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Effect of ethanolic extract of *Senecio biafrae* on puberty onset and fertility in immature female rat

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ABSTRACT
Senecio biafrae is a medicinal plant widely used by traditional healers in the western region of Cameroon for the treatment of woman infertility. In the course of the evaluation of its effect on puberty onset and some physiological parameters of fertility, different doses of its ethanolic extract were orally administered to immature female rats for 30 days. Body, ovarian, uterus weight; uterine, ovarian proteins or cholesterol level as well as data on puberty onset were recorded.
A linear increase in the growth rate of all animal was observed throughout the experimental period. From the 27th day till the end of treatment, the body weight gain of rats treated with 8 mg/kg was significantly increased ($p<0.05$) compared to that of control animals. The age of animals at vaginal opening (VO) was significantly reduced ($p<0.05$) in those treated with the dose of 8 mg/kg (41.54 ± 0.50) compared to control animals (43.33 ± 0.73). Ovarian weight and uterine protein level of animals treated at 64 mg/kg significantly increased ($p<0.05$). A reduction in the number of corpora lutea with doses of 32 and 64 mg/kg as well as an increase in the resorption index with doses 8 and 64 mg/kg were also noticed.
Overall, the present results provide evidence on the estrogenic effect of the ethanolic extract of *Senecio biafrae* leading to the acceleration of puberty onset in immature female rat.
Key words: Fertility; *Senecio biafrae*; puberty; ovaries; uterus

INTRODUCTION

Medicinal Plants, which are important for the development of modern drugs, have been used for many years in daily life to treat diseases all over the world [1, 2]. Indeed, many of these plants are used to treat various reproductive ailments such as women infertility which is a public health concern in Sub-Saharian Africa [3]. *Senecio biafrae* (Asteraceae) is one of these plants [4-6]. It naturally occurs in the African forest zones, from Guinea to Uganda, and is mainly cultivated in Cameroon and Nigeria where it constitutes a food of choice [7]. It is very rich in proteins (29%), food fibers and mineral elements such as manganese, sodium, potassium, magnesium and calcium [8]. It is also known for its therapeutic virtues, notably in Nigeria where it is used in the treatment of diabetes or pulmonary defects [9-11]. In West and Northwest regions of Cameroon, ethnobotanical studies revealed its utilization in the treatment of cases of women infertility [4, 5, 12]. One of these studies, undertaken among traditional healers in Baham subdivision, indicated that the concoction was mainly prepared by maceration in palm wine and administered twice daily during 30 days to the patient [6]. Moreover the high frequency of utilization of this plant by traditional healer of various villages in this area to cure women infertility may constitute a proof of its efficiency. However, to the best of our knowledge, no scientific studies have been undertaken on the effect of *S. biafrae* on mammalian reproductive function.

The present study therefore aimed at evaluating the effect of the ethanolic extract of *S. biafrae* on puberty onset and fertility induction in immature female rats. These animals have long been used as a model system for studying *in vivo,* the potentialities of pharmacological compounds [13, 14] and medicinal plants extracts or derived compounds [15-18] to induce ovarian folliculogenesis. In those various studies, the

gonadotrophic-like effects of the preparation was characterized by the following biological parameters: - increase in the weight of the ovary and uterus ; - opening and cornification of the vagina; - formation of corpora lutea or changes in the histology of the ovary, uterus and vagina; - induction of ovulation; -increase in ovarian estradiol, progesterone, protein levels, -decrease in ovarian cholesterol level. Thus, the precocity of the puberty onset in treated animals was evaluated through the determination of their age at vaginal opening and the inducing effect of the extract on animal fertility evaluated through the determination of ovarian and uterine weight, protein or cholesterol levels; number of corpora lutea, implantation sites and other gestational parameters.

MATERIALS AND METHODS

Plant extract preparation

The fresh leaves and stems of *Senecio biafrae* (Oliv. & Hiern) J. Moore were collected in January 2010 in Baham subdivision (western region of Cameroon) and identified in the National Herbarium of Cameroon under voucher specimen code 32999/SRF/Cam. These parts were washed and dried at room temperature. The dried plants were ground in a mortar and the powder obtained was used to prepare the ethanolic extract by maceration in ethanol 95° for 24 h. The extracts thus obtained were concentrated by recovering the solvent by Rotary Evaporator. The concentrated extract was then evaporated to dryness at 45°C in a ventilated oven. The dried extract was weighted and stored at 4°C in a refrigerator. The yield of extraction was 5.6 %. This dried extract was further used to prepare, in distilled water, the extracts to be administered to animals at concentration of 2.8 mg/ml (extract 1), 11.2 mg/ml (extract 2) and 22.4 mg/ml (extract 3). Together with distilled water (control group), these preparations (extract 1, 2, 3) were orally administered to animals in a volume of 10 ml/kg body weight, thus corresponding to doses of 0, 8, 32, and 64 mg/kg respectively. The dose of 8 mg/kg was obtained by reconstitution from the recipe mainly used by traditional medicine practitioners in the ethnopharmacological survey performed in Baham subdivision (Western region of Cameroon), and the two other doses (32, 64 mg/kg) were its multiples [6].

Animals

The animals used in this study were immature female albino Wistar rats, 21-22 days old, weighing 30–45 g. They were bred in the animal house of the Biochemistry Department (University of Dschang), housed under natural conditions of light (12h cycle) and temperature (22 ± 2°C) and fed a standard laboratory diet and tap water *ad ibitum.*

Experimental procedure

A total of fifty-two immature female rats were randomized into 4 groups of thirteen animals each, based on their body weight. They received by oral route, either distilled water or different doses of the ethanolic extract for 30 consecutive days and were weighed, throughout the experimental period, at 2 days interval. After two weeks of treatment, the vaginal opening of each rat was checked every day and the vaginal smear collected, from the day of the opening up to the end of the experiment, permitted to characterize the phases of the estrous cycle, its length and that of the complete cycle. The vaginal smears were stained by May-Grünwald solution (1 % w/v) followed by Giemsa solution (1 % w/v) and staged by light microscopy.

At the end of the experimental period, 6 animals in each group were randomly sacrificed under chloroform anesthesia. The ovaries and uteri were removed, blotted, weighed and stored at -20°C until use. They were then 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. After centrifugation (4000 x g, 15 min), their supernatants were collected and used for protein [19] and cholesterol [20, 21, 22] assays.

The remaining rats (7 per group) were matched, the following day and during two weeks, with males of proven fertility. Vaginal smears were collected on a daily basis in order to assess the presence of sperm. A laparoscopy was undertaken, under diazepam (5 mg/ml, 5 mg/kg) and Ketamin (50 mg/ml, 80 mg/kg), ten days after the day of mating to count the number of implantation sites in uterine cords and the number of corpora lutea in ovaries. After delivery, the fetuses were weighed and their number recorded. After delivery, the fetuses were weighed and their number recorded. From these data, the number of resorption sites (number of implantation site – number of alive fetuses), implantation index ([total

number of implantation sites/number corpora lutea] x 100), resorption index ([total number of resorption sites/total number of implantation sites] x 100), preimplantation loss ([number of corpora lutea – number of implantations/ number of corpora lutea] x 100), postimplantation loss ([number of implantations x number of live fetuses/number of implantations] x 100), antifertility activity ([number of females without living fetuses/total number of females] x 100), antiimplantation activity ([number of females without implantation sites/ total number of females] x 100), and gestation rate ([number of females with living fetuses at birth/total number of gestational females] x 100) were calculated [23].

Statistical analysis

The data from biological assays were registered as Mean ± SEM (Standard Error on the Mean). The statistical differences between the values were shown by ANOVA (Analysis of Variance) test. The Fisher PLSD test was used for the comparison of means. The analysis of percentages was done by X^2 (Chi-square) test. The Kruskall-Wallis test was used for non parametrical data, and the Mann Whitney test used when their differences were significant [24].

RESULTS

Effect on body weight gain

The effect of the ethanolic extract of Senecio *biafrae* on the body weight gain of female rats during the treatment is presented in figure 1. There was a linear increase, at various rates, in their growth. A slight decrease in the body weight gain of the animals treated at high dosages (32 and 64 mg/kg), as compared to that of control animals, was indeed noticed after 13 days of treatment. Animals receiving the 8 mg/kg dose instead gained more corporal weight ($p<0.05$) than the control group starting from the 27th day of treatment till the end of the experiment.

Effect on the age and estrus cycle phases at vaginal opening

Figure 2 illustrates the average age of animal at vaginal opening and the percentage of those presenting vaginal aperture at a given age. Female rats receiving the extract at the lowest dose (8 mg/kg) presented vaginal opening two days earlier ($p<0.05$) as compared to control animals (43.33 ± 0.73 *vs* 41.54 ± 0.50) (Figure 2A). This precocity is well shown in figure 2B where 77 % of animals receiving the therapeutic dose of the extract presented the vaginal opening at the age of 42 d, versus 38 % for the animals of the control group. In addition, the vaginal opening was observed in all the animals of that group when they were 45 days old while the complete opening of the vagina of control group animals was obtained at the age of 50 d. Most of the animals presented an estrus (43 % – 70 %) or metoestrus (21 % - 39 %) phase of the cycle on the day of their vaginal opening (Table 1).

Effect of *Senecio biafrae* on the lenght of the estrous cycle

No change in the estrous cycle length of animals of various experimental groups as well as in the duration of the estrus, metestrus and diestrus phases of their cycle was observed (Table 2). However, a significant decrease ($p<0.05$) in the length of the proestrus phase of animals treated at the dose of 32 mg/kg as compared to that of the control animals was recorded.

Effect on ovarian weight, protein or cholesterol level and corpora lutea

The changes obtained after 30 days of oral administration of various doses of the ethanolic extract of *S. biafrae* are presented in figure 3. No significant variation in ovarian cholesterol level was recorded (Figure 3B). When administered at the dose of 64 mg/kg and comparatively to the control group, the extract significantly increased ($P<0.05$) the ovarian weight (Figure 3A). Ovarian protein was significantly reduced ($P<0.05$) in all animals treated with the plant extract whatever the dosage (Figure 3C), while the reduction in the number of corpora lutea was significantly ($P<0.05$) obtained in animals treated at the dose of 32 mg/kg (Figure 3D).

Effect on uterine weight and proteins

No variation in the uterine weights of animal was recorded. Uterine protein was significantly increased by 45.5 % ($P<0.05$), 86.5 % ($P<0.01$) and 87.8 % ($P<0.01$) after administration of the extract at doses of 8, 32 and 64 mg/kg respectively (Figure 4).

Effect on some fertility and gestational parameters

Table 3 shows that the intragastric administration of ethanolic extract of *S. biafrae* at doses of 8, 32 and 64 mg/kg had no significant effect on the

number of pregnant rat, implantation sites and fetal weight. A significant reduction in the number of corpora lutea was recorded with the highest dose (P<0.05). A slight increase in the number of resorption sites at the dose of 8 mg/kg was obtained. This resulted in an increase in antiimplantation and antifertility activities (14 % *vs* 0 % for control group) and a significant increase (P<0.05) in resorption index (27 % *vs* 0 %) at this dosage..

Table 1: Frequency (%) of estrous cycle phases at vaginal opening in female rats treated with various doses of *Senecio biafrae*.

	Phases of the cycle				Total
Doses	Proestrus	Estrus	Metestrus	Diestrus	
0 mg/kg	19.05	42.9	33.3	4.8*	100
8 mg/kg	7.7*	53.8	38.5	0*	100
32 mg/kg	0*	69.2	23.1	7.7*	100
64 mg/kg	21.4	50	21.4	7.1*	100

*Values significatively different at p < 0.05 of those of the estrus phase (Fisher and Khi square tests)

Table 2: Effect of *Senecio biafrae* on the length (days) of the various phases in the estrous cycle.

Doses	Phases of the cycle				
	Proestrus	Estrus	Metestrus	Diestrus	Cycle Length
0 mg/kg	0.83 ± 0.17	1.38 ± 0.19	0.57 ± 0.11	1.22 ± 0.16	4.00 ± 0.16
8 mg/kg	0.58 ± 0.10	1.55 ± 0.23	0.42 ± 0.12	1.46 ± 0.21	4.01 ± 0.17
32 mg/kg	0.33 ± 0.12*	1.57 ± 0.31	0.46 ± 0.12	1.59 ± 0.15	3.95 ± 0.21
64 mg/kg	0.50 ± 0.11	1.41 ± 0.14	0.68 ± 0.1	1.41 ± 0.15	4.00 ± 0.13

The length of the phases is expressed as days. Each value represents the mean ± SEM for 13 animals.*Values significantly different at p < 0.05 from those of the control group (ANOVA and Fisher PLSD).

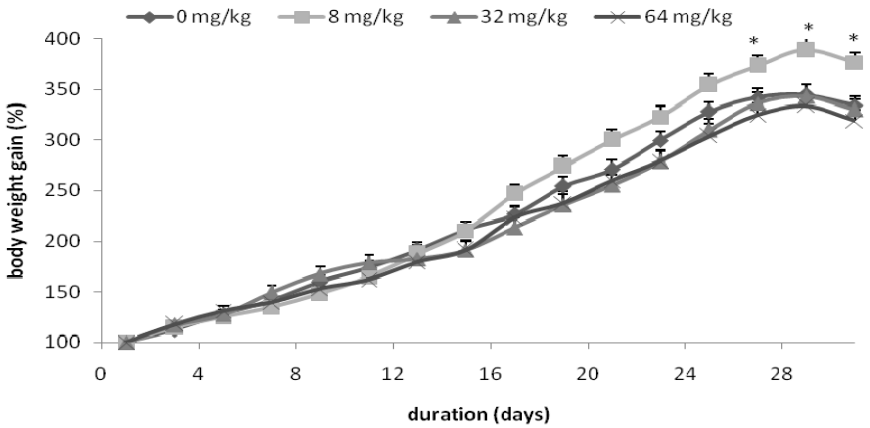

Figure 1: Body weight gain of rats after administration of various doses of ethanolic extract of *Senecio biafrae*. Each point represents mean ± SEM. *Values statistically different from that of the control group of each day at P < 0.05 (ANOVA and Fisher PLSD).

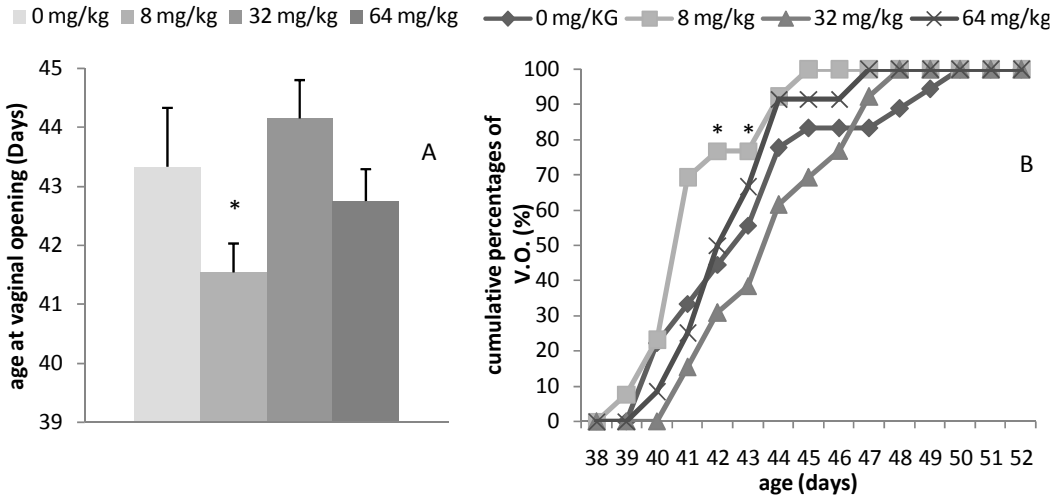

Figure 2: Age of animals at the day of vaginal opening (A) and Cumulative percentages of vaginal opening relatively to animal ages (B) after oral administration of *Senecio biafrae*.
*Values significantly different at $p < 0.05$ from those of the control group (Kruskal-Wallis, Mann-Whitney tests (A) exact Fisher, and Khi Square tests (B)); each histogram in A represents the mean ± SEM of the values for 13 animals.

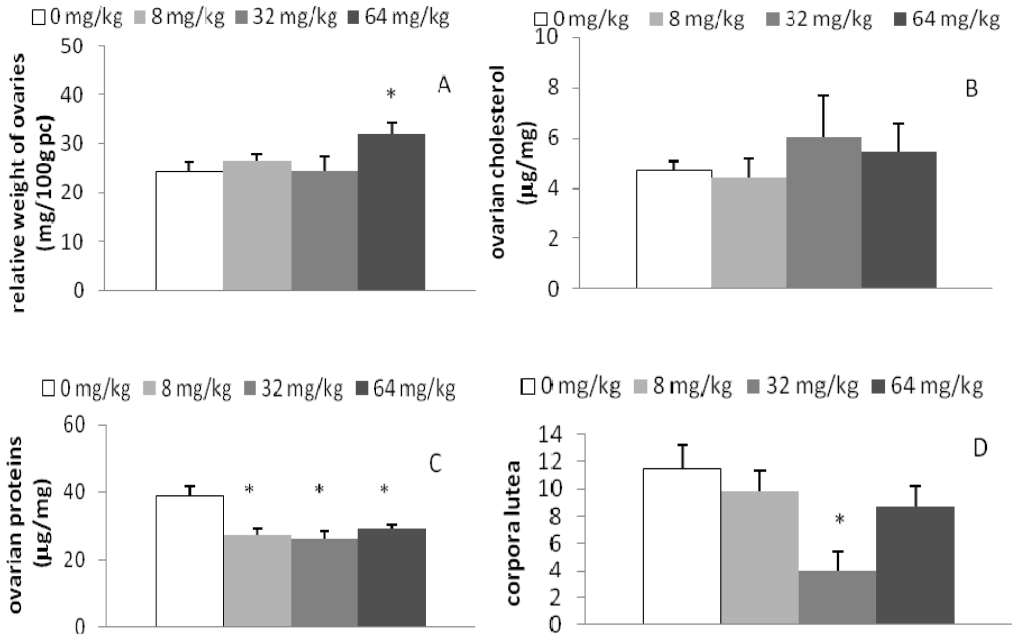

Figure 3: Effect of the ethanolic extract of *Senecio biafrae* on the relative weight of the ovaries (A), the ovarian cholesterol (B) and proteins (C) levels, the number of corpora lutea (D).
*Values significantly different at ($p < 0.05$) from those of the control group (ANOVA and Fisher PLSD). Each histogram represents the mean ± SEM of the values for 6 animals.

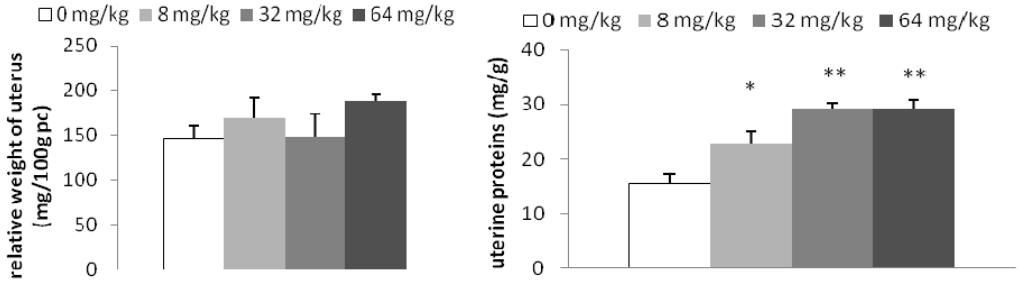

Figure 4: Effect of *Senecio biafrae* ethanolic extract on uterine relative weight and proteins level.
*Values significantly different at ($p < 0.05$) from those of the control group (ANOVA and Fisher PLSD); each histogram represents the mean ± SEM of the values for 6 animals.

Table 3: Effect of the ethanolic extract of Senecio biafrae on some fertility and gestational *parameters*

Studied parameters	Dosage (mg/kg/Day)			
	0	8	32	64
N°Corpora Lutea	11.90 ± 0.31	11.33 ± 0.71	11.43 ± 0.90	10.43 ± 0.75*
N°Implantation sites	10.80 ± 0.44	9.67 ± 0.56	9.71 ± 0.57	9.57 ± 0.57
N° Fetuses Alive	9.50 ± 0.50	7.00 ± 1.71	8.86 ± 0.70	7.14 ± 1.32
N°Resorption sites	1.30 ± 0.47	2.67 ± 1.74	0.857 ± 0.404	2.43 ± 1.45
Mean weight of fetuse (g)	4.87 ± 0.085	4.95 ± 0.23	4.84 ± 0.19	5.33 ± 0.22
Fixation rate (%)	90.93 ± 3.36	85.68 ± 3.41	86.83 ± 5.22	92.45 ± 2.85
Preimplantation Loss (%)	9.07 ± 3.36	14.32 ± 3.41	13.17 ± 5.22	7.55 ± 2.85
Postimplantation Loss (%)	0.12 ± 0.04	0.27 ± 0.13	0.09 ± 0.05	0.24 ± 0.13
Antiimplantation Activity (%)	0	14.29	0	0
Antifertility Activity (%)	0	14.286	0	0
Resorption Index (%)	12.04	27.59*	8.824	25.37*
Gestation Rate (%)	100	83.333	100	100

Each value represents the mean ± SEM for 7 animals.*Values significantly different at $p < 0.05$ from those of the control group (ANOVA; Fisher PLSD and Khi Square tests).

DISCUSSION

Senecio biafrae, which is the plant of interest in this study, is used by some African populations for its nutritional and pharmacological properties [7-10]. Their estrogenic and inducing effects on some physiological parameters of the onset of puberty (age and phase of the estrous cycle at vaginal opening) has been evaluated. The choice of these parameters was not only directed by the influence of the gonadotropic hormones (FSH, LH, PMSG, GnRH) in the induction of the follicular growth and the precocious onset of puberty in immature female rats, but also by the clinical usage of these hormones in the treatment of various forms of infertility (ovulatory defects or hypogonadal infertility) [25]. The ethanolic extract of *S. biafrae* led to an early onset of vaginal opening in animals treated with the therapeutic dose (8 mg/kg). This result could be attributed to the implication of some compounds of the plant extract in the precocious maturation of the ovarian follicles. In various mammals, precocious puberty onset can be induced in a prepubertal animal by repeated injections of GnRH, FSH, LH or of an analogous compound of a general excitatory neurotransmitter of the central nervous system, like glutamate or aspartate which induced the pulsatile releases of GnRH [26]. This shows that the ethanolic extract of *S. biafrae* could contain a molecule acting as one of these compounds on the onset of puberty. The reduction in the age at vaginal opening observed in animals receiving the

therapeutic dose of the extract could then result from a high follicular production of estrogens, following the maturation of the hypothalamo-pituitary-ovarian axis, through a pulsatile secretion of GnRH and gonadotropins. The ethanolic extract of *S. biafrae* could also contain some phytoestrogens, or estrogenic compounds which could directly induce precocious vaginal opening. As puberty is also the result of an increase in body weight [26], the significant increase in the body weight of the animals treated with the therapeutic dose could also explain the precocity in the pubertal onset.

The opening of the vagina during the pubertal age of the rats results from the increase in the secretion of estradiol by ovarian follicles. The vaginal cells are also keratinized in this high estrogenic environment. That is why the vaginal smear of pubertal rat on the day of vaginal opening corresponds to the estrus phase of the cycle. As can be seen in Table 1, the main phase of the cycle observed at vaginal opening in all treated animals was estrus or metestrus, which are the vaginal cornified cells containing phases of the cycle [27]. The short length of the estrous cycle of rats also makes them ideal for investigation of changes occurring during the reproductive cycle [27]. In animals treated with 32 mg/kg of *S. biafrae*, the proestrus phase of their cycle was significantly reduced. This could be linked to the acceleration of ovarian follicular growth, given the high estrogenic environment of the ovarian cells, following the induction of pulsatile secretion of GnRH and gonadotropins by the extract.

Estrogens and estrogen-like compounds (phytoestrogens) exert their biological effects following their fixation to their receptors in their main target organs (ovary, uterus, hypothalamus, bone,...) which leads to a chain of reactions, culminating in the biosynthesis of biomacromolecules (DNA, RNA, and proteins) and the increase in the weight of ovary and uterus [1, 28, 29]. But, at a high dosage, these estrogenic compounds, by a feed-back effect on their hypothalamic receptors, inhibit the pulsatile secretion of GnRH and thus of gonadotrophins (FSH, LH) which finally result in the inhibition of folliculogenesis and reduction in estradiol synthesis and secretion [30]. The data of ovarian and uterine parameters presented in this study have shown an increase of more than 45 % in uterine proteins with all the doses, and an increase in the ovarian weight at the dose of 64 mg/kg, thus confirming the estrogenic effect suggested. As concerns the number of corpora lutea, its decrease with the doses of 32 or 64 mg/kg could be linked to the reduction in the ovulation rate of these animals following the negative feed-back, at the level of the hypothalamus, of high estrogens level induced by the plant ethanolic extract. This down regulation of estradiol synthesis and secretion may lead to the reduction in ovarian protein level with all the doses.

The significant level in the resorption index with doses 8 and 64 mg/kg may result from the increase in the contraction of uterine smooth muscle which may be related to the estrogenic potential of the plant extract [31].

Globally, this study has shown the implication of some compounds of the ethanolic extract of *S. biafrae* on the rapid maturation of ovarian follicle cells thus leading to a precocious puberty onset. The mechanisms of this stimulation are multiple (induction of GnRH synthesis or secretion, induction of gonadotropins synthesis or secretion, effects of estrogens-like compounds or specific amino acids present in the extract). Further studies are required for its elucidation. However, the consumer may be aware of the negative effect of the long term usage (30 days) of this ethanolic extract. The suitable duration of its administration may be 20 days or less as this duration correspond to the period of significant increase of vaginal opening in rats treated with the therapeutic dose.

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