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Eremomastax speciosa potentializes the PMSG-inducing effect on some physiological and biochemical parameters in PMSG-primed immature rats

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Research Article

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Summary

The present study evaluated the effect of the aqueous extract from leaves of *E. speciosa* on some physiological and biochemical parameters of reproduction and the onset of puberty in pregnant mare serum gonadotropin (PMSG)-primed immature female rats. High pressure liquid chromatography (HPLC) was used to quantify the phenolic compounds in the methanol/methylene chloride (1:1) extract, the ethanolic and ethyl acetate fractions and the aqueous residue of *E. speciosa*. *E. speciosa* (0, 8, 32 or 64 mg/kg) were administered for 15 days to 24 non-PMSG-primed and 24 primed rats with 0.01 IU of PMSG. At the end of the treatment period, animal were sacrificed and their body, ovarian, uterine weight, ovarian protein or cholesterol level, as well as data on puberty onset were recorded. Of the 16 polyphenolic compounds quantitatively revealed in the extracts and fractions of *E. speciosa* after HPLC analysis, quercetin, rutin, apigenin and eugenol were the most abundant. Non-primed rats showed a significant increase ($P < 0.05$) in the uterine relative weight at the dose of 8 mg/kg when compared with the other treatments. The uterine proteins and the ovarian cholesterol ($P < 0.05$), respectively, showed a reduction at doses of 64 mg/kg and 32 mg/kg in non-primed rats. However in PMSG-primed rats, a significant decrease ($P < 0.05$) was observed in ovarian cholesterol at 64 mg/kg. In conclusion, *E. speciosa* potentializes the PMSG-inducing effect on folliculogenesis in PMSG-primed rats.

Introduction

Medicinal plants are used to cure several diseases, including infertility problems (Tsobou *et al.*, 2016). Some plants are rich in compounds that exhibit regulatory effects on reproductive function, acting directly or indirectly on the hypothalamic–pituitary–ovarian axis by induction or inhibition of ovarian activity (Telefo *et al.*, 1998). Therefore, their use can bring direct answers to some health problems such as reproductive disorders (Mbemya *et al.*, 2017). The use of medicinal plants such as *E. speciosa* in response to reproductive problems can be seen as an alternative to manufactured drugs, especially in developing countries in which they are expensive and/or inaccessible (Rates, 2001).

E. speciosa is a medicinal plant used in Cameroon to normalize the menstrual cycle, cure female infertility, dysentery, anaemia, haemorrhoids and urinary tract infection (Oben *et al.*, 2006). Phytochemical analysis of aqueous extract from leaves suggests the presence of alkaloids, flavonoids, saponins and tannins (Tagne *et al.*, 2014). These metabolites could present significant important antioxidant activities important for the regulation of the female reproductive activity. *In vivo*, this activity is often evaluated on reproductive organs of immature female rats (16–23 days) that have long been used as a model system for studying effects of pharmacological compounds (Tohei *et al.*, 2000) and medicinal plants (Lienou *et al.*, 2012; Goka *et al.*, 2016) on ovarian folliculogenesis. *In vitro* activity, conversely, has been assessed through activation and development of ovarian preantral follicles using diverse medicinal plants such as *Justicia insularis* (Mbemya *et al.*, 2017; 2018), *Amburana cearensis* (Gouveia *et al.*, 2016; Barberino *et al.*, 2016); *Auxemma oncocalyx* (Leiva-Revilla *et al.*, 2016; 2017). In both *in vitro* and *in vivo* studies, biological activity was due to the presence of gonadotropin-like compounds of the extract.

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Related to the gonadotropin-like effect of the plant mixture, its oral administration to female immature animals initially primed with a dose of PMSG, with no significant effect on their ovarian and uterine weights, could modify the anatomical and biochemical parameters of the rats. Moreover, priming of the immature rat with PMSG would stimulate an advance in the physiological maturation to a stage at which only short-term treatment (15 days) with any exogenous stimulating compound would lead to puberty onset. This approach could be useful for confirmation of the follicle-stimulating hormone (FSH)-like effect of aqueous extract of *E. speciosa*, as the time needed as for *in vivo* experimental protocols on the study of medicinal plants used to cure some forms of infertility is generally long.

Accordingly, this study was conducted to evaluate the effect of an aqueous extract of *E. speciosa* on the age at puberty onset in PMSG-primed immature female rats, as well as its FSH-like effect through some physiological parameters.

Materials and methods

PMSG and plant extract preparation

The concentration of lyophilized PMSG (0.01 IU/ml) used in this study was selected based on a previous study. This concentration represents the highest dose of PMSG (HD) used without effects on ovarian and uterine weights and which did not induce vaginal opening in immature female rats after 5 days of injection (Goka *et al*., 2016).

Fresh leaves of *E. speciosa*, identified at the National Herbarium of Cameroon under voucher specimen code 23604/SFR/CAM (Tagne *et al*., 2014), were collected in a town in Western Cameroon (Dschang subdivision, Menoua division, 5°26′N, 10°03′E, altitude 1345 m). Leaves were dried at 24°C in the shade and subsequently ground in a mortar; 10 g of the powder obtained was boiled in 50 ml of distilled water for 24 h. After maceration, the extract was filtered before being dried in a ventilated oven at 45°C. The powder extract was stored in the refrigerator at −20°C for further use. The dose of 8 mg/kg was obtained by reconstitution based on information gathered from traditional medicine practitioners in an ethnopharmacological survey performed in Dschang (Telefo *et al*., 2011) and the two other doses (32 and 64 mg/kg) are its multiples.

Polyphenol analysis

The phenolic compounds were quantified in the methanol/methylene chloride, aqueous residue, ethanolic and ethyl acetate fractions of *E. speciosa* using high pressure liquid chromatography (HPLC). The sample was dissolved in pure water at a 3% concentration and centrifuged at 2647 *g* for 10 min. The supernatant was filtered through a cellulose acetate membrane filter (0.20 µm or 0.45 µm; Schleicher and Schuell) and used for analysis. A 25 µl portion of the filtrate was injected into the HPLC system and eluted. Analysis was performed on an Agilent Technologies 1200 HPLC system fitted with a SUPELCOSIL LC-18 column (length 250 mm, diameter 4.6 mm, packaging size 5 µm). The column temperature was settled equal to 20 °C. The mobile phase consisted of an aqueous solution of 0.5% volume acetic acid ('A') and acetic nitrile ('B'). At the start and finish for the first 2 min of the run, 100% A was used. From 2–60 min after the run had started, a linear ramp was used, targeting 40% A and 60% B. The flow rate settled to equal to 1 ml/min. Polyphenols were measured using a ultraviolet (UV) light detector (280 nm). Beforehand, the retention times of the polyphenolic compounds of interest were measured using single polyphenol standard solutions at a concentration of 1%.

Animals

Animals used in this study were female immature Wistar rats, bred in the animal house of the Department of Biochemistry (University of Dschang, Cameroon). At the beginning of each experiment, the animals were 21–23 days old, weighing 30–35 g. They were housed under uniform husbandry conditions of light (12 h cycle) and temperature (22 ± 2°C) and fed with standard laboratory diet and tap water *ad libitum*.

PMSG-inducing effect potentiation by E. speciosa extract on animal growth, puberty onset and some biochemical parameters of reproduction

To evaluate the potentiation effect of PMSG-inducing effect on animal growth, puberty onset and some biochemical parameters of reproduction by the aqueous extract of the plant, 48 animals were divided into two experimental groups: non-PMSG-primed and HD-primed animals. The non-PMSG-primed group received distilled water (control group), and 8, 32, and 64 mg/kg doses of the extract, whereas the other group receive an injection of PMSG prior to oral administration of the extract at the same dosages (0, 8, 32, and 64 mg/kg) over 15 days. Each rat was checked every day for vaginal opening. At the end of the experimental period, animals in each group were randomly sacrificed by anaesthesia using chloroform. Their ovaries and uteri were removed, blotted, weighed and stored at −20°C until use. The ovaries were homogenized and the supernatants used for quantification of protein and total cholesterol.

Preparation of the uterine and ovarian supernatants and biochemical analysis

Ovaries and uteri were homogenized in Tris–sucrose buffer (0.25 M sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.4) at 1% and 2% respectively. The homogenate was then centrifuged at 6000 *g* at 4°C (Beckman model J2–21) for 15 min, and the supernatants collected were used for protein (Bradford, 1976) and total cholesterol (Richmond, 1973) assays.

Statistical analysis

Data were analysed using the SPSS system (version 23.0). Analysis of variance (ANOVA) was used to determine the sources of variation (or to detect the significance of treatment) in mean values of ovarian and uterine weights. Student–Newman–Keuls and Fisher's least significant difference (LSD) tests were used for comparison between means whenever experimental factors were significant for ANOVA. The percentages of rats showing vaginal opening were analysed by Levene test to verify homogeneity and the ANOVA was performed by Tukey test with 5% probability for comparison between means.

Results

HPLC analysis for phenolic compounds

The quantitative evaluation of phenolic compounds in the aqueous residue of *Eremomastax speciosa* showed the following quantities expressed in milligrams per litre (mg/l): 3,4-OH benzoic 13.33, apigenin 279.23, caffeic 12.66, catechine 64.51, gallic 2.99, *o*-coumaric

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Table 1. Constituents found in each fraction of *E. speciosa* using HPLC

	Eremomastax speciosa extracts			
Polyphenols	Methanol/ methylene chloride (mg/l)	Aqueous residue (mg/l)	Ethanolic (mg/l)	Ethyl acetate (mg/l)
3,4-OH Benzoic	–	13.33	–	2.51
Apigenin	44.36	279.23	21.00	–
Caffeic	1.43	12.66	–	6.89
Catechine	4.86	64.51	–	14.53
Eugenol	–	–	204.06	–
Gallic	–	2.99	–	4.10
o-Coumaric	2.23	23.36	–	4.80
OH-Tyrosol	9.58	78.58	4.96	19.79
p-Coumaric	1.39	35.86	9.55	10.23
Phenol	0.63	–	6.91	–
Quercetin	183.17	93.21	107.50	699.33
Rutin	174.52	556.09	59.43	–
Sinringic	1.48	3.50	–	–
Theobromine	–	9.45	–	1.31
Tyrosol	1.99	32.64	–	3.97
Vanillic	1.80	13.85	6.13	5.42

23.36, OH-tyrosol 78.58, *p*-coumaric 35.86, quercetin 93.21, rutin 556.09, sinringic 3.50, theobromine 9.45, tyrosol 32.64, and vanillic 13.85 (Table 1). These results showed very high quantities of apigenin and rutin while the lowest values were reported for gallic and theobromine (Table 1).

Effect of E. speciosa *on body weight gain*

The effect of *E. speciosa* on body weight of non-PMSG-primed and primed female rats is presented in Fig. 1. A linear increase in the growth rate of primed (*A*) and non-primed animals (*B*) was observed. However, the body weights of non-primed animals treated at the dose of 8 mg/kg were significantly ($P < 0.05$) lower than those of the control group on days 11, 13 and 15.

Effect of E. speciosa *on uterine weight and proteins*

PMSG-primed rats treated at the dose of 8 mg/kg showed higher ($P < 0.05$) uterine weight when compared with the other treatments (Fig. 2A). Regarding the uterine proteins, a decrease ($P < 0.05$) was observed at the dose of 64 mg/kg when compared with the control treatments (Fig. 2B).

Effect of E. speciosa *on ovarian weight and proteins*

The oral administration of *E. speciosa* to non-PMSG-primed immature female rats did not affect the ovarian weight, however a significant decrease ($P < 0.01$) was observed on primed rats at 8 mg/kg and 64 mg/kg dosages when compared with control group (Fig. 3A).

No variation was observed in ovarian proteins in both primed and non-primed animals (Fig. 3B).

Effect of E. speciosa *on ovarian cholesterol*

As can be seen from Fig. 4, in non-PMSG-primed rats, the ovarian cholesterol significantly decreased ($P < 0.01$) at 32 mg/kg dosage compared with the control. Whereas in primed animals, the cholesterol level increased significantly at 8 mg/kg dosage compared with the control group, while it decreased ($P < 0.05$) at 64 mg/kg.

Effect of E. speciosa *on days of vaginal opening*

No vaginal opening was observed in non-primed immature female rats. Table 2 shows the percentages of PMSG-primed rats presenting vaginal opening. The vaginal opening was observed on day 6 at 8 and 32 mg/kg dosages (3 and 1 opening respectively). The remaining rats treated at 8 mg/kg opened on the 10th day of treatment. Only one rat treated at 64 mg/kg dose was open on that 10th day.

Discussion

The present study showed a significant difference in body growth rate, uterine weight and proteins, total ovarian cholesterol and induction of vaginal opening in female rats after oral administration of different doses of aqueous extract of *E. speciosa*.

HPLC–phytochemical screening of polyphenols of the methanol/methylene chloride extract, its ethyl acetate and ethanol fractions and the aqueous residue of *E. speciosa* revealed the presence of several polyphenolic compounds among which apigenin and rutin presented high amounts. Chemically known as 4′,5,7,-trihydroxyflavone, apigenin is a yellow crystalline powder belonging to the flavone class (Ali *et al.*, 2017). It is present in many fruits and vegetables and has diverse biological effects including antioxidant and antigenotoxic properties (Liu, 2004; Ali *et al.*, 2014) and modulation of immune cell function (Nicholas *et al.*, 2007). According to Singh *et al.* (2012), the cancer preventive effect of apigenin is due to its potent antioxidant and anti-inflammatory activities. It has also been demonstrated that apigenin promotes metal chelation, scavenges free radicals and stimulates phase II detoxification enzymes in cell culture and *in vivo* tumour models. (Middleton *et al.*, 2000). Rutin (3,3′,4′,5,7-pentahydroxyflavone-3-rhamnoglucoside) is a flavonoid of the flavonol class that is widespread in plants and fruits (Hosseinzadeh and Nassiri-Asl, 2014). Like quercetin and eugenol (Miean and Mohamed, 2001), which are antioxidants that protect the body against oxidative stress, rutin has a wide range of pharmacological properties that have been exploited in human medicine and nutrition. In recent years, Hsu *et al.* (2014) reported that rutin serves as a potential agent for glycaemic control through enhancement of insulin-dependent receptor kinase activity, thereby inducing the insulin signalling pathway, and therefore causing increased glucose transporter 4 translocation and increased glucose uptake. Many endogenous or synthetic neurotransmitters influence the hypothalamic–pituitary–gonadal axis and can consequently affect menstrual cycling. Similarly, the emotional state may disrupt reproductive functioning through the effects of stress hormones on the hypothalamic–pituitary–gonadal axis (Baldwin and Mayers, 2003). These various pharmacological effects of phenolic compounds encountered in the medicinal plant may explain their multiple therapeutic usages in traditional medicine (Telefo *et al.*, 2011; Mbemya *et al.*, 2017).

A previous study showed that subcutaneous injection of 0.01 IU PMSG to immature female rats over 5 days led to a significant increase in ovarian and uterine weights as well as vaginal opening (Goka *et al.*, 2016). PMSG is a natural glycoprotein with FSH-like

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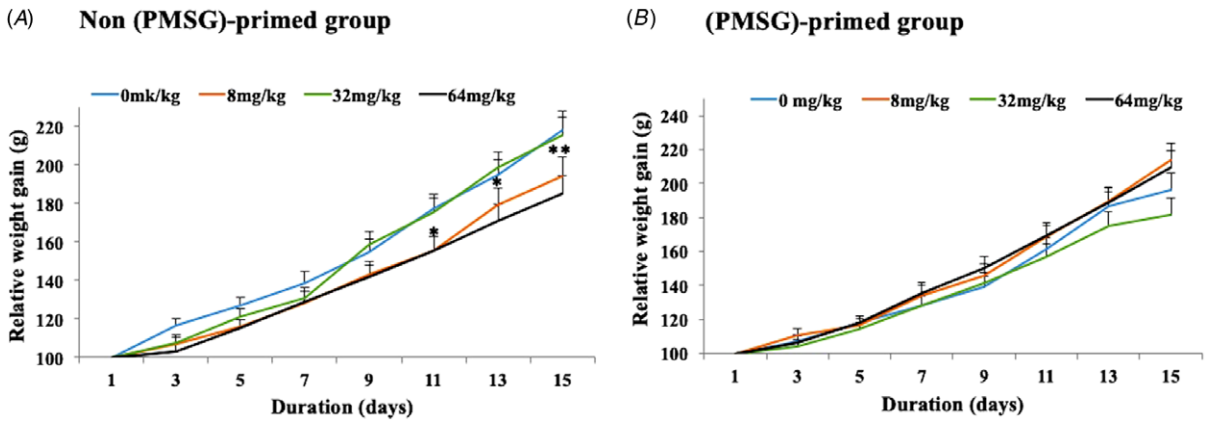

Figure 1. Body weight gain of non-PMSG-primed (*A*) and primed (*B*) rats after oral administration of 8, 32 and 64 mg/kg doses of *E. speciosa*. Each point represents the mean ± SEM of six animals per group. Values statistically different from that of the control group each corresponding day, respectively at $^{*}P < 0.05$ and $^{**}P < 0.01$.

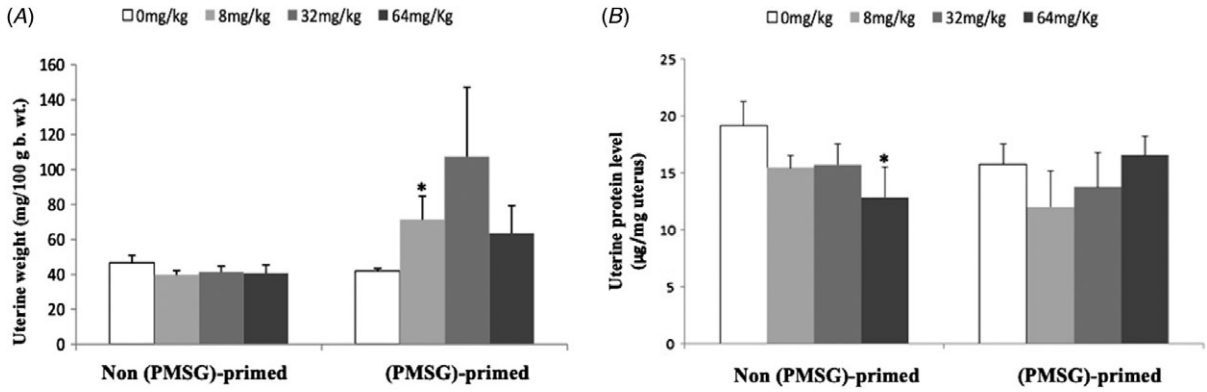

Figure 2. Effect of *E. speciosa* on uterine relative weight (*A*) and proteins level (*B*). Each histogram represents the mean ± SEM of six animals per group. Values significantly different ($^{*}P < 0.05$) from those of the control group.

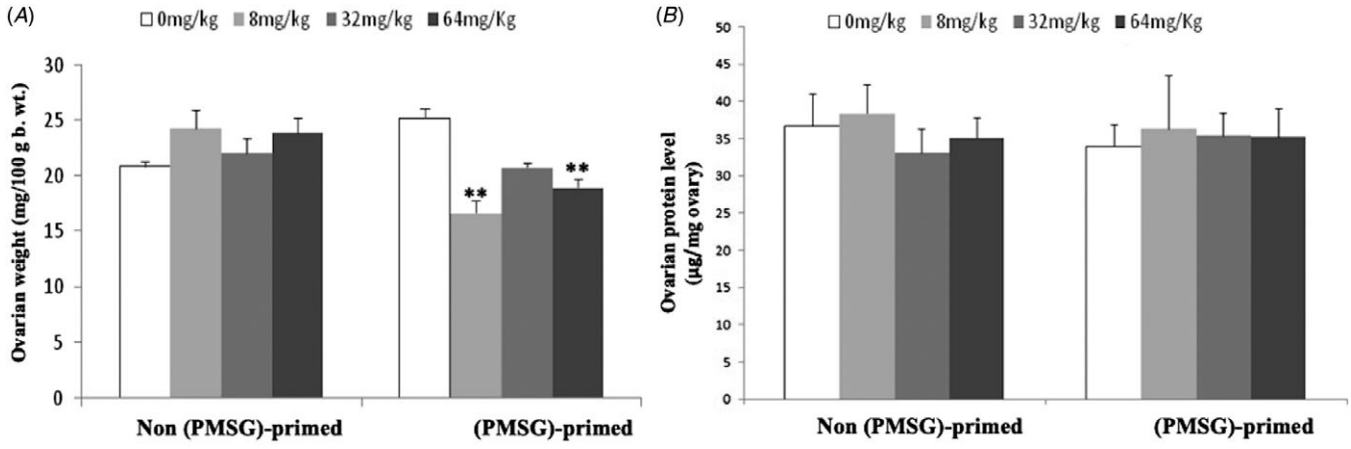

Figure 3. Effect of *E. speciosa* on ovarian relative weight (*A*) and proteins level (*B*). Each histogram represents the mean ± SEM of six animals per group. Values significantly different at ($^{**}P < 0.01$) from those of the control group.

and weak LH-like activities (Moore and Ward, 1980). Worthington and Kennedy (1979) showed that the use of PMSG during 6-week intervals increased the weight of the uterus and the ovary, stimulating the conversion of follicles into corpora lutea and increased the number of follicles (Worthington and Kennedy, 1979). The application of PMSG in immature rats increases ovulation and prevents the atresia of periantral and antral follicles (Braw and Tsafriri, 1980).

In non-PMSG-primed animals, the daily oral administration of an aqueous extract of *E. speciosa* led to a significant reduction in

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Table 2. Percentages of PMSG-primed rats showing vaginal opening

Starting days of vaginal opening	*Eremomastax speciosa* (mg/kg)			
	0	8	32	64
6	0.0 (0/6)^{A,a}	50.0 (3/6)^{A,a}	16.7 (1/6)^{A,a}	0.0 (0/6)^{A,a}
8	0.0 (0/6)^{A,a}	83.3 (5/6)^{B,a}	16.7 (1/6)^{A,a}	0.0 (0/6)^{A,a}
9	0.0 (0/6)^{A,a}	83.3 (5/6)^{B,a}	16.7 (1/6)^{A,a}	0.0 (0/6)^{A,a}
10	0.0 (0/6)^{A,a}	100.0 (6/6)^{B,a}	16.7 (1/6)^{A,a}	0.0 (0/6)^{A,a}
15	0.0 (0/6)^{A,a}	100.0 (6/6)^{B,a}	16.7 (1/6)^{A,a}	16.7 (1/6)^{A,a}

Uppercase letters in the same row and lowercase letters in the same column indicate differences, $P < 0.05$. Total number of rats: $n = 6$.

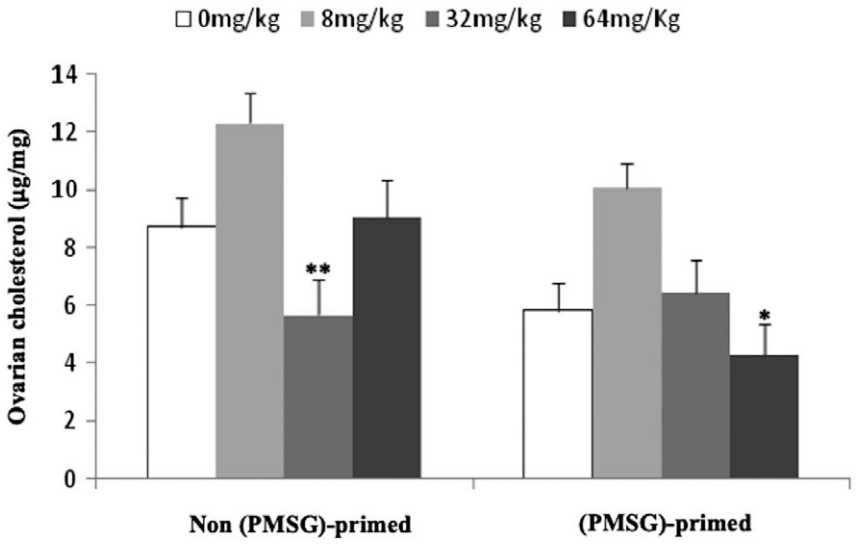

Figure 4. Variation of the ovarian cholesterol in non-primed and PMSG-primed immature rats. Each histogram represents the mean ± SEM of six animals per group. Values statistically different from that of the control group of each day at $^{*}P < 0.05$, $^{**}P < 0.01$.

body weight on days 11, 13 and 15, and was even not dose dependent. Uterine proteins also showed a significant reduction. These results could be due to anti-oestrogenic compounds in *E. speciosa*. Caffeic acid (3,4-dihydroxycinnamic acid) and gallic acid (3,4,5-trihydroxybenzoic acid) found in the aqueous residue of *E. speciose* are phenolic compounds known for their anti-oestrogenic activities (Hidalgo *et al.*, 2012; Rezaei-Seresht *et al.*, 2019). Mechanisms suggest an affinity for the oestrogen receptors and display agonist effects. Furthermore, De Oliveira *et al.* (2012) also suggested that polyphenols from *E. speciosa* could have anti-oestrogen activity, which by binding to oestrogen receptors would interact at the early stages of oestrogen synthesis.

In primed animals, the increase in the relative uterine weight could be due to the anabolic effect of FSH-like compounds from *E. speciosa*. Our results rattest the presence of FSH-like compounds in the extract and may justify its traditional used. FSH has a well established role in reproduction. In females, FSH acts via the FSH receptor located in ovarian granulosa cells to control follicle development and steroidogenesis (Simoni *et al.*, 2019). Sun *et al.* (2006) proposed that rising FSH levels with age may directly induce bone loss independent of oestrogen deficiency. In contrast, contrary to the proposed FSH-induced bone loss, Allan *et al.* (2010) demonstrated that FSH has dose-dependent anabolic effects on bone via an ovary-dependent mechanism, which is independent of LH activity and does not involve direct FSH actions on bone cells. How polyphenols from *E. speciosa* act on the uterus awaits further investigations.

In the present study, a decrease in the ovarian weight was observed at 8 mg/kg and 64 mg/kg in primed rats. This is an unexpected result and could be because PMSG and FSH-like compounds present in *E. speciosa* have probably affected FSH receptors at the granulosa cell level through downregulation. Zhang and Roy (2004) reported that *in vivo*, exogenous compounds like those with FSH-like activity downregulated FSH receptors in the hamster ovary and could be associated with a reduction in ovarian weight and activity. Dierich *et al.* (1998) showed that impairing FSH signalling *in vivo* resulted in reduction of the ovarian and uterine weights. *In vitro* studies, conversely, have shown that *in situ* culture of rats granulosa cells with high concentrations of FSH (>10 ng/ml) caused downregulation of its own receptor (Xiao *et al.*, 1992).

Cholesterol is the main precursor of steroids hormones during their biosynthesis (Moon *et al.*, 2016). Its reduction in both groups at doses of 32 (non-primed) and 64 mg/kg (PMSG-primed animals) clearly showed its utilization in the biosynthesis of estradiol. Similar results have been reported by others authors (Lienou *et al.*, 2012; Telefo *et al.*, 2012).

As reported previously, the main external phenomenon at puberty in immature rats is vaginal opening (Navarro *et al.*, 2004). FSH and LH stimulate proliferation of follicular cells and the production of estradiol by ovarian cholesterol catabolism (Skibola, 2004). Overall, hormonal signalling culminates in opening and cornification of the vagina, and to an increase in ovarian and uterine weights (Counis *et al.*, 2001). In non-PMSG-primed animals, the administration of *E. speciosa* extract at all doses did not lead to vaginal opening,

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while in primed animals, at dose of 8 mg/kg, on day 15, the vaginal orifices of all the animals were opened. This effect demonstrated that antioxidant polyphenol compounds from *E. speciosa* such as apigenin (Ali *et al*., 2017) and rutin (Jahan *et al*., 2016) can influence the hypothalamic–pituitary–gonadal axis and can consequently induce puberty. The same result has been obtained at the 8 mg/kg dose, with the ethanolic extract of *Senecio biafrae* (Lienou *et al*., 2010).

In conclusion, this study confirmed the presence of FSH-like compounds in the aqueous macerate of *E. speciose*, which acts in synergy with PMSG to allow physiological and biochemical modifications of parameters of reproduction and also precocious puberty of immature rats. Globally, a therapeutic dose of 8 mg/kg improved ovarian activity of rats and therefore justifies its used by traditional healers.

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Conflict of interest. None of the authors have any conflict of interest to declare.

Ethical standards. This study was performed according to the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Community guidelines (Radzikowski, 2006).

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