations compared with the indomethacin-only treated group.

Indomethacin competitively inhibits COX-2 activity by binding to the active site on the enzyme for arachidonic acid pathway synthesis [12]. This competitive inhibition, therefore, limits the concentration of COX-2 and the resultant availability of arachidonic acid for prostaglandin synthesis. This study showed that *E. speciosa* and *E. polysperma* plant fractions counteracted the effect of indomethacin by increasing the concentration of COX-2 in the ovaries and the serum.

Ovarian COX-2 expression is a valuable marker for follicular progression and rupture to the ovulatory stage [44]. Follicular maturation and rupture require adequate blood flow [45,46], consequently, indomethacin impedes blood flow to the ovaries and has been implicated in aplastic anemia [47]. *E. speciosa* and *E. polysperma* leaves induced erythropoiesis and reversed hemolytic anemia in experimental rats [34,48]. Erythropoiesis enhances ovulatory function through the action of increased blood supply to developing oocytes [49]. Studies on *E. speciosa* and *E. polysperma* leaves and other medicinal plants showed that *E. speciosa* and *E. polysperma* enhance the development of oocytes and the *Phoenix dactylifera* plant improved oocyte maturation [50]. In vitro and in vivo studies revealed that *E. polysperma* and *Carica papaya* leaves have an antisickle effect, thus acting as a blood tonic [51,52].

This research showed the inhibitory action of *E. speciosa* and *E. polysperma* leaf fractions on indomethacin-generated ovarian ROS by reducing the oxidant status of MDA and NO through scavenging ROS. The free-radical scavenging activity of *E. speciosa* and *E. polysperma* extract is attributed to their rich phenolic content [53]. Polyphenolic compounds have been quantified in methanol and ethyl acetate fractions of *E. speciosa* plants [54] with *E. speciosa* fractions showing abundant phenolic rutin compounds [55]. In addition, rutin in the *Fagopyrum esculentum* plant inhibited lipid peroxidation by radical scavenging [56]. Phenolic compounds have been reported to stimulate the catalytic activity of COX-2 [57].The ovarian histology showed the ameliorative action of methanol and ethyl acetate fractions of *E. speciosa* and *E. polysperma* fraction with improved ovarian structure in treated ovaries compared with indomethacin administration only. The newly formed corpus luteum in *E. speciosa*- and *E. polysperma*-treated ovaries showed evidence of reversed toxicity effect of indomethacin. A newly formed CL is indicative of the progression in the ovarian cycle to the ovulatory stage [58]. This lends supportive evidence to the action of *E. speciosa* and *E. polysperma* leaves on the ovaries. The findings also showed that abnormal ovaries were restored to normalcy following treatment with the Genistein plant [59].

Conclusion

This study showed that the methanol and ethyl acetate fractions of *E. speciosa* and *E. polysperma* exert their protective action in the ovaries of ovulating rats by reversing the effect of indomethacin on COX-2 and ovarian MDA and NO concentrations.

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Authors' contributions: O.E.M. conceived and planned the experiments in consultation with E.U.E. I.A.I. contributed to plant-sample preparation. O.E. M. carried out the experiment assisted by R.M. R.M., I.A.I., and F.U. contributed to the interpretation of the results. O.E.M. took the lead in writing the paper assisted by R.M. and F.U. The paper writing was consulted with E.U.E.

This work was primarily carried out in the International Center of Chemical and Biological Sciences – Karachi, Pakistan.

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Conflicts of interest

There are no conflicts of interest.

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