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Potentiation of FSH-like effects of the extracts and fractions of *Senecio biafrae* (Oliv. & Hiern) J. Moore (Asteraceae) by a subthreshold dose of PMSG

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ABSTRACT

The FSH-like effect of *Senecio biafrae* can be potentiated by reproductive hormones to accelerate the awakening of the Hypothalamus-Pituitary-Gonad axis in immature animals. This research evaluated the synergistic effect between Pregnant Mare Serum Gonadotrophin (PMSG) and the extracts and fractions of *S. biafrae* (EFSb) in boosting sexual maturation. Various PMSG doses were administered to im-

mature female rats during 5 consecutive days. Its highest dose without effect on ovary and uterus was co-administered with 4, 8, 32, 64 and 128 mg/kg doses of aqueous, ethanol and methanol/methylene chloride extracts as well as hexane, ethyl acetate and n-butanol fractions of *S. biafrae* during five days. Ovarian and uterine masses, proteins and cholesterol, serum FSH, LH, estradiol, progesterone levels and ovarian follicle numbers were evaluated. The subthreshold value of PMSG was 0.011 UI/rat/day. Its co-administration with EFSb led to a significant drop ($p<0.05$) in ovarian mass, but to an increase tendency in ovarian proteins and cholesterol. Uterine mass and proteins significantly increased in almost all treated animals. FSH, LH and estradiol significantly increased while progesterone was decreasing. Primary and secondary follicle presented high counts in almost all treated groups ($p<0.05$). Co-administration of EFSb and PMSG led to successful stimulation of the reproductive axis.

INTRODUCTION

Infertility is caused by many factors among which hormonal disorders. In female mammals, an unbalanced production or release of these hormones can lead to many dysfunctions among which perturbation of the reproductive cycle, anovulation or cycle blockade, hindering the process of fertilization and culminating in partial or total sterility [1, 2]. Conventional medicine proposes many endogenous hormones derivatives or structural analogous in an attempt to solve the problem. Clomifene citrate and many other synthetic analogous of sex hormones are constantly used for managing menstrual disorders or anovulation in infertile women [3 - 7]. These hormones are also used in stimulation protocols for follicle rapid maturation and superovulation through artificial insemination and *ovum pick-up* techniques in donors or embryo transfer in recipients through Assisted Reproductive Technologies (ARTs) in human or livestock [8 - 10]. The unavailability or harmlessness of these treatments to poor people in rural regions principally in developing countries led to a regained interest in alternative medicine and principally treatments using medicinal plants. Among the plants currently investigated for fertility/antifertility effects, *Senecio biafrae* has already been revealed as a reservoir of estrogenic and/or FSH-like compounds [11 - 15]. As many trials are still ongoing on *S. biafrae* and others medicinal plants revealed by ethnopharmacological surveys made by our research team [16 - 18], it appeared necessary to find a research protocol that would allow rapid evaluation of their pharmacological effects, shortening the duration. The best model for evaluating the effects of medicinal plants on female reproductive parameters is the immature female rat [19, 20]. However, the time for the animal to reach puberty is about 20 to 30 days. So, for evaluating compounds of weak estrogenic effects, the stimulation period appears very

long and as the animal is growing throughout the administration period, important amounts of substances are needed to meet the dose to be administered. It then appeared necessary to reduce the stimulation period by a technique that would accelerate the sexual maturation of the animal so that short-time stimulation with the plant product can lead to precocious puberty, showing its estrogenic/FSH-like activity. PMSG is the equine homologous of the human chorionic gonadotrophin (hCG) used in many stimulation protocols as it possesses strong FSH-like and weak LH-like activities [21]. It has been successfully used to prestimulate immature female rats in previous study; the highest dose of PMSG without effect on ovarian and uterine masses was 0.01UI [22]. As these findings were limited to ovarian and uterine masses and vaginal opening, investigations on more parameters and doses were necessary. Ovarian and uterine proteins are product molecules of the effect of estrogenic compounds on the reproductive axis, further leading to an increase in the masses of the organs [23, 24]; these can be of interest for refining the PMSG dosage without effect on ovary and uterus development. The present research was designed to determine the best PMSG priming dose and evaluate its co-administration with EFSb to immature female rats for designing a best protocol for FSH-like effects evaluation in medicinal plants.

MATERIALS AND METHODS

Animals

The immature female albino Wistar rats (21–23 days old, weighing 25–35 g) used in the experiment were bred in the animal house of the Biochemistry Department (University of Dschang, Western region of Cameroon), 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. Experimental protocols used in this study strictly conformed to the internationally accepted standard ethical guidelines for laboratory animals use and care as described in the European Community guidelines EEC Directive 86/609/EEC, of 24th November 1986 [25].

Preparation of the PMSG solution

The PMSG lyophilisat (Prospec protein specialist, CAT: HOR-272) was diluted in NaCl 0.9% to obtain an initial solution (200 IU/mL). Successive dilutions of this solution was done to obtain the different solutions (0.01, 0.011, 0.012, 0.013, 0.014, 0.015, 0.016, 0.017, 0.018 and 0.019 IU/mL) used in this study.

Extracts and fractions of S. biafrae

The fresh leaves and stems of *S. biafrae* were collected in April 2017 in Baham subdivision (Western Cameroon) and identified at the National Herbarium of Cameroon under voucher specimen code 32999/SRF/Cam. These plant parts were washed and dried at room temperature in the shade. The EFSb were prepared as described in Lienou *et al.* [15]. The six powders (from water, ethanol, methanol/methylene chloride [extracts], hexane, ethyl acetate and n-butanol [fractions]) were further suspended in distilled water for their administration to immature female rats at the considered dosages (4, 8, 32, 64 and 128 mg/kg of body weight).

Treatments

2.4.1. PMSG dose-response effects on ovary and uterus

During the first phase of this study, the highest dose of PMSG without effect on ovarian and uterine weights was determined using a modified protocol of Goka *et al.* [22]. Through the experiment, 55 immature female rats were subdivided into 11 experimental groups of 5 animals each and subcutaneously injected, for 5 consecutive days, PMSG doses of 0.01, 0.011, 0.012, 0.013, 0.014, 0.015, 0.016, 0.017, 0.018 and 0.019 IU/rat/day while control animals received the same amount of NaCl 0.9% (0.2 mL) through the same treatment period. The animals were weighed and checked for vaginal opening on a daily basis. Twenty four hours after the last injection, the animals were euthanized by intra-abdominal injection of thiopental sodium (6 $mg.mL^{-1}$, 30 $mg.kg^{-1}$). Their ovaries and uteri were carefully removed, cleaned of adherent tissue and weighed. These organs were further homogenized in Tris–sucrose buffer (0.25 mol/L sucrose, 1 mmol/L Ethylene Diamine Tetra acetic Acid and 10 mmol/L Tris–HCl, pH 7.4) at 1 and 2% respectively. After centrifugation (4000×g, 15 min), their supernatants were collected and used for protein assay [26].

Potentiation effect of PMSG on EFSb effects in immature rats

A total of 186 immature female rats was randomly divided into 6 groups of 30 animals each and one control group of six animals. Each group of 30 was randomly divided into five subgroups of six animals each. These subgroups were daily subjected, during 5 days, to oral administration of 4, 8, 32, 64 and 128 mg/kg doses of the plant extracts or fractions and intraperitoneal administration of 0.011 UI/rat/day of PMSG while the control group received distilled water by oral gavage and the same dose of PMSG during the same period. The day following the last administration, the animals were eu-

thanized by intra-abdominal injection of thiopental sodium (80 mg/kg). Their blood was collected by cardiac puncture; their ovaries and uteri were removed, blotted and weighed. The blood was centrifuged (2500×g, 15 min) and the serum collected was stored at -20°C for hormonal dosages (FSH, LH, E2 and P4). The left ovary and the entire uterus of each animal were homogenized in Tris–sucrose buffer (0.25 mol/L sucrose, 1 mmol/L Ethylene Diamine Tetra acetic Acid and 10 mmol/L Tris–HCl, pH 7.4) at 1 and 2% respectively. After centrifugation (4000×g, 15 min) of the homogenates, their supernatants were collected and used for protein (as previously described) and cholesterol (from ovarian supernatant only) assays [26, 27, 28, 29]. The right ovary of each animal was fixed in Formaldehyde (10%) and conserved during 48 h for different growing stages follicles counting. Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), estradiol (E2) and progesterone (P4) assays were performed using the direct (for FSH and LH) and indirect (E2 and P4) competitive binding immunological techniques (ELISA). The reagents used to perform these analyses were obtained from GBC (General Biological Corporation, HSIN CHU, 30077, Taiwan, R.O.C) and the hormone levels were obtained by reading the absorbance to Microtiter well reader (Lab Systems Multiskan RC, 351, FIN-00881, Helsinki, Finland) at 450 nm wavelength.

Ovarian histology

The right ovary of each rat was removed from formaldehyde, progressively dehydrated with ethanol (70%, 80%, 90% and 100%) followed by xylene (100%). Each organ was further embedded in paraffin wax and serially sectioned at 7 µm thickness every 60 µm using a Leica rotary microtome (Leica RM 2125, Leica Microsystems Nussloch GmbH, Deutschland), and strips of sections were gently lowered onto the surface of a warm water bath at 40 °C. The floated sections were mounted on microscopic slides and put in an oven maintained at 60 °C for 30–40 min to firmly fix the tissue on the slide. They were further progressively colored in haematoxylin and eosin dyes and dried. All sections were examined microscopically at 100 and 400X magnification and the mean number of primary, secondary and antral follicles in each ovarian cortex was calculated.

Statistical analysis

The data from biological assays were registered as Mean ± SE (standard error). The statistical differences between the values were shown by ANOVA (Analysis of Variance) test. The Fisher Low Significant Difference (LSD) and Student-Newman-Keuls (SNK) tests were used for the comparison of means and

the significance of the differences was established at the 5% level ($p>0.05$) [30].

RESULTS

PMSG dose-response effects on ovary and uterus:

The intraperitoneal administration of different doses of PMSG to immature female rats through 5 consecutive days led to various observations. It appears obvious that high dosages of PMSG led to earlier puberty in experimental animals showed by the vaginal opening in all treated animals (Table 1). But considering the other parameters (ovarian and uterine proteins and relative weights), the significant modifications were obtained starting from the 0.012 UI/kg/day dosage. This shows that the highest dosage without effect on ovarian and uterine weights and proteins is 0.011 UI /rat/day.

Effect of the co-administration of EFSb and PMSG during 5 days on reproductive parameters in immature female rats

Effects on ovarian mass, cholesterol and proteins

Except the 8 and 32 mg/kg doses of the methanol/methylene chloride extract, a general drop was experienced in ovarian masses of all the animals treated with the extracts and fractions independently on the dosage, as compared to control animals (Fig. 1). The most important drops were shown by the 32 and 128 mg/kg dosages of the aqueous extract, the 32 and 64 mg/kg dosages of the hexane fraction and the 128 mg/kg dosage of the ethyl acetate fraction. Relatively, ovarian proteins increased significantly ($p<0.01$) with almost all the doses of the aqueous extract and the three fractions; animals treated with ethanol extract experienced a significant drop at 8 ($p=0.12$) and 128 mg/kg ($p = 000$) dosages. The highest values of protein rates were registered at low dosages (4, 8 and 32 mg/kg) with the n-butanol fraction, while high doses (64 and 128 mg/kg) were best ranked with the ethyl acetate fraction. Ovarian cholesterol significantly rose ($p<0.01$) in almost all treated animals as compared to control animals; the highest value was registered with the 64 mg/kg dosage of the ethanol extract. The hexane fraction was the only one which presented a significant decrease at almost all the dosages. The lowest cholesterol rates were obtained in animals treated with the hexane fraction.

Effect of EFSb on uterine mass and proteins

The uteri relative masses significantly increased in treated animals of all the extracts and fractions,

principally, the ethyl acetate and n-butanol fractions as compared to control animals, in except of the 4 mg/kg dosage ($p = 0.007$) of the hexane fraction and the 32 mg/kg dosage of the ethyl acetate fraction ($p = 0.000$) which experienced a significant drop.

Uterine proteins globally increased in the experimental groups receiving the ethanol extract (32 and 64 mg/kg dosages), ethyl acetate and hexane 4 and 32 mg/kg dosages, and principally the n-butanol fraction for which the increase was observed in all the administered doses. Significant drops were experienced in this parameter only with the aqueous extract 32 and 128 mg/kg dosages (Fig. 2).

Effect of EFSb on hormones levels

The animals co-administered with PMSG and EFSb presented different hormonal profiles (Fig. 3):

Serum FSH significantly increased at 4 mg/kg, 8 mg/kg and 128 mg/kg dosages of the aqueous ($p = 0.011$), methanol/methylene chloride extract ($p = 0.014$) and ethyl acetate fraction ($p = 0.014$) respectively. However, this has significantly dropped in animals administered the 4 mg/kg dosage of the methanol/methylene chloride extract ($p = 0.018$) or the ethyl acetate ($p = 0.045$) and n-butanol ($p = 0.038$) fractions. A significant drop ($p = 0.001$) was also experienced at the 128 mg/kg dosage of that same extract when compared to the control group (Fig. 3A).

Comparing the LH values of the PMSG + EFSb-treated animals and the control ones, it appears a global increase in LH levels, which was significant ($p<0.01$) with the aqueous (8 and 32 mg/kg), ethanol (4 and 8 mg/kg) and methanol/methylene chloride (64 mg/kg) extracts as well as the ethyl acetate and n-butanol ($p<0.05$) 8 and 128 mg/kg dosages. The 8 mg/kg dosage showed the highest LH rates in almost all the treated groups (Fig. 3B).

As concerns estradiol, its serum level was significantly raised ($p<0.01$) in almost all the co-administered animals as compared to control animals. However, the positive influence of the treatment appeared the most in the aqueous extract 8 mg/kg-treated animals which was 53 times higher than the same dosage of the ethyl acetate fraction treated-animals (Fig. 3C).

Progesterone values significantly dropped ($p<0.001$) in almost all the treated groups as compared to the control; only the 8 mg/kg dosage of the hexane fraction presented a significant increase ($p = 0.026$) in that parameter. The most important drop was obtained with that same dosage of methanol/methylene chloride extract administered animals (Fig. 3D).

Effects of EFSb on the number of follicles at different growing stages

The number of primary follicles significantly rose at different doses in animals treated with all the extracts and fractions as compared to the control group ($p<0.01$). The 8 mg/kg, 32 mg/kg and 64 mg/kg were ranked as the ones with greater primary follicles numbers respectively for the ethanol extract, the ethyl acetate and n-butanol fractions (Fig. 4A).

As compared to the control, the number of secondary follicles has significantly raised ($p<0.001$) in animals treated with all the dosages of the ethanol and methanol/methylene chloride extracts (Fig. 4B). A significant increase was also recorded with the fractions at the doses of 4 ($p = 0.000$) and 8 ($p = 0.001$) mg/kg for hexane, 32 and 128 mg/kg for the ethyl acetate ($p = 0.000$); as well, the 32 ($p=0.003$), 64 and 128 ($p=0.000$) mg/kg doses were also significantly raised with the n-butanol fraction. However, a significant drop was experienced with the 128 mg/kg dosage of the aqueous extract ($p = 0.003$) and the 64 mg/kg dosage of the hexane ($p = 0.002$) and ethyl acetate ($p = 0.019$) fractions.

Only the 4 mg/kg dosage of the ethyl acetate fraction led to an increase ($p = 0.001$) in the number of tertiary follicles (Fig. 4C). A general decrease tendency was noticed in all the experimental groups when compared to the control group: all the doses of the aqueous extract as well as the hexane and n-butanol fractions led to a significant decrease in the parameter ($p<0.001$ or $p<0.01$).

DISCUSSION

The present study was undertaken to investigate the potentiation property of PMSG on the aqueous, ethanol and methanol/methylene chloride extracts as well as the hexane, ethyl acetate and n-butanol fractions of the leaves and stems of *S. biafrae* on reproductive parameters of immature female rats. PMSG is a natural glycoprotein possessing strong FSH-like and weak LH-like activities. It is produced by the chorion of pregnant mares and is commonly used associated with progesterone derivatives to induce ovulation in livestock prior to artificial insemination [21]. A subcutaneous administration of PMSG to immature female rats during 5 consecutive days led to a significant increase in ovarian and uterine masses and proteins in animals treated with doses greater than 0.011 IU per rat. In response to its FSH-like activity, several ovarian follicles in immature female rat mature through the fixation of FSH or analogous to their granulosa cells receptors, thus regulating the expression of some genes related to proliferation and steroidogenesis in ovarian cells [31]. According to the results obtained, 0.011

UI of PMSG can be administered to immature female rat to accelerate its sexual maturation in such way that a compound with enough FSH-like activity can complete it, leading to puberty, opening and cornification of the vagina in female rat in 5 days. The 4 and 128 mg/kg dosages were then added to the commonly used dosages (8, 32 and 64 mg/kg) of EFSb to verify whether the use of 0.011 UI of PMSG would lead to an increase or reduction in the dosage for attaining the same effects [11 - 15].

The co-administration along 5 days of EFSb and 0.011 UI of PMSG to immature female rats led to various and controversial effects on some of the folliculogenesis parameters evaluated through the present study. The ovarian masses were significantly reduced in various doses of all EFSb. However, the significant drop was not correlated with a decrease in ovarian proteins levels except in animals treated with the ethanol extract. These results show a possible synergistic effect between the active substances of the extract and the PMSG through its FSH-like effect [21, 32 - 34]. The association with the active compounds of the plant surely led to an adverse effect at the level of the ovary, manifested by the significant reduction in ovarian parameters. The result can be due to an important production/action of compounds of estrogenic activity contained in the plant extract, functioning as weak estrogens, leading to a down regulation effect [13, 15]. The effect is more obvious with the increase in ovarian proteins and uterine parameters. The rates of the reproductive hormones as well as the scores of the ovarian follicles categories tend to confirm that observation.

These results show the estrogenic effect of the plant associated with the synergistic FSH-like effect between the extracts/fractions and the PMSG on the ovarian and uterine physiology. However, the significant increase in ovarian cholesterol rate suggest that the estrogenic compounds would be of exogenous origin because ovarian cholesterol is the precursor of the biosynthesis of steroid hormones [35], thus, its high rate shows that it seems to have not been used for that purpose. A prolonged GnRH/FSH-like effect leads to inhibition in the production and release of steroid hormones at the level of the ovary. In fact, a continuous secretion/action of GnRH leads to an inhibition in the release of endogenous gonadotrophins, culminating in an inhibition of ovarian steroidogenesis [36, 37]. This effect can be noticed with almost all the doses of EFSb, suggesting a high concentration of FSH/GnRH-like compounds in the plant. The increase in estradiol rate was correlated to a drop in progesterone with many administered dosages of EFSb. The result suggests a stimulation, at these particular dosages, in the activity of the enzymes of the biosynthesis of estradiol from progesterone. The enzymes generally synthesized at high speed during the follicular phase of the estrus cycle catalyze the aromatization of androgens (coming from a common way and primary passing through progesterone production) into

estrogens. Many enzymatic ways then assure the conversion of progesterone into estradiol [35, 38]. These reactions can be stimulated by pituitary FSH or exogenous FSH-like compounds [39]. However, besides the genomic action of sex steroids, the importance of rapid, non-genomic signaling initiated at the cell-membrane is increasingly recognized. Receptors involved in rapid estrogen signaling include the membrane-localized forms of ERα and ERβ, and possibly G-protein-coupled receptor 30 (GPR30). Rapid gestagen signaling has been attributed to membrane G-protein-coupled gestagen receptors mPRα, mPRβ, and mPRγ and membrane-localized forms of nuclear PR [40]. This can contribute to explain the controversial tendency in the rates of both ovarian hormones (E2 and P4) at the level of the follicular tissue and in their expression on ovarian and uterine protein synthesis and growth in response to the anterior pituitary command.

The feasibility of using PMSG-primed immature rats to shorten the generally long period (20 to 30 days till the puberty) commonly used for evaluating the fertility properties of medicinal plants in immature female rats was also assayed throughout this study. When compared to previous research where 8, 32 and 64 mg/kg dosages were administered to immature female rats along 20 consecutive days [13 - 15], the following observation can be released:

The decrease in ovarian masses of almost all the extracts and fractions was observed only with the 64 mg/kg of the ethyl acetate fraction when administered over 20 days. This same fraction and the n-butanol one were the only fractions which presented a significant increase in ovarian proteins and cholesterol but the present research shows a significant increase in these parameters in almost all the treated groups. As concern uterine mass and proteins, the present research shows a significant decrease in uterine mass after 5 days instead of the increase revealed after 20 days of consecutive administration. The significant decrease observed with administration of the 8 and 32 mg/kg doses of aqueous and methanol/methylene chloride extracts as well as the hexane fraction observed in uterine proteins rate were not observed in previous research where, on the contrary, there was an increase tendency.

The treatment during 20 days led to significant reductions in the rates of pituitary hormones of animals treated with the three extracts. The present research showed an increase tendency in the parameters for the animals treated with different EFSb. However, the increase observed in the rate of the hormones of 20 days hexane fraction-treated animals did not appear in the present research. The estradiol rates which were low after 20 days were increased in the present research. A controversial situation was observed with the progesterone rates. Only the hexane fraction presented the same tendency in the parameter. The results of follicles count (Fig. 5) showed many differences when compared with the results

of previous tests: the increase in primary and secondary follicle numbers as well as the decrease in tertiary follicles were not noticed after the 20-days treatment.

The global observation is that there are many differences and controversial observations when comparing the treatment of 20 consecutive days and the co-administration of EFSb and 0.011 UI/rat/day of PMSG along 5 days. Of course, the FSH-like effects of the association could have been greater than the one of *S. biafrae* only. As it is well known that differences in the reproductive parameters can be due to slight difference in hormone rates in growing animals, a situation which can be exacerbated by the onset of puberty in immature animals, as the phenomenon leads to great changes in animal's physiology. Thus, the attainment of puberty could be responsible of the variations observed in PMSG-primed rats treated with different EFSb. The variations in the behavior of different treated groups are certainly due to the difference in active compounds concentrations in the administered extracts or fractions. Goka *et al.* [22] investigated the highest dose of PMSG without effect on ovarian and uterine masses and obtained 0.01 UI/kg/rat. More research was then necessary to refine the priming dose of PMSG by checking between the dose without effect (0.01 UI/kg/rat) and the 0.02 UI/kg/rat dose which expressed an effect, as the five days duration has already been specified for that purpose through previous research [22]. So, the present work shows 0.011 UI/kg/rat as the best priming dose of PMSG to be administered for five consecutive days to get almost the same results of the 20 days - treatment but only in some extracts and fractions according to the behavior of the evaluated reproduction parameters. Nevertheless, more research is needed to really match the results of the two treatment durations.

CONCLUSION

The results of the present research are of value as it developed a new protocol for evaluating FSH-like effects of medicinal plants extracts and fractions, especially in the case of *Senecio biafrae*. More research is then necessary to refine the priming dose of PMSG by checking between the dose without effect (0.01 UI/kg/rat) and the 0.02 UI/kg/rat dose which expressed an effect, as the five days duration has already been specified for that purpose through previous research [22]. So, the present work shows 0.011 UI/kg/rat as the best priming dose of PMSG to be administered for five consecutive days to get almost the same results of the 20 days - treatment but only in some extracts and fractions according to the behavior of the evaluated reproduction parameters. Nevertheless, more research is needed to really match the results of the two treatment durations.

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The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization: **LLL, PBT**; Formal analysis: **PBT**; Investigation: **LLL, SMCG, GTM, NJD**; Methodology: **PBT, LLL**; Project administration: **PBT**; Resources: **LLL**; Software: **PBT, LLL**; Supervision: **ARNN, PBT**; Original draft: **LLL**; Writing - review & editing: **All the authors**

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455 **Table 1.** Effect of different doses of PMSG on ovarian and uterine masses and proteins and on oviduct mass

	Control	0.01UI	0.011UI	0.012UI	0.013UI	0.014UI	0.015UI	0.016UI	0.017UI	0.018UI	0.019UI
Ovarian Relative weight (mg/kg bw)	0.063 ± 0.008	0.294 ± 0.073	0.240 ± 0.018	0.503** ± 0.254	0.354* ± 0.001	0.324* ± 0.032	0.189 ± 0.069	0.581** ± 0.073	0.524** ± 0.062	0.150 ± 0.021	0.127 ± 0.017
Uterine relative weight (mg/kg bw)	0.324 ± 0.020	0.299 ± 0.086	0.441 ± 0.108	1.718** ± 0.749	0.735* ± 0.038	1.173** ± 0.568	0.627* ± 0.050	2.192** ± 0.852	0.764 ± 0.105	0.442 ± 0.083	5.106*** ± 1.367
Oviduct relative weight (mg/kg bw)	0.165 ± 0.011	0.106* ± 0.003	0.114* ± 0.008	0.153 ± 0.030	0.141 ± 0.024	0.157 ± 0.004	0.163 ± 0.007	0.142 ± 0.001	0.179 ± 0.009	0.156 ± 0.004	0.086* ± 0.021
Ovarian proteins (µg/mg)	0.125 ± 0.039	0.854* ± 0.168	0.734 ± 0.379	0.917* ± 0.010	1.585** ± 0.180	1.417** ± 0.044	0.543 ± 0.133	4.431*** ± 0.290	2.838*** ± 0.543	0.660 ± 0.285	0.841 ± 0.266
Uterine	4.260 ±	5.641 ±	8.751 ±	13.157* ±	10.380 ±	22.303** ±	27.460** ±	36.318** ±	18.068* ±	12.264* ±	174.808*** ±

proteins (µg/mg)	0.095	0.575	1.875	3.082	0.678	12.164	4.765	11.241	2.592	2.426	42.361
Vaginal openings	0	0	0	1	2	3	3	3	4	4	5

456 *, ** and *** Values significantly different respectively at $p<0.05$, $p<0.01$ and $p<0.001$ from those of the control group (ANOVA and Fisher LSD). Each value represents

457 the mean ± SE of the values for 5 animals.

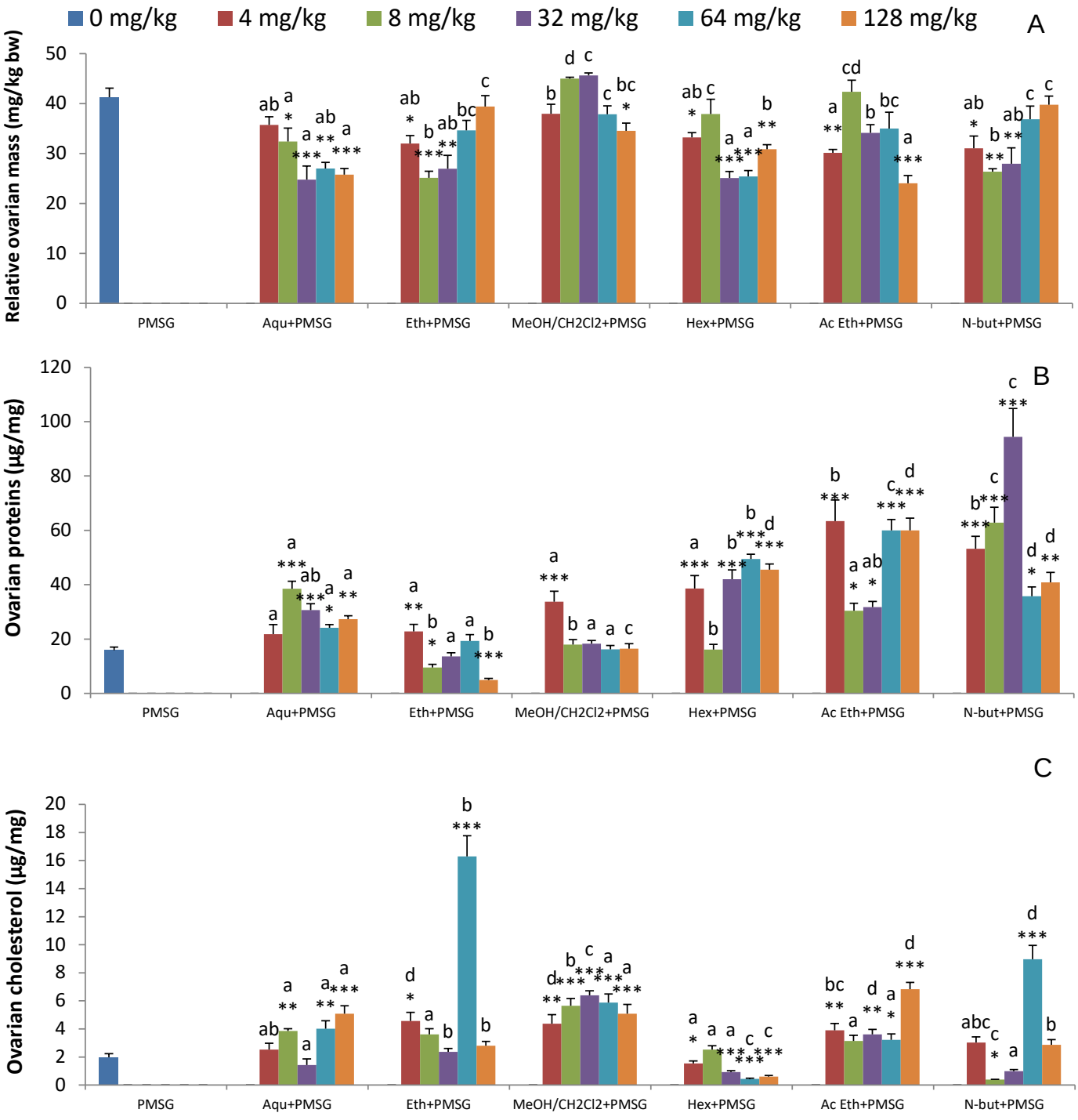

Figure 1. Effect of EFSb co-administered with PMSG on the ovarian mass (A), proteins (B) and cholesterol (C) levels.

*, ** and *** Values significantly different respectively at $p<0.05$, $p<0.01$ and $p<0.001$ from those of the control group (ANOVA and Fisher LSD); different letters a, b, c,... represent differences between the same doses of different extracts or fractions (SNK test). Each histogram represents the mean ± SE of the values for 6 animals.

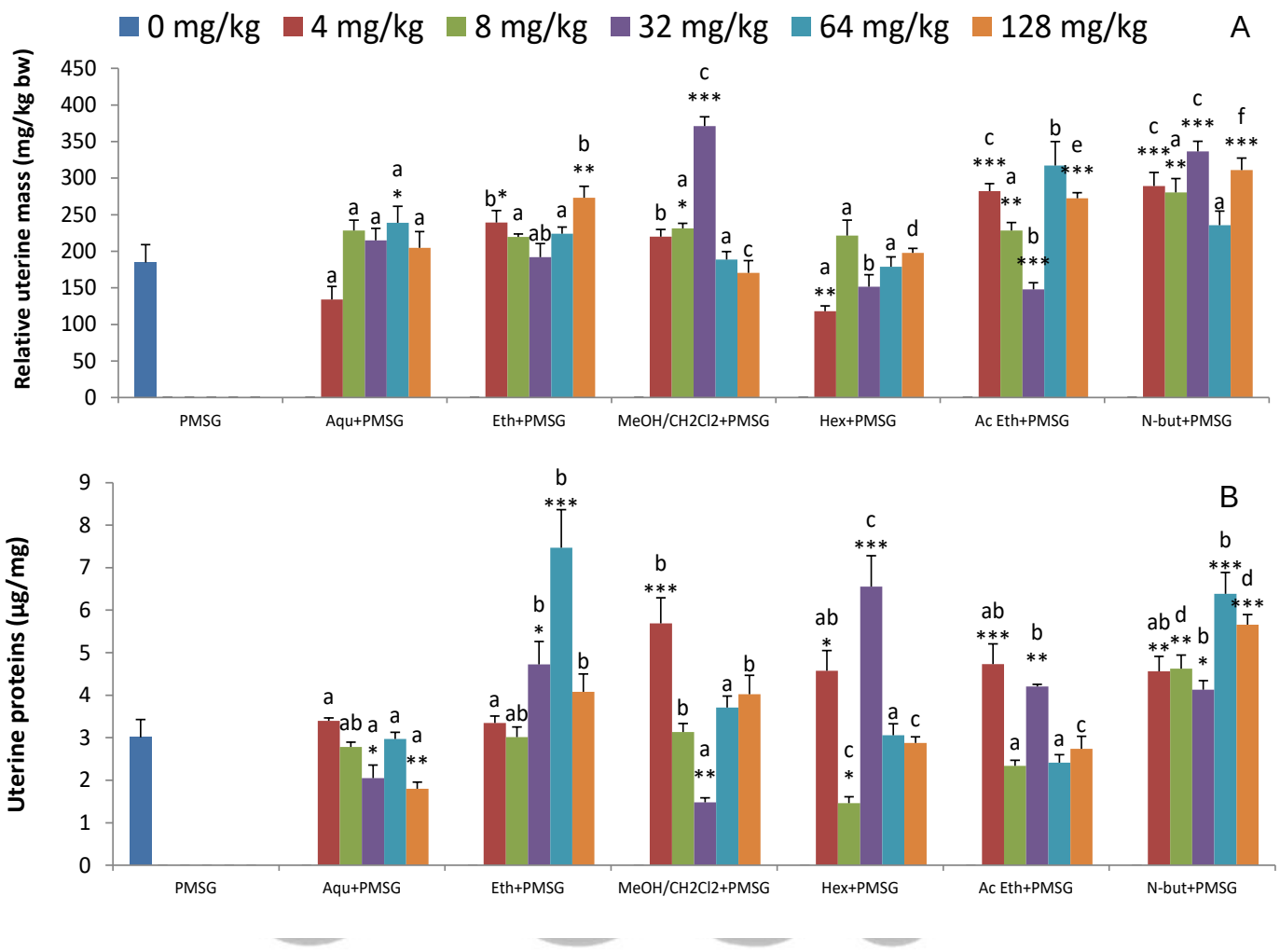

Figure 2. Effect of EFSb co-administered with PMSG on uterine mass (A) and proteins level (B).

*, ** and *** Values significantly different respectively at $p<0.05$, $p<0.01$ and $p<0.001$ from those of the control group (ANOVA and Fisher LSD); different superscript letters a, b, c,... represent differences between the same doses of different extracts or fractions (SNK test). Each histogram represents the mean ± SE of the values for 6 animals.

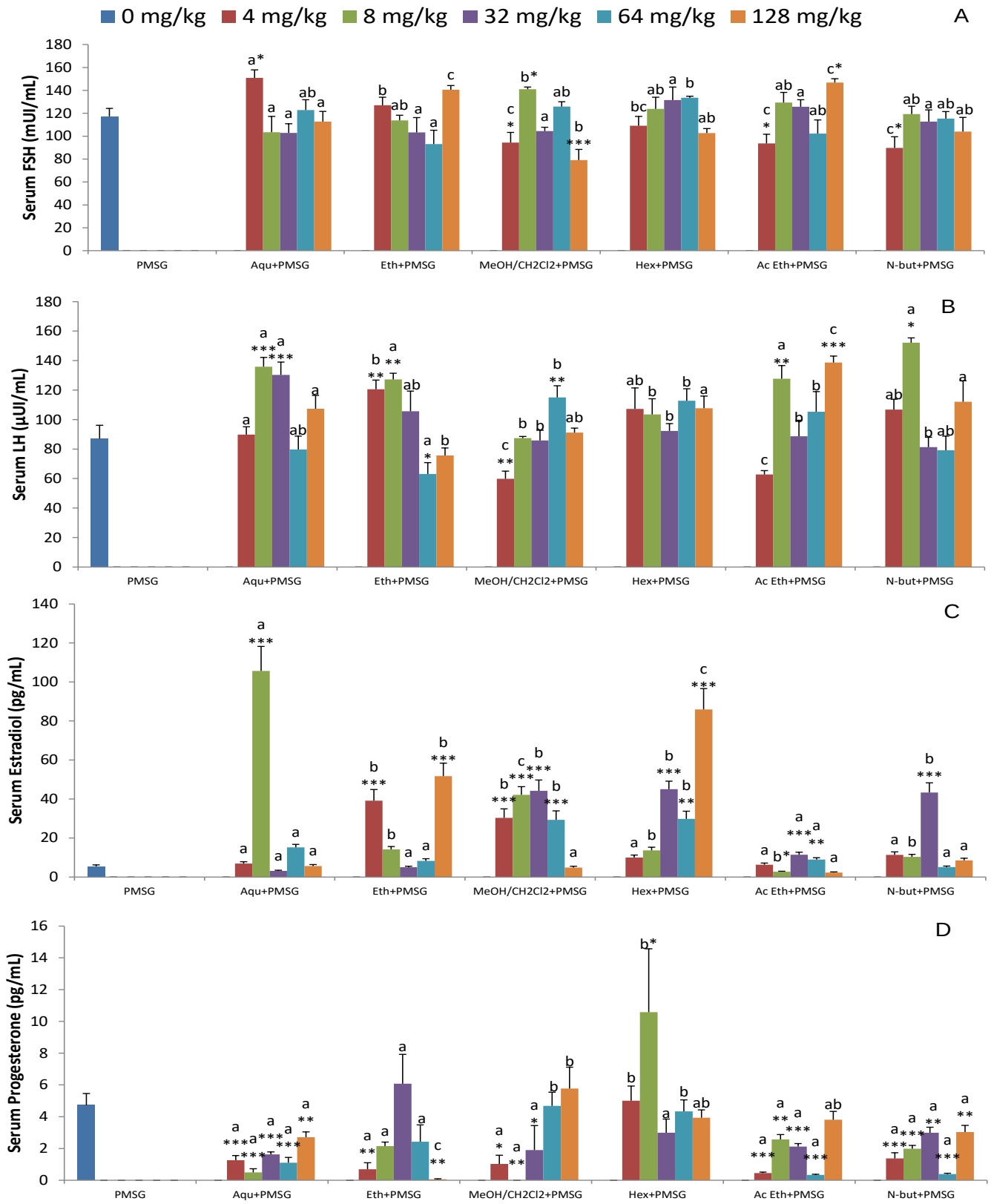

Figure 3. Effect of EFSb co-administered with PMSG on the levels of serum FSH (A), LH (B), estradiol (C) and progesterone (D).

, ** and *** Values significantly different respectively at p<0.05, p<0.01 and p<0.001 from those of the con-

trol group; different letters [a, b, c,...] represent differences between the same doses of different extracts or fractions (ANOVA and Fisher LSD). Each histogram represents the mean ± SE of the values for 6 animals. The values of the FSH rates are positively correlated to the LH values ($p<0.01$) while the P4 values are negatively correlated to E2 values ($p<0.01$).

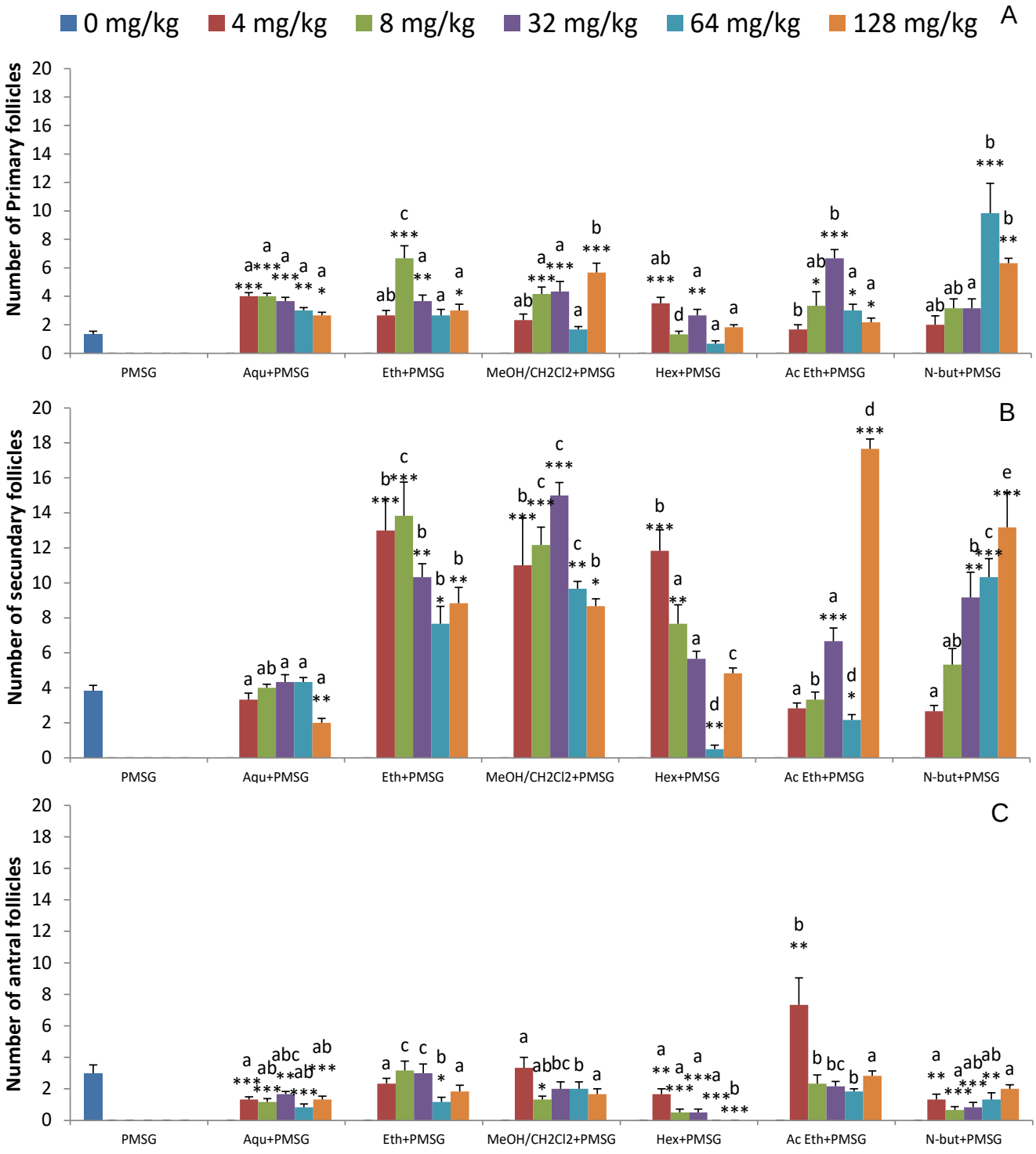

Figure 4. Effect of EFSb co-administered with PMSG on the numbers of primary (A), secondary (B) and tertiary (C) follicles.

*, ** and *** Values significantly different respectively at $p<0.05$, $p<0.01$ and $p<0.001$ from those of the control group; different letters [a, b, c,...] represent differences between the same doses of different extracts or fractions (ANOVA and Fisher LSD). Each histogram represents the mean ± SE of the values for 6 animals. The values of the FSH rates are positively correlated to the LH values ($p<0.01$) while the P4 values are negatively correlated to E2 values ($p<0.01$).

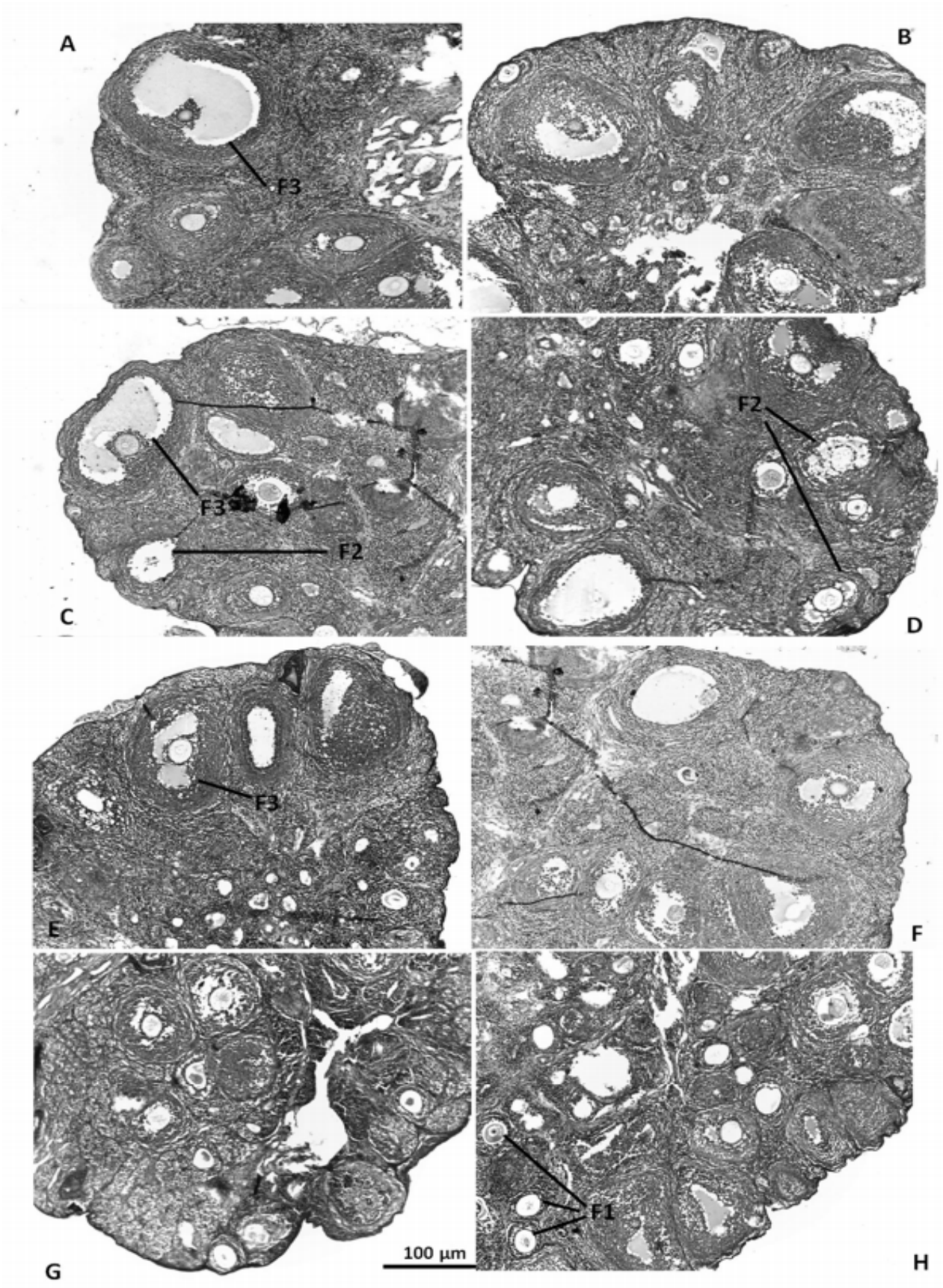
A
F3
B
C
F3
F2
D
F2
E
F3
F
G
100 µm
H
F1

Figure 5. Representative Images of ovarian sections stained using haematoxilin - eosin staining system. The photographs show sections presenting primary (F1), secondary (F2) and antral follicles (F3). Magnification: 100x

A= PMSG control,
B= 4 mg/kg dose of Ethyl acetate fraction + PMSG,
C= 32 mg/kg dose of Ethanol extract + PMSG,
D= 32 mg/kg dose of MeOH/CH2Cl2 extract + PMSG,
E= 64 mg/kg dose of Aqueous extract + PMSG,
F= 4 mg/kg dose of hexane fraction + PMSG,
G= 64 mg/kg dose of n-butanol fraction + PMSG,
H= 128 mg/kg dose of n-butanol fraction + PMSG