
Anti-endometriotic Effects of the Mixture of *Erigeron floribundus* (Asteraceae) and *Tragia benthamii* (Euphorbiaceae) Extracts in Peritoneal Endometriosis in Wistar Rats

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Abstract

Endometriosis is a frequent pathology in gynecology. This study was aimed at investigating the curative effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* aqueous extracts on experimental endometriosis in rat. Twenty endometriotic rats were randomly distributed into four groups of five animals each and orally treated during four weeks with either distilled water (10 ml/kg), raloxifene hydrochloride (10 mg/kg) or aqueous extract of plant mixture (130 or 260 mg/kg). Vaginal smear was daily checked throughout the experiments and, blood and sexual organs were collected after sacrifice. Body and sexual organ weights, sexual hormones, prostaglandin E2, oxidative stress markers and uterine histology were measured. Results showed a disrupted estrus cycle, an increase in MDA concentration, implant weight and implantation surface in the untreated endometriotic rats. On the contrary, in the plant extracts-treated endometriotic animals and raloxifene, the estrus stage frequency was increased while the endometriosis lesions were significantly ($p < 0.001$) regressed. Plant extracts also decreased the level of estradiol, progesterone, MDA and catalase, and increased the activities of SOD and peroxidase compared to the untreated rats.

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Prostaglandin E2 concentration and the uterine architecture remained statistically unchanged after treatments. In conclusion, the aqueous extract of the mixture of *E. floribundus* and *T. benthamii* alleviates the endometriotic alterations in rats.

Keywords: Endometriosis ; *Erigeron floribundus* ; *Tragia benthamii* ; estrus cycle ; sexual hormones ; rat.

1. Introduction

Endometriosis is a frequent gynecological disease defined as the growth of endometrial cells outside the uterus. Most often, endometriosis lesions are found among others in the pelvic cavity and peritoneal membrane which are affected by menstrual hormonal changes [1, 2]. The prevalence of this estrogen-dependent inflammatory pathology ranges from 5 to 10 % in women at reproductive age in the general population and the main clinical features are chronic pelvic pain, dyspareunia and infertility [3, 4]. Endometriosis is found in 25 to 40 % of women with pelvic pain and in about 50 % of cases of female infertility [5]. The causes of endometriosis are multifactorial resulting from the mixed effects of environmental and genetic factors [6]. Several theories have been proposed to explain the pathogenesis of endometriosis. They include the implantation theory of Sampson, the coelomic metaplasia theory of Mayer and the theory of induction [7]. But, none of them has brought its definite pathophysiological mechanism. It is however clear that endometriosis is characterized by inflammation, excessive secretion of estrogens and progesterone resistance [8]. Recent studies showed that prostaglandin E2 and oxidative stress may play a central role in the pathophysiological mechanisms of endometriosis [9, 10]. Current treatment options of endometriosis include surgical intervention and/or hormonal therapies. The hormonal therapy aims at suppressing the excess of estrogen and is based on oral contraceptives, androgenic agents, progestogens and GnRH analogs [11, 12, 13]. Other therapeutic options include non-steroidal anti-inflammatory drugs. During the development of endometriosis, PGE₂ regulates cells proliferation, apoptosis and angiogenesis [9]. Although efficient in some cases, the restricted accessibility and numerous side effects are important setbacks of these therapeutic strategies. For these reasons, there is an increasing interest in developing novel solutions to counter the problem. In this vein, many studies suggested the curative effects of some medicinal plants on endometriosis lesions. These include *Radix puerariae*, *Urtica dioica*, *Achillea cretica* and *Persea americana* [3, 14, 15, 16]. *Erigeron floribundus* and *Tragia benthamii* are two medicinal plants traditionally used in combination to heal pelvic pain, dysmenorrhea and female sexual dysfunctions. Previous studies indicated that they possess analgesic [17], antiplasmodial [18], cytotoxicity, anti-proliferative [19], anti-inflammatory [17, 20] and antioxidant [21] properties. The present study was carried out to investigate the curative effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on endometriosis in rat.

2. Materials and Methods

2.1. Animals

Female Wistar rats were reared in the animal house of the Research Unit of Animal Physiology and Phytopharmacology (URPAP) of the Department of Animal Biology, Faculty of Science, University of Dschang, Cameroon. They were maintained at room temperature with a natural light/dark cycle and standard

laboratory rat diet without soybean products. They were given tap water *ad libitum*. The estrus cycle of female rats was monitored through vaginal smear screening and only rats with normal estrus cyclicity (at least three consecutive regular cycles out of a total of five) were used in the study. The research proposal was presented and validated by the scientific committee of the Department of Animal Biology, University of Dschang, which follows the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European community guidelines; EEC Council Direction 2010/63/EU of 22 September 2010 [22].

2.2. Plants harvesting and extract preparation

Fresh plants of *T. bentharii* and *E. floribundus* (also called *Conyza sumertrensis*) were harvested in Baham, West Region of Cameroon. Botanical authentication was done at the Cameroon National Herbarium (HNC) in comparison with the specimens deposited under Vaucher numbers 9747/SRF/Cam and 33115/HNC for *T. Bentharii* and *E. floribundus*, respectively. The entire plants were cut into small pieces, shade-dried for one week and powdered. Aqueous extract was prepared according to folk indications. One hundred and forty-four grams (144 g) of powder of the mixture (ratio 1:1) was boiled in 4.5 L of distilled water for 15 min. The resulting solution was allowed to cool down at room temperature and filtered with whatman paper number 4; the filtrate was oven-dried at 45°C for two days. From this process, 32.78 g of aqueous extract were obtained giving an extraction yield of 22.76 %. This extract was kept at 4°C until use.

2.3. Experimental design

2.3.1. Endometriosis induction

Endometriosis was induced by auto-transplantation of endometrial tissues as proposed by Jones [23] with minor modification by Amaral and his colleagues [24]. Thirty-five adult female rats with regular estrus cycle were anesthetized with diazepam (10 mg/kg: i.p.) and ketamine (50 mg/kg: i.p.). The abdomen was opened through a 3 cm midline incision to expose the uterus. The left uterine horn was ligated at both the uterotubal junction and the cervical end and 1 cm segment was removed. The excised horn was maintained in sterile saline solution (NaCl 0.9 %) and opened longitudinally. The prepared piece was then grafted onto the muscle in the right inguinal region using mononylon thread 3-0. The abdominal wall was finally closed with two layers of sutures using the same thread. All procedures were carried out under sterile conditions and animals followed three days antibiotic (penicillin G; 2000 UI/kg/day) treatment to prevent post-surgery infections. Following induction, animals received 17-β estradiol at 10 mg/kg body weight on days 4, 8 and 12 to promote the growth of the ectopic endometrium [25, 26]. Twenty-one days after the auto-transplantation of endometrial tissues, animals underwent a laparotomy checking and the length and width of the implant were measured. The implant surface area was estimated as follows [1]:

$$\text{Surface area (cm}^2\text{)} = \text{length of implant (cm)} \times \text{width of implant (cm)}$$

2.3.2. Treatment and sacrifice

After checking for the occurrence of endometriosis, rats were randomly distributed into four groups of five

animals each and daily treated during 4 weeks as follows: group 1 received distilled water (10 ml/kg) and constituted the negative control; group 2 received raloxifene hydrochloride (10 mg/kg) and served as positive control. Groups 3 and 4 were test groups which received plants aqueous extract at 130 and 260 mg/kg respectively. All treatments were administered orally and the gavage volume daily adapted to the body weight. Throughout the treatment period, vaginal smear was checked under optic microscope (40X) in order to identify the estrus stage. The dose of raloxifene was chosen according to Jin and his colleagues [3]. One day after the last treatment, animals were sacrificed under diazepam/ketamine anesthesia. Blood was collected via catheterization of the abdominal artery and centrifuged at 3000 rpm for 15 minutes and the supernatant was kept at -20°C for measurement of sexual hormones, prostaglandin E₂ and oxidative stress markers. The surface area of the implant was calculated and the implant was excised and weighed. The ovaries and uterus were also collected, cleaned of fat tissues, weighed and fixed in 10 % formalin for histological analysis.

2.4. Biochemical analysis

Plasma levels of estradiol and progesterone were assessed by enzyme-linked immunosorbent assay (ELISA) according to the instructions of commercial kits (Accubind, Monobind Lake Forest, USA). Plasma prostaglandin E₂ was determined by ELISA test using reagent kits, according to the instructions of the manufacturer (Prostaglandin E₂ ELISA Kit-Monoclonal, Cayman Chemical, USA). Also, catalase, lipid peroxidation, superoxide dismutase and peroxidases were quantified using referenced methods [27, 28, 29, 30].

2.5. Histological analysis

The preparation of histological slides followed the standard procedure described by Cannet [31]. Briefly, uterus fixed in 10 % formalin were dehydrated in ethanol and embedded with paraffin. Paraffin-embedded tissues were cut at 5 µm thickness and hematoxylin-eosin staining procedure was followed. Prepared slides were analyzed with a light microscope (Olympus) and the uterine epithelial height was then measured through a computer connected to the microscope.

2.6. Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by post hoc Tukey HSD was performed to estimate statistical differences between data. Analysis was done using STATISTICA software Version 8.0 (StatSoft, Inc., Tulsa, USA). A probability of $p < 0.05$ was considered significant.

3. Results

3.1. Effects of treatments on the estrus cycle of rats with endometriosis

The effectiveness of endometriosis was shown by the disrupted estrus cycle in the untreated rats. No alleviating effect was observed after different treatments as indicated in table 1. Raloxifene and the dose of 260 mg/kg of the plants extract blocked the cycle at the estrus stage (Table 1).

Table 1: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on the frequency and regularity of estrus cycle in rats with endometriosis

Treatment	Number of checked estrus cycle	Frequency of estrus stage (%) appearance			
		Proestrus	Estrus	Metestrus	Diestrus
Untreated	7	20.71	18.57	26.43	34.29
Raloxifene 10 mg/kg	7	18.57	50.00	7.86	23.57
Aqueous extract					
130 mg/kg	7	20.00	25.00	32.86	22.14
260 mg/kg	7	17.86	65.71	6.43	10.00

3.2. Effects of treatments on body and sexual organ weights

As shown in table 2, the dose-dependent increase in body weight gain and uterine weight observed in the endometriotic rats treated with the plant mixture was lower than that recorded in raloxifene and untreated groups. A 39.06% increase was noticed in the uterine relative weight of rats treated with the dose 260 mg/kg of the aqueous extract compared to the untreated group.

Table 2: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on the body and sexual organ weights of rats with endometriosis

Treatments	Body weight		Uterine weight			Ovarian weight	
	Initial (g)	Final (g)	Bw gain (%)	Absolute (g)	Relative (mg/100g bw)	Absolute (g)	Relative (mg/100g bw)
Untreated	177.6±21.5	191.4±17.66	8.40±2.1	0.32±0.01	179.57±15.80	0.080±0.0	45.40±5.61
	6	6	6			1	
Raloxifene 10 mg/kg	157.6±19.7	171.4±11.29	8.42±2.9	0.24±0.02	153.02±21.57	0.067±0.0	42.30±3.05
	1	3	3			2	
Aqueous extract							
130 mg/kg	159.6±7.07	165.4±10.93	3.49±1.8	0.23±0.02	143.32±10.07	0.081±0.0	50.07±9.69
			4			2	
260 mg/kg	176.8±4.73	189.4±7.54	6.92±2.8	0.48±0.16	249.71±68.29	0.090±0.0	48.10±8.30
			7			1	

3.3. Effects of treatments on the implant weight and implantation size

In the untreated endometriotic females, the implant weight had an average of 25 mg/100 g bw and a surface area of 0.6 cm². After treatments, a dose-dependent significant decrease (P<0.001) was recorded in both implant weight and implantation area of plant extract-treated rats. Raloxifene used as positive control also showed reducing effects when compared to the untreated females.

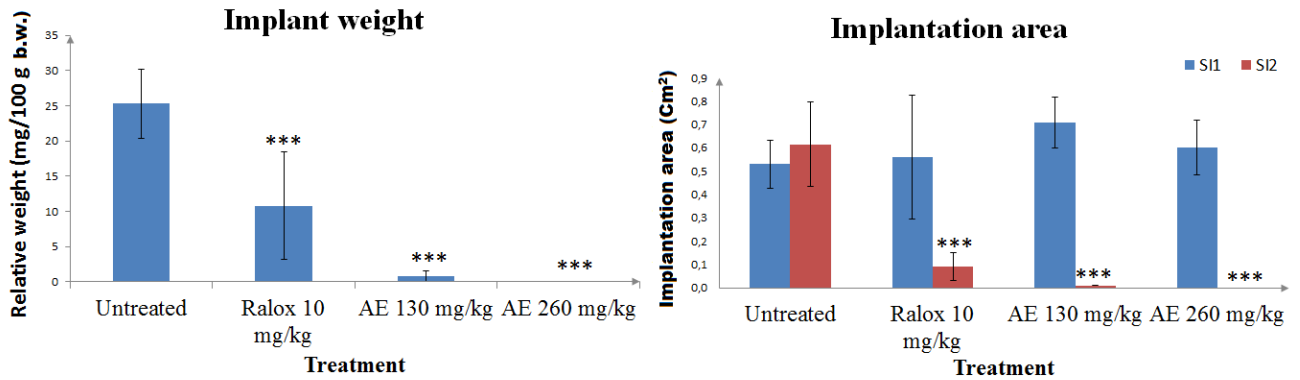


Figure 1: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on the implant weight and implantation surface in rats with endometriosis

S1: surface area at confirmatory laparotomy, S2: surface area after treatment; Bw: Body weight; Ralox: raloxifene, AE: aqueous extract, ***P<0.001: significantly different compared to untreated group.

3.4. Effects of treatment on plasmatic estradiol, progesterone and prostaglandin E₂ concentrations

In endometriotic rats, raloxifene and plant mixture (at 130 mg/kg) significantly increased (p<0.001) the plasma estradiol (E₂) level, compared to the untreated female rats (Figure 2). In rats administered with the doses of 130 mg/kg and 260 mg/kg of plant mixture, the progesterone (P₄) concentration was respectively decreased (p<0.001) by 46.73 and 37.49 % compared to the untreated animals. In all groups, plasmatic prostaglandin E₂ (PGE₂) was statistically (p>0.05) unchanged after treatments (Figure 2).

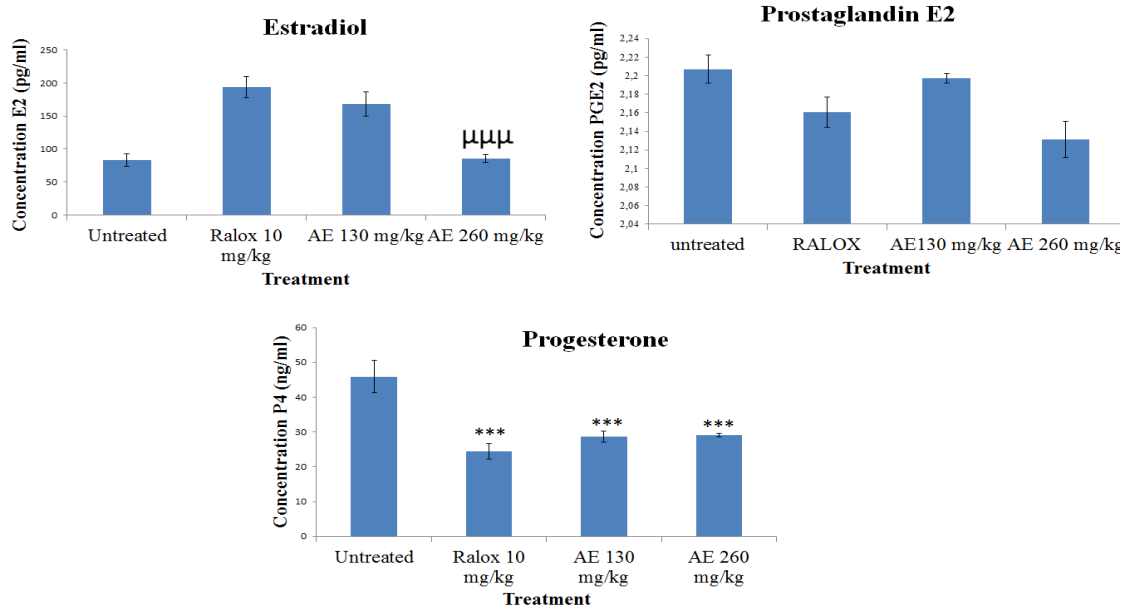


Figure 2: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on plasmatic levels of estradiol, progesterone and prostaglandin E₂ in rats with endometriosis

AE: Aqueous extract; Ralox: raloxifene; μμμ $P < 0.001$: significantly different compared to raloxifene group; *** $P < 0.001$: significantly different compared to untreated group.

3.5. Effects of treatments on plasmatic levels of oxidative stress markers

The effects of treatments on oxidative stress markers are summarized in table 3. Similar to raloxifene, plants extract (260 mg/kg) reduced MDA concentration and catalase activities. Also, plant extracts increased superoxide dismutase and peroxidase activities (Table 3).

Table 3: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on the plasma levels of oxidative stress parameters

Oxidative stress markers	Untreated	Raloxifene 10 mg/kg	Aqueous extract 130 mg/kg	260 mg/kg
MDA (uM/g PTT)	0.22 ± 0.01	0.19 ± 0.02	0.25 ± 0.02	0.11 ± 0.01
SOD (U/Min/g PTT)	0.17 ± 0.04	0.21 ± 0.02	0.22 ± 0.03	0.24 ± 0.05
Catalase (U/g PTT/Min)	1.16 ± 0.17	0.93 ± 0.07	0.65 ± 0.04	0.70 ± 0.11
PEROX (mM/Min/g PTT)	11.16 ± 1.18	10.63 ± 1.13	15.67 ± 1.04	12.55 ± 1.08

PTT= total protein

3.6. Effects of treatments on the uterine histology

Generally, no remarkable alteration of the normal architecture of the uterus was noticed in all endometriotic rats (figure 4). However, a non-significant increase ($p>0.05$) of the epithelial height was registered in raloxifene and the two plants extract-treated rats when compared to the untreated endometriotic animals (Figure 3).

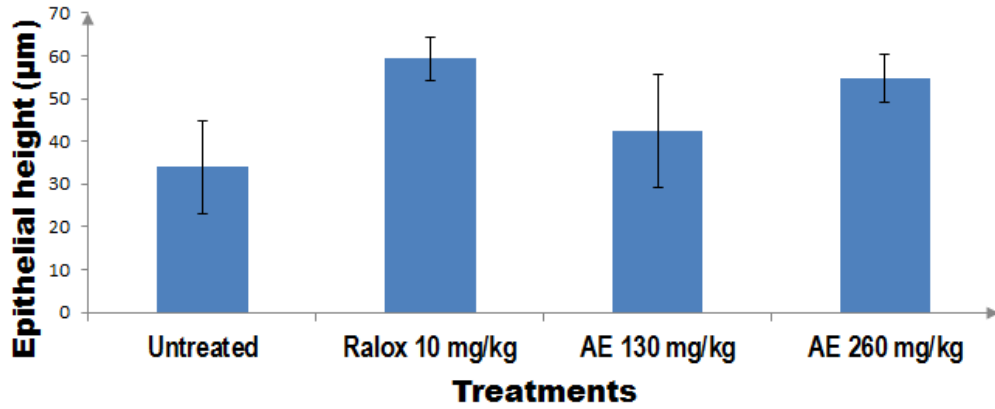


Figure 3: Effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on the endometrial epithelial height

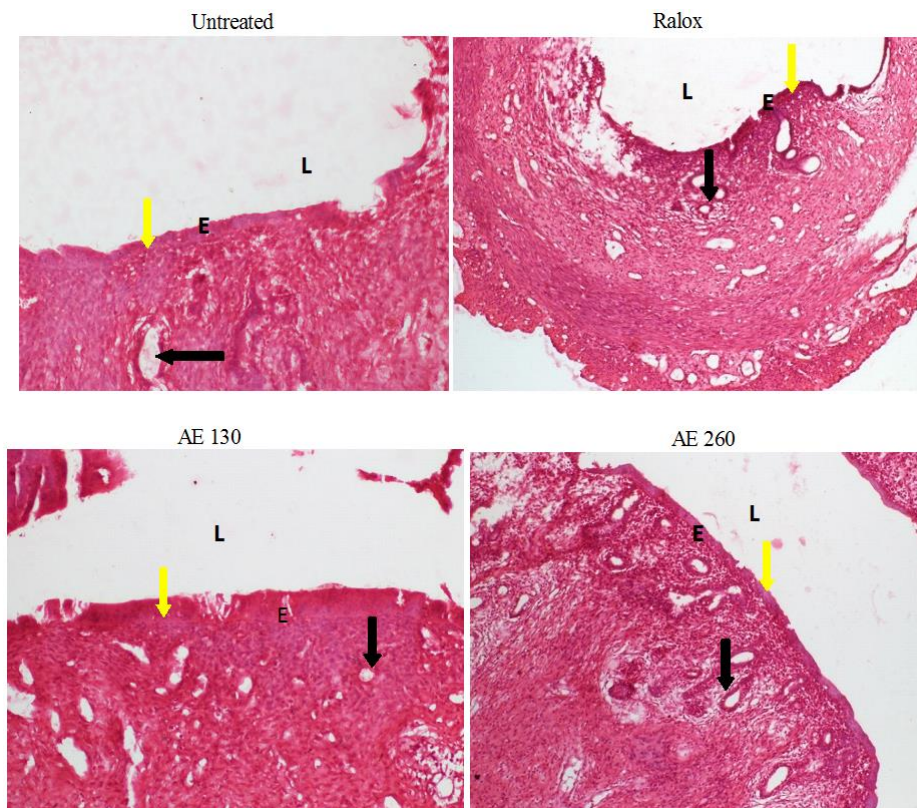


Figure 4: Microphotographs (×100, hematoxylin and eosin staining) of the uterus.

L= lumen, E= epithelium, black arrow = gland, yellow arrow = cubic cells. AE: aqueous extract; Ralox: raloxifene.

4. Discussion

The aim of the present study was to investigate the curative effects of the mixture of *Erigeron floribundus* and *Tragia benthamii* on endometriosis in rat. Endometriosis dysregulated the estrus cycle which was marked by an alteration in the frequency and chronology of estrus stages. Endometriosis is an estrogen-dependent inflammatory pathology associated with an abnormally high blood prolactin concentration. Although prolactin concentration level was not measured in the present study, it is however reported that elevated level of prolactin prevents luteinizing hormone (LH) secretion and interferes with hypothalamic function by blocking estrogen receptors, leading to anovulation and dysmenorrhea [32]. Assay of prolactin in further studies may therefore be of good interest to explain the estrus cycle disruption observed in the present study. Our findings are consistent with previous studies reporting a disruption in the estrus cycle in rats with endometriosis [33]. Jones and his colleagues found that in mice, endometriosis lesions provoke a slight disruption in estrus cyclicity. In the endometriotic rats treated with the plants extract or raloxifene, the estrus cycle remained disturbed and was blocked at estrus stage. Raloxifene is an estrogen receptor modulator used in female to induce ovulation [34]. Similar to raloxifene, plants effect may suggest resumption or disturbance of the ovulation process in rats. These results are similar to those of Mustapha and his colleagues [35] and Lemuel and his colleagues [36] who found that *Rhynchosia sublobata* and *Cleome gynandra* induced acyclicity in rats with persistent estrus phase. As suggested by Mustapha and his colleagues [35], these effects could be attributed to flavonoids. These compounds were also found in *Erigeron floribundus* [17] and may justify present results. However, further studies are needed to confirm this hypothesis. After sacrifice and sample collection, no change on the relative weight of the uterus and ovaries was noticed in all groups. These data suggest that the presence of endometriosis did not affect sexual organ development [26]. On the contrary, the relative weight and implantation surface of the endometriomas remained elevated in the untreated endometriotic rats. Indeed, the proliferative process observed under endometriosis is due to the high estrogen levels, especially estradiol (E₂) which was found to stimulate the growth of endometriosis lesions [8, 16]. On the contrary, raloxifene as well as the plants extract significantly ($p < 0.001$) suppressed the growth of the endometriomas. A number of studies have documented the effects of raloxifene on experimental endometriosis [3, 37]. Raloxifene binds to the estrogen receptors with similar affinity to 17 β -estradiol and its actions are tissue-specific. It acts as an agonist in the skeleton, on serum lipid metabolism, and on a number of coagulation factors, but as an estrogen antagonist in the breast and uterus [2, 38]. Although the exact mechanism of action has not been determined, it has been reported that raloxifene inhibits estrogen-induced endometrial proliferation [39]. The effects of the plant extracts corroborated the cytotoxic, anti-inflammatory and antioxidant properties already reported on these plants. Indeed, Kuete and his colleagues demonstrated that crude extract of *T. benthamii* exhibit cytotoxic effects on leukemia CCRF-CEM cells [19]. The anti-inflammatory properties of the aqueous extract of *Erigeron floribundus* was characterized by a significant decreased in the rat paw oedema volume at 50 mg/kg and above [17]. Moreover, the antioxidant activity of *Erigeron floribundus* essential oil (determined by DPPH radical scavenging ability and showing an IC₅₀ value about 250 times higher than that of the positive control Trolox) was reported by Petrelli and his colleagues Higher antioxidant activity was detected in the ABTS radical cation scavenging activity assay [21].

Also, these effects of the mixture may be attributed to its contents in flavonoids, which was reported to display antiproliferative activities [14, 20]. Raloxifene and the dose of 130 mg/kg of the aqueous extract of *Erigeron floribundus* and *Tragia bentharii* mixture increased the plasma concentration of estradiol. Results observed with raloxifene could arise from the fact that, this molecule does not interfere with estrogen synthesis process, rather, it blocks the estrogen receptors [37], preventing estrogens utilization, resulting in its accumulation. This hypothesis could explain the results obtained in the present study. Many studies reported that endometriosis is intimately associated with steroid metabolism and associated pathways [2, 40]. Results of the plant extracts could reflect the presence of active molecules interfering with steroidogenesis and/or estrogen metabolism. Some reports on the chemical constituents of *E. floribundus* and *T. bentharii* demonstrated that they possess phytoestrogens [19, 41] which, depending on the dose and/or the model, could exhibit estrogenic or anti-estrogenic activities [42]. Following treatments, the plant extract and raloxifene normalized the activities of antioxidant enzymes and decreased plasma level of MDA. These effects were associated with an increase in the uterine epithelial layer height. No change was observed in the concentration of prostaglandins E₂. These results suggest that the mixture of *E. floribundus*/*T. bentharii* extract reduced oxidative stress and lipid peroxidation in endometriotic rats, which is consistent with their reported antioxidant and anti-inflammatory properties [9, 10, 43].

5. Conclusion

The present findings demonstrated that the aqueous extract of the mixture of *E. floribundus* and *T. bentharii* suppressed endometrioma growth in rat through antioxidant, anti-inflammatory activities and regulation of the sexual hormone levels. These data are in accordance with the traditional use of these plants. However, fertility improving capacity and toxicity of these plants need to be assessed.

6. Recommendations

Even though the results of the present study confirm the traditional claim related to the use of these plants in the management of endometriosis, we recommend to the community to be careful with regard to the dosage that should be clearly defined after toxicity tests.

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