HOSTED BY

Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage: www.elsevier.com/locate/apjtd

Document heading doi: 10.1016/S2222-1808(14)60805-7

Comparative effects of the crude methanol/methylene chloride extract and fractions of *Senecio biafrae* (Oliv. & Hiern) J. Moore on some fertility parameters in immature female Wistar rats

Landry Lienou Lienou[1], Bruno Phelix Telefo[1*], Charlotte Nangue[2], Bale Bayala[3], Stephanie Chekem Goka[1], Didiane Mefokou Yemele[1], Richard Simo Tagne[1], Nathalie Jiatsa Donfack[4], Gildas Tetaping Mbemya[1], Ana Paula Ribeiro Rodrigues[4]

[1]*LABPMAN, Department of Biochemistry, Faculty of Science, University of Dschang, P.O Box: 67 Dschang, Cameroon*

[2]*Pathological Analysis Department of the Central Hospital of Yaounde, Cameroon*

[3]*Laboratory of Animal Physiology, University of Ouagadougou, UFR/SVT, 03 P.O. Box 7021 Ouagadougou 03, Burkina Faso*

[4]*LAMOFOPA, Faculty of Veterinary sciences, State University of Céara, Fortaleza, Brasil*

PEER REVIEW

Peer reviewer

Dr. Mahmoud E F Abdel-Haliem, Associate professor, Molecular Biology, Botany Department, Faculty of Science – Zagazig University, Egypt.

E-mail: Farrag1999@yahoo.com

Comments

This is a good study in which the authors using the leaf and stem extracts of *S. biafrae* of methanol/methylene chloride, and obtained good results of the two extract; due to the polarity of the solvent used for extraction, could indicate the type of secondary metabolite that is active in the plant.

Details on Page 410

ABSTRACT

Objective: To assay the comparative effect of *Senecio biafrae* leaves and stems methanol/methylene chloride extract as well as its hexane, *n*-butanol and ethyl acetate fractions on reproductive function of immature female rat.

Methods: Various doses of the methanol/methylene chloride extract and fractions were orally administered for 20 consecutive days to immature female rats. The parameters of the rats such as body, ovarian and uterus weight, uterine, ovarian and serum proteins, ovarian cholesterol as well as follicle-stimulating hormone, luteinizing hormone, estradiol and progesterone levels were recorded.

Results: Levels of ovarian cholesterol and protein as well as uterine protein were doubled principally in animals treated with the ethyl acetate fraction not matter what dose was administered. The animals treated with crude extract presented a significant increase ($P < 0.05$) of 108%, 79% and 135% in serum estradiol level at the dose of 8 mg/kg, 32 mg/kg and 64 mg/kg respectively.

Conclusions: Results of the present study provide evidence on the inducing effect of the methanol/methylene chloride extract of *S. biafrae* and its fractions on immature female rat's fertility parameters in serum, ovary and uterus.

KEYWORDS

Crude extract, Fractions, *Senecio biafrae*, Fertility, Ovary, Uterus

1. Introduction

Senecio biafrae (*S. biafrae*), a plant used in the African pharmacopoeia, grows in equatorial forests and wet areas of Africa. It is very important for inhabitants of the localities where it is found. The plant is used as food, for traditional rites and for therapeutic purposes[1-4]. In the Western region of Cameroon, *S. biafrae* is principally used for fertility improvement and the treatment of some reproductive tract ailments that can impair fertility of women[1]. In view of the valorization of the therapeutic virtues of *S. biafrae*, its methanol/methylene chloride extract and fractions were subjected to many studies concerning their pharmacological effects on some basic fertility parameters in immature female rats.

Previous work on the ethanolic and aqueous extracts of the

*Corresponding author: Bruno Phelix Telefo, University of Dschang, Faculty of Science, Department of Biochemistry, Laboratory of Biochemistry of Medicinal Plants, Food Sciences and Nutrition, P.O.Box: 67 Dschang, Cameroon.

Tel: +237 77 20 42 67 / Fax: + 237 33 45 19 35

E-mail : bphelix@yahoo.co.uk

Foundation Project: Supported by International Foundation for Science (Grant No. F/4731-1).

Article history:
Received 19 Sep 2014
Received in revised form 20 Oct 2014
Accepted 3 Jan 2015
Available online 20 Mar 2015

plant administered for 30 d to immature female rats showed the stimulating effects on the sexual maturation of the animals[5,6]. The extracts used in these previous studies were formulated based on the procedures used by the traditional healers of the Baham subdivision (Western Cameroon) during the preparation of recipes. These recipes were prepared by maceration in water or palm wine[1].

Many studies aiming at elucidating the mechanism of action of medicinal plants are usually undertaken on their methanol/methylene chloride extracts. This is justified by the fact that the mixture of these two solvents was shown to have a better extraction power on the active secondary metabolites of plants at cellular level[7]. Literature also shows that *S. biafrae* contains many secondary metabolites of different polarities such as dihydroisocoumarins, terpenoids, sesquiterpenes, amino acids *etc*. that can play many therapeutic roles[8-10]. So the activity, depending on the polarity of the solvent used for extraction, could indicate the type of secondary metabolite that is active in the plant.

Moreover, the duration of 30 days of treatment used during previous work has been shortened to 20 d. It is well known that puberty, which culminates with vaginal opening, occurs in female Wistar rats between their 40th and 50th day after birth[11]. Consequently, the appearance of the estrous cycle during this period is linked to high variability in the biochemical and physiological parameters of reproduction in female rats, depending on the cycle phases. A reduction in the duration of the treatment would obviously lead to a better evaluation of the biological effect of the plant extract and fractions. For a better view of the mode of action of the active compounds in *S. biafrae*, additive parameters such as follicle count and serum reproductive hormone levels have also been included. Hence to assay the comparative effect of *S. biafrae* leaves and stems on reproductive function of immature female rat, the present study was conducted using methanol/methylene chloride extract as well as its hexane, *n*-butanol and ethyl acetate fractions.

2. Material and methods

2.1. Preparation of the extracts

The fresh leaves and stems of *S. biafrae* were collected in October 2012 in the Baham subdivision (Western Region of 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. The dried plants were ground in a mortar and the powder obtained was used to prepare the methanol/methylene choride extract by maceration in the mixture of the two solvents (1:1) for 72 h. At the end of the maceration period, the solution was filtered with a Whatman paper and concentrated by recovering the solvent at 65 ˚C in a rotary evaporator. The concentrated solution was then completely dried in a ventilated oven at 45 ˚C. The yield of extraction was 9%. One fifth of the crude extract powder was kept at -20 ˚C for the assays and the rest was dissolved and successively extracted with pure hexane, ethyl acetate and *n*-butanol solvents. The extraction was repeated with each solvent until it appears clear, meaning that the considered solvent cannot extract compounds again, and the residue was transferred to a different solvent for extraction. The solutions obtained by extraction with the same solvent were further mixed, concentrated and dried using the same previous process (rotary evaporator followed by oven). It allowed to obtain hexane, ethyl acetate and *n*-butanol fractions. After the extraction with the *n*-butanol solvent, there was no more residue, showing that all the plant active constituents have been extracted by the three solvents and should be found in the three partitions obtained.

The four powders (from methanol/methylene chloride, hexane, ethyl acetate and *n*-butanol) were further suspended in distilled water to prepare the extracts at different concentrations for their administration to the animals at the needed dosages, *i.e*, 8, 32, and 64 mg/kg of body weight.

2.2. Phytochemical screenings

Preliminary phytochemical screenings of various active compounds from the crude methanol/methylene chloride extract and its fractions were performed using different techniques based on color changes in the solution.

2.2.1. Test for polyphenols

A mass of 0.5 g of the powdered sample was boiled in 20 mL of distilled water and then filtered using a filter paper. A volume of 5% (w/v) $FeCl_3$ was added to the filtered samples and observed for the presence of brownish green or blue black color showing the presence of polyphenols[12].

2.2.2. Test for steroids

The crude plant extract (1 mg) was taken in a test tube and dissolved with chloroform (10 mL). Then, an equal volume of concentrated sulphuric acid was carefully poured along the side of the test tubes. The positive result of the test was materialized by the red coloration of the upper layer combined with the green fluorescence coloration, at the bottom of the tube, of the sulphuric acid layer[12,13].

2.2.3. Test for terpenoids

About 1 mL of acetic anhydride and 2 mL of concentrated sulphuric acid were added into beakers containing 1 mL of previously filtered extract. The reddish brown color formed on the interface indicated the presence of terpenoids[14].

2.2.4. Test for coumarins

A volume of 5 mL of each previously filtered extract was introduced in a test tube and covered by a filter paper saturated in NaOH. Then the test tube was introduced in water bath and boiled for 10 min. After that, the filter paper was recovered and exposed to UV light. The presence of coumarines was indicated by a green bright or yellow color[15].

2.2.5. Test for flavonoids

Few quantity of each extract was dissolved in water and filtered. A volume of 2 mL of a 10% aqueous solution of sodium hydroxide was later added to produce a yellow coloration. A change in color from yellow to colorless after further addition of diluted hydrochloric acid was an indication for the presence of flavonoids[15].

2.2.6 Test for anthranoids

A mass of 50 mg of plant material was boiled for 2 min with 2 mL of 0.5 mol/L KOH and 0.5 mL of 5% H_2O_2. After cooling, the mixture was filtered and the filtrate treated with 6 drops of acetic acid and the resulting solution was mixed with 5 mL of toluene. The upper layer (toluene) was separated with a pipette and transferred to a test tube with 2 mL of 0.5 mol/L KOH. The red color that appeared in the aqueous layer showed the presence of anthranoids[16].

2.3. Animals

The animals used in this experiment were immature female albino Wistar rats (21-23 d old, weighing 25-35 g). They were obtained from the animal house of the Biochemistry Department (University of Dschang). These animals 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*.

2.4. Ethical consideration

Experimental protocols used in this study strictly conformed with the internationally accepted standard ethical guidelines for laboratory animal use and care as described in the European Community guidelines EEC Directive 86/609/EEC, of 24th November 1986[16]. They were designed and submitted to our local ethical committee which validated it before the beginning of the experiments.

2.5. Treatments

A total of 65 immature female rats were randomly divided into 5 groups: four groups of fifteen animals each and one control group of five animals. Each group of fifteen animals was randomly divided into three subgroups of five animals each. These subgroups were daily subjected, during 20 d, to oral administration of increasing doses (8, 32, 64 mg/kg) of the plant crude extract and organic fractions while the control group received distilled water. The animals were weighed every two days of treatment.

At the day following the last administration, the animals were sacrificed by thiopental sodium injection. Their blood was collected by cardiac puncture; their ovaries and uteri were removed, blotted and weighed. The blood was later on centrifuged (1 800 r/min, 15 min) and the serum collected was stored at -20 ˚C for the levels of proteins and hormones [follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol and progesterone)[17]. 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 Tetraacetic Acid and 10 mmol/L Tris–HCl, pH 7.4) at 1 % and 2 % respectively. After centrifugation (2 880 r/min, 15 min) of the homogenates, their supernatants were collected and used for protein and cholesterol (for ovarian supernatant) assays[17-20]. The right ovary of each animal was fixed in Bouin's liquid and conserved for 48 h for histological analysis and counting of the follicles at different growing stages. Figure 1 shows photography of some ovarian cortical regions under microscopy (100× magnification). It shows different stages of growing follicles (primary, secondary and tertiary follicles).

The FSH, LH, estradiol and progesterone assays were performed using the direct (for FSH and LH) and indirect (Estradiol and Progesterone) 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 (LabSystems Multiskan RC, 351, FIN-00881, Helsinki, FINLAND) at 450 nm wavelength.

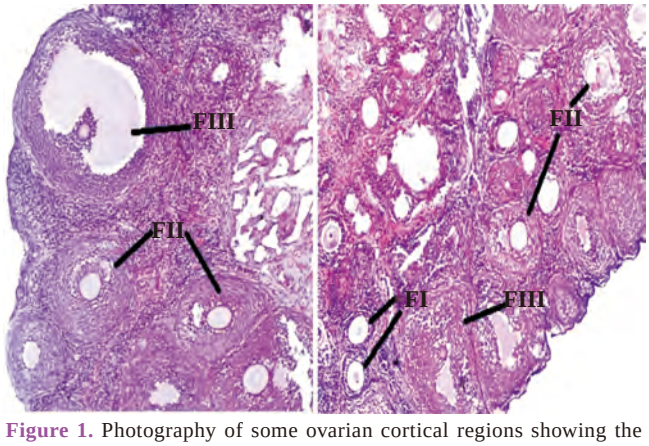

Figure 1. Photography of some ovarian cortical regions showing the different stages of growing follicles.

FI: The primary follicles; FII: The secondary follicles; FIII: The antral follicles. 100× magnification.

2.6. Ovarian histology

The right ovary of each rat was removed from Bouin's liquid, progressively dehydrated with ethanol (70%, 80%, 90% and 100%) followed by xylene (100%). Each tissue block was further embedded in paraffin wax and serially sectioned at 7 µm thickness every 60 µm using 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 fix the tissue firmly on the slide. They were further coloured progressively in haematoxylin and eosin dyes and dried. All sections were examined microscopically at 100× magnification and the mean number of primary, secondary and antral follicles in the ovarian cortex was calculated for each specimen.

2.7. Statistical analysis

The data from biological assays were registered as mean ± SEM. The statistical differences between the values were shown by ANOVA test. The Fisher LSD test was used to compare the means and the significance of the differences was established at the level of 5% ($P < 0.05$)[21].

3. Results

3.1. Phytochemical screening

The results of the phytochemical screening of the plant extract and fractions are presented in Table 1. The crude extract of *S. biafrae* and its fractions showed the presence of steroids, polyphenols and coumarines; while no traces of triterpenoids, flavonoids and anthranoids were found.

Table 1

Phytochemical evaluation for some secondary metabolites in the Plant.

Active compounds	Crude methanol/methylene chloride extract	Hexane fraction	Ethyl acetate fraction	*n*-butanol fraction
Steroids	+++	++	+	-
Triterpenoids	-	-	-	-
Polyphenols	+++	+	+	++
Flavonoids	-	-	-	-
Coumarines	+++	+	++	+
Anthranoids	-	-	-	-

+: Presence of the metabolite, and the number of symbols show its abundance; -: Absence of the metabolite.

3.2. Effect on the body weight gain

The effect of the crude extract and its fractions on the body weight of immature female rats during the treatment period is presented in Figure 2. There was a linear increase, at various rates, in the growth of the animals of all groups. When compared to control animals, those receiving 8 mg/kg of ethyl acetate fraction presented a significant drop ($P < 0.05$) in their body weight gain from the beginning till the 17th day of treatment (Figure 2D). No significant variation, as compared to body weight gain of control animals, was observed in other treated groups at all administered doses. As concerns the monitoring of their food intake, no significant variation was observed between the different experimental groups (data not shown).

3.3. Effect on ovarian weight, protein and cholesterol levels

The effects of the extract and fractions of *S. biafrae* on the ovarian

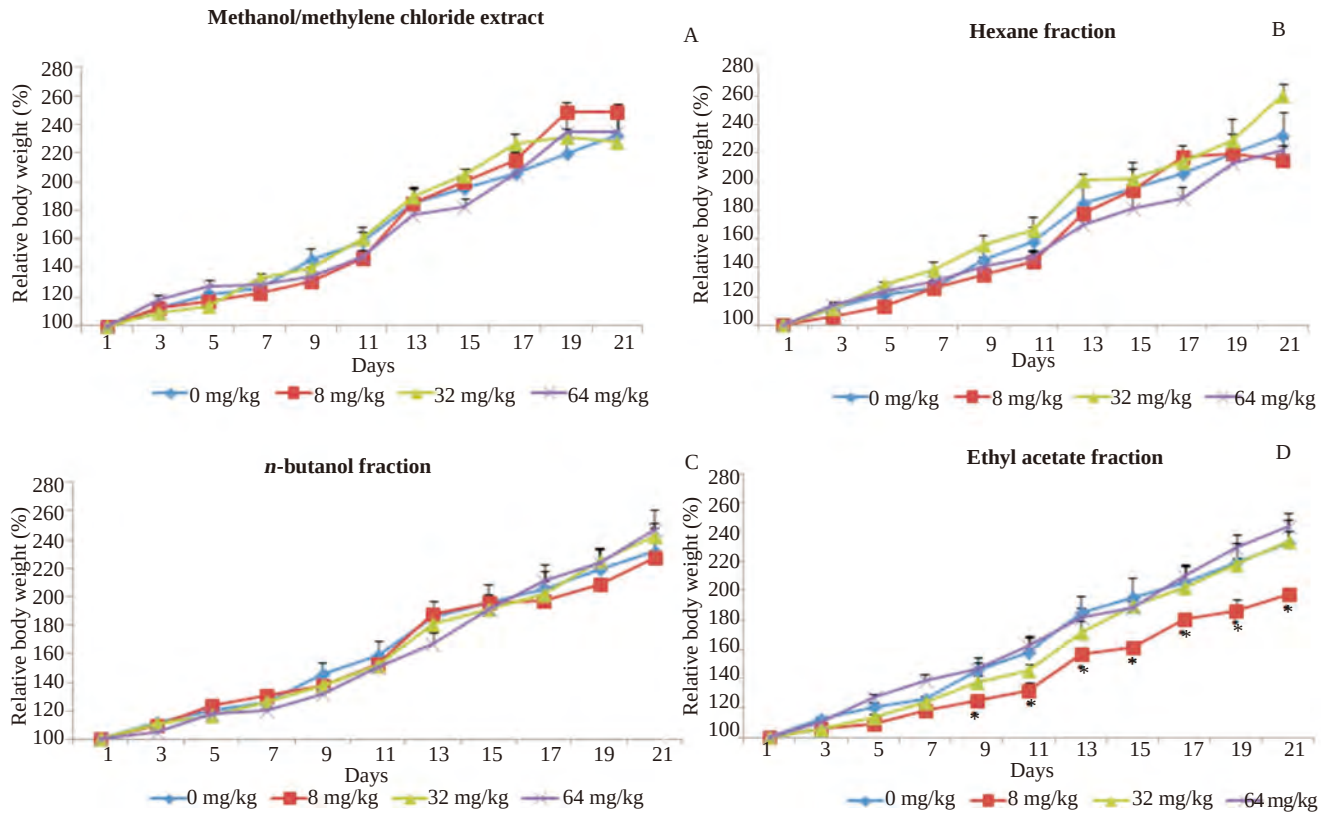

Figure 2. Evolvement of the body weight gain in the animals during the administration period.
*: The dose is significantly different at $P < 0.05$ from the control at the corresponding value.

weight, proteins and cholesterol levels are presented in Figure 3. A significant decrease ($P < 0.05$) was observed in the ovarian weight of animals treated with the 64 mg/kg of the ethyl acetate fraction as compared to the control group animals (Figure 3A). The same fraction showed a significant increase ($P < 0.05$) in ovarian proteins at all the doses (115% for 8 mg/kg, 161% for 32 mg/kg and 115% for 64 mg/kg). Ovarian protein levels also significantly increased in animals treated with 8 mg/kg ($P < 0.05$), 32 and 64 mg/kg ($P < 0.01$) of the *n*-butanol fraction (Figure 3B). As concerns ovarian cholesterol (Figure 3C), it significantly increased by 30% in animals treated with the crude extract at the dose of 32 mg/kg ($P < 0.01$) then by 107% and 126% in those receiving the ethyl acetate fractions at the doses of 32 and 64 mg/kg respectively ($P < 0.05$).

3.4. Effect on the different stages of follicular growth

Relatively to the ovarian histology (Figure 4), a significant increase ($P < 0.01$) of 142% in the number of primary follicles of 64 mg/kg *n*-butanol treated animals was observed. As concerns crude extract treated animals, significant increases in the number of their secondary (8 mg/kg, $P < 0.05$) and tertiary follicles (64 mg/kg, $P < 0.01$) were also observed. The ethyl acetate fraction showed a significant decrease ($P < 0.05$) of 160% in the number of their secondary follicles at 32 mg/kg dose as compared to the control. The number of antral follicles significantly increased in animals receiving 64 mg/kg of the crude extract ($P < 0.01$) as well as in those treated with 8 and 64 mg/kg of the hexane fraction ($P < 0.05$).

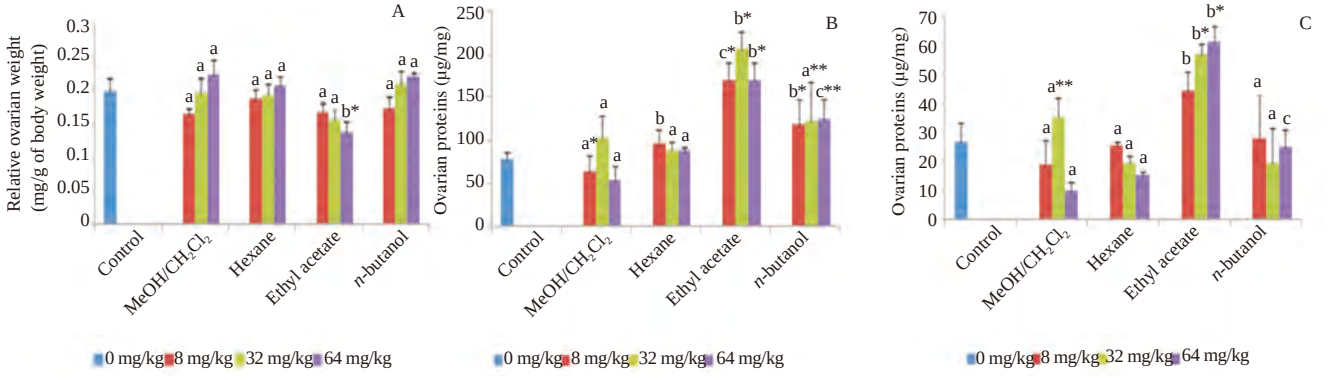

Figure 3. Effect of the crude extract and fractions on the ovarian weight (A), the ovarian proteins (B) and cholesterol (C) levels.
$MeOH/CH_2Cl_2$: Methanol/methylene chloride; *, **: Values are significantly different at $P < 0.05$ and $P < 0.01$ respectively from those of the control group (ANOVA and Fisher LSD). The same letter (a-a) shows a similarity in the values of the same dose between many extracts or fractions while different letters show (a-b, a-c or b-c) show a difference between them. Each histogram represents the mean ± SEM of the values for 5 animals.

3.5. Effect on the uterine weights and proteins

The effect of the fractions and extract on the uterine weights and proteins is shown in Figure 5. A significant decrease ($P < 0.01$) in uterine weights of animals treated with 64 mg/kg of ethyl acetate fraction, as compared to that of control animals, was obtained (Figure 5A). Relatively to animals of the ethyl acetate and *n*-butanol subgroups, the uterine proteins (Figure 5B) significantly increased at all administered doses. The same trend was observed with hexane fraction at doses of 32 mg/kg ($P < 0.05$) and 64 mg/kg ($P < 0.001$) respectively. The crude extract did not show any significant variation.

3.6. Effect on the serum hormonal and proteins levels

The effect of the plant extract and fractions on the hormonal (FSH, LH, estradiol and progesterone) and protein levels are presented in Figure 6. A significant decrease ($P < 0.05$) in serum FSH level of animals treated with 32 mg/kg dosage of the crude extract or *n*-butanol fraction of *S. biafrae* was observed. Animals treated at the same dose of the hexane fraction presented a significantly increased serum FSH level ($P < 0.001$).

As concerns serum LH levels (Figure 6B), it significantly increased ($P < 0.05$) in animals treated with the hexane fraction at 32 mg/kg while significant decreases ($P < 0.01$) were obtained in crude extract and ethyl acetate fraction treated animals (32 and 64 mg/kg), as well as *n*-butanol fraction treated animals ($P < 0.05$) (8 and 32 mg/kg).

A significant increase in serum estradiol levels (Figure 6C) was observed, whatever the administered dose, in crude extract treated animals while the level of those receiving the hexane fraction (all doses, $P < 0.001$), ethyl acetate fraction (32 and 64 mg/kg, $P < 0.001$) and *n*-butanol fraction (8 and 32 mg/kg, $P < 0.001$) were significantly reduced as compared to their respective control.

Although some decreases were observed in the serum progesterone levels of crude extract (32 mg/kg, $P < 0.001$) and ethyl acetate (64 mg/kg, $P < 0.001$) treated animals (Figure 6D), this hormonal parameters globally and significantly ($P < 0.001$) increased in all treated animals that were administered with hexane and *n*-butanol fractions at all doses.

Significant decreases in serum proteins were observed in animals treated with ethyl acetate ($P < 0.001$), *n*-butanol ($P < 0.001$) and hexane ($P < 0.01$ at 8 and 64 mg/kg) fractions. The decreases observed in the ethyl acetate fraction treated animals were the

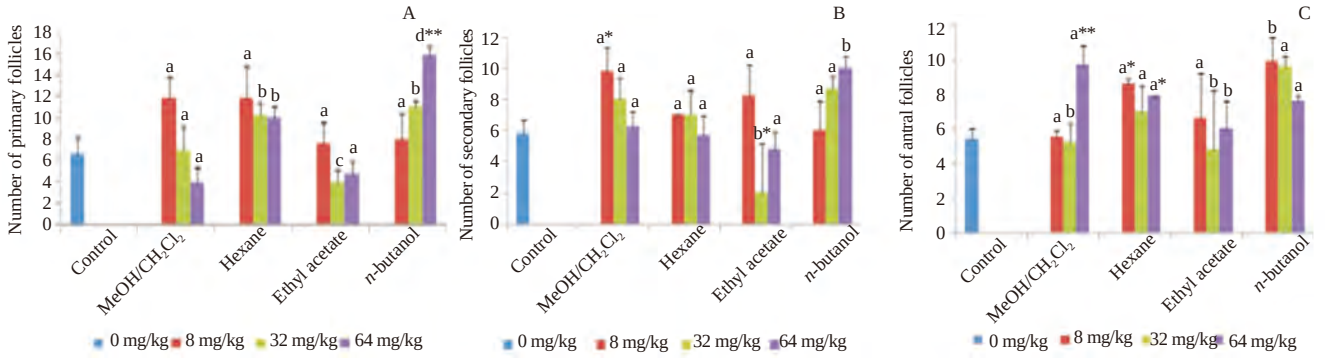

Figure 4. Effect of the crude extract and fractions on the number of primary (A), secondary (B) and tertiary (C) follicles in the ovarian cortex of the animals after the treatment.

$MeOH/CH_2Cl_2$: Methanol/methylene chloride; *, **: Values are significantly different at $P < 0.05$ and $P < 0.01$ respectively from those of the control group (ANOVA and Fisher LSD). The same letter (a-a) shows a similarity in the values of the same dose between many extracts or fractions while different letters show (a-b, a-c or b-c) show a difference between them. Each histogram represents the mean ± SEM of the values for 5 animals.

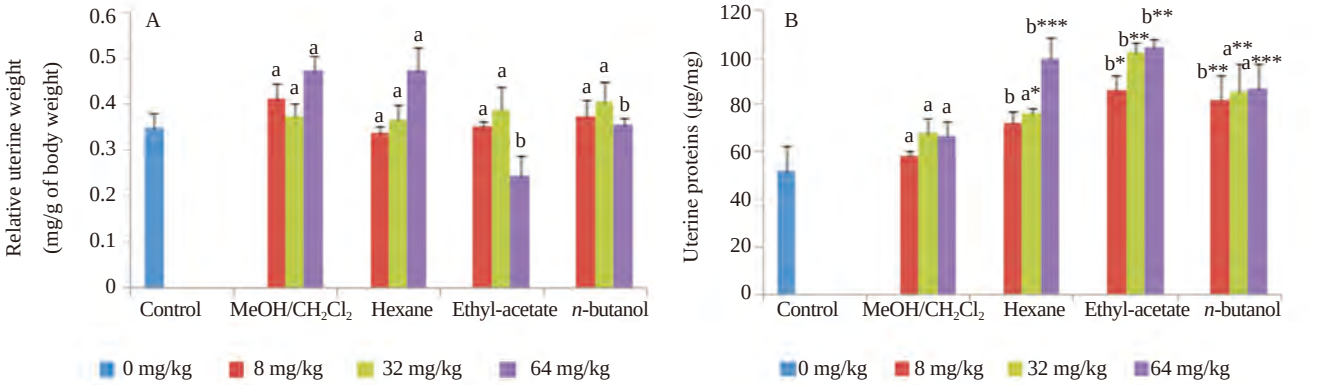

Figure 5. Effect of the crude extract and fractions on the uterine weight (A) and proteins (B).

$MeOH/CH_2Cl_2$: Methanol/methylene chloride; *, ** 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). The same letter (a-a) shows a similarity in the values of the same dose between many extracts or fractions while different letters show (a-b, a-c or b-c) show a difference between them. Each histogram represents the mean ± SEM of the values for 5 animals.

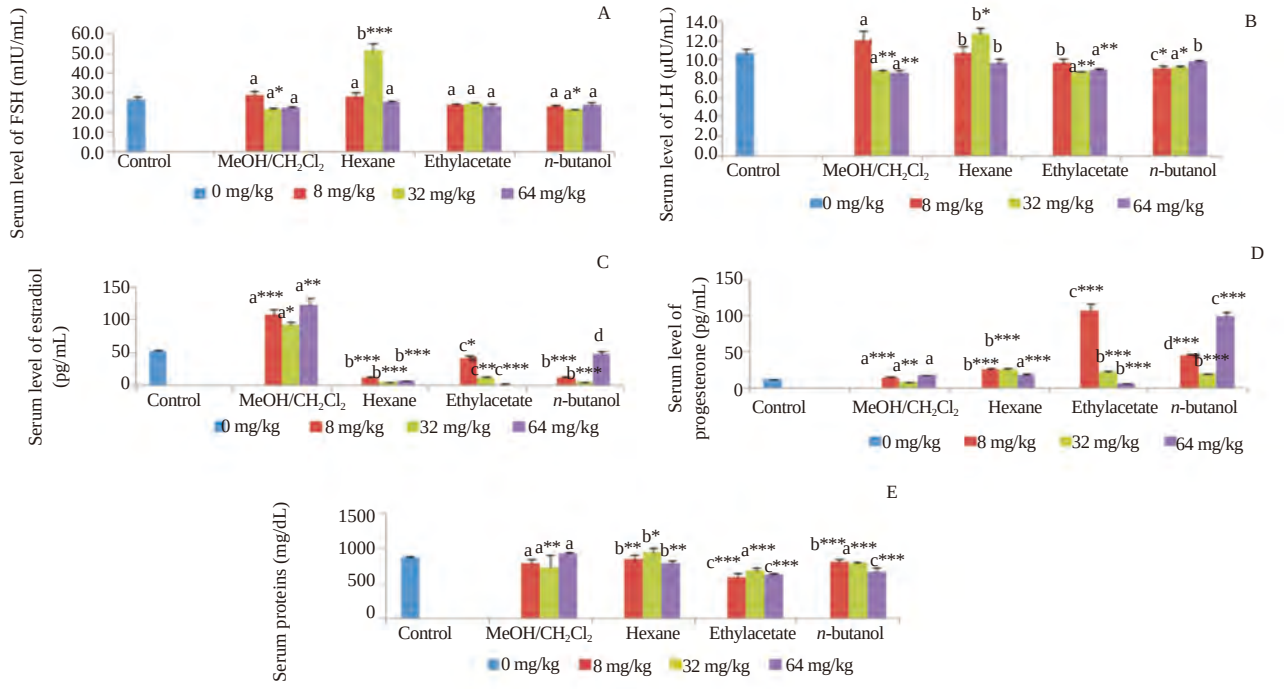

Figure 6. Effect of the crude extract and fractions on the serum levels of FSH (A), LH (B), estradiol (C), progesterone (D) and proteins (E). $MeOH/CH_2Cl_2$: Methanol/methylene chloride; *, ** and ***: Values significantly different respectively at $P < 0.05$, $P < 0.01$ and $P < 0.001$ from those of the control group (ANOVA and Fisher LSD). The same letter (a-a) shows a similarity in the values of the same dose between many extracts or fractions while different letters show (a-b, a-c or b-c) show a difference between them. Each histogram represents the mean ± SEM of the values for 5 animals.

lowest recorded, whatever the administered doses (Figure 6E).

4. Discussion

Infertility is a real human health problem worldwide. Many therapeutic solutions exist for its treatment, and one of them is the utilization of natural substances from medicinal plants[22]. Pharmacological investigations applied on effective medicinal plants could then become the best mean for the understanding of the mechanism of action of natural products on the sexual maturation and regulation of the reproductive axis in women[23]. This axis is under the control of reproductive hormones like FSH and LH produced by the pituitary gland, estrogens and progesterone produced by the ovary and also gonadotropin-releasing hormone released by hypothalamic neurons in the brain. In the normal scheme of stimulation/regulation, the release of gonadotropin-releasing hormone by hypothalamus stimulates the releases of FSH and LH at the level of the anterior pituitary. These later hormones fix to their receptors on the ovarian follicular cells to stimulate the synthesis and release of estrogens and progesterone[24,25]. Some results of the present work militate in favour of these observations. Indeed, the increase observed in the FSH and LH levels with the 32 mg/kg dose of the hexane fraction was linked to an increase in the progesterone level showing that this fraction stimulates ovarian steroidogenesis at that given dose. The same stimulating potential of the studied preparations was observed in the 32 mg/kg crude extract treated animals where significant increases in serum estradiol level coupled to a significant decrease in serum progesterone level were observed. These results could be related to the stimulating effect of *S. biafrae* constituents in the conversion of cholesterol to progesterone and later on to estradiol. The enzymatic processes in ovarian steroidogenesis are regulated by LH and FSH which control the entry of cholesterol in follicular cells and the expression of many steroidogenic enzymes (aromatase for example)[24]. The steroid hormones produced (estradiol in particular) would exert their biological effect following their fixation to specific receptors in their main target organs (ovary, uterus, hypothalamus, bone, *ect*.), leading to a chain of reactions that culminate in the biosynthesis of biomacromolecules (DNA, RNA, and proteins)[26-28]. The significant increases in ovarian and uterine proteins observed in crude extract treated animals and all the resulting fractions groups, whatever the dose administered, confirm the ovarian inducing potential of these preparations.

Estrogens, as their concentration increases, fix their hypothalamic receptors, leading to a reduction in the release of FSH and LH. This reduced gonadotropin release would then be followed, at the level of ovarian cells, with the decrease in steroid hormones synthesis, a mechanism known as down-regulation[25,28,29]. In the present study, significant decreases in serum LH (at higher doses of all preparations except for hexane) and FSH (crude extract at 32 mg/kg and *n*-butanol fraction) levels were recorded. These reductions in pituitary hormones were coupled to reduction in serum estradiol levels of the animals treated with the fractions and particularly with the hexane one which presented, with all the doses, a significant decrease in serum estradiol. This down regulating effect of the fractions of *S. biafrae* was confirmed by the significant increase in ovarian cholesterol of the 32 and 64 mg/kg ethyl acetate treated animals, which was coupled to a significant decrease in serum estradiol and progesterone levels. These results show a possible blockade of the ovarian steroidogenesis in these animals leading to accumulation of cholesterol in their ovarian cells. It confirms the explanation suggested earlier *i.e.*, the decrease in steroidogenic enzymes expression resulting from the low amount of gonadotrophic hormones (FSH and LH) at the level of their ovarian cell receptors. The overall observations on the hormonal profile of the treated

animals may lead to the following observation: the crude methanol/methylene chloride extract showed a good steroidogenic effect but its fractionation, instead of presenting an increased effect, led to contrary results due to a probably high concentration of the active estrogenic compounds in the fractions, principally the ethyl acetate and *n*-butanol ones.

Folliculogenesis is an important index of sexual capability in animals. In the late phases of this phenomenon, the follicle recruitment matches with the development of aromatase activity and appearance of LH receptors in the granulosa cells. It also leads to reduction in the expression of some insulinlike growth factors linking proteins and increase in inhibin release[29]. These parameters either lead to atresy of some follicles or growth of others. The increase in tertiary follicles number observed with the hexane fraction was linked to an increase in FSH and LH releases. This shows a possible inducing effect of that fraction on the recruitment process during folliculogenesis. The progressive dose-dependent decrease of preantral follicles (primary and secondary follicles) in the crude extract treated animals was correlated to an increase in their antral follicle numbers. These results prove the dose-dependent increasing effect of the crude extract on ovarian folliculogenesis. The *n*-butanol fraction, contrary to the crude extract, presented at 64 mg/kg significant increase and decrease in preantral and antral follicles respectively. These double effects could be linked, as suggested earlier, to the down regulating potential of their chemical constituents. At the same dosage (64 mg/kg), the plant's crude extract instead led to acceleration in follicular maturation, so that there was a reduction in the pre-antral follicle numbers in favour of antral follicles whose number increased. These results could be related to the FSH-like or estrogenic potentials of compounds contained in the crude extract.

Steroids, polyphenols and coumarines were found in the crude extract and fractions of *S. biafrae* contrary to anthranoids, flavonoids and triterpenoids that were missing. Secondary metabolites generally show many pharmacological effects. They often act as agonists or antagonists of neurotransmitter systems or form structural analogs of endogenous hormones[30,31]. Among polyphenolic compounds encountered in medicinal plants, phytoestrogens can stimulate the development of the ovary by acting as agonists of natural estrogens[32,33]. The concentration of these compounds, which surely differs from the crude extract and its fractions, could be responsible for the high variability in the effects observed. Moreover, the synergistic effect shown by these compounds in the crude extract could have also been lost in the fractions.

The methanol/methylene chloride extract of *S. biafrae* and its fractions from hexane, ethyl acetate and *n*-butanol solvents showed interesting dose-dependent activities on fertility parameters of immature female rats. The ovarian inducing potential of the plant's crude extract and particularly its ethyl acetate and *n*-butanol fractions were noted. These important hormonal activities found in the above fractions of the crude extract prove that the active compounds, responsible for the *S. biafrae* ovarian inducing effects, are surely of intermediate polarities and are more concentrated there. For future studies, a reduction in the dose of the crude extract and its resulting fractions is highly suggested. This change will help in seeking whether the down regulating effect observed in this study is dose-dependent or not. The duration of the treatment should be further shortened because hormones and proteins levels did not show a same profile due to their different half-life and synthesis length. The evaluation of the activities or expressions of major enzymes of ovarian steroidogenesis can also bring an understanding in the mechanism of action of the compounds responsible for the effect obtained.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Our special thanks go towards the International Foundation for Science for laboratory equipment and reagents facilities we benefited through the grantee F/4731-1. We also Thanks Dr Ngoula Ferdinand of the Faculty of Agronomy and Agricultural Sciences of the University of Dschang who photographed the ovarian sections.

Comments

Background

The leaf and stem extracts of *S. biafrae* is used as a medical drug in Cameroon's traditional medicine to improve female fertility. The literature reports the ethanolic and aqueous extract of the plant, showed their stimulating effects on the sexual maturation of the animals during 30 d in immature female rats. The comparative effect of its leaves and stems methanol/methylene chloride extract as well as its fractions in hexane, *n*-butanol and ethyl acetate solvents were assayed on immature female rat reproductive function.

Research frontiers

Studies are being performed in order to to determine the effect of methanol/methylene chloride extracts from leaf and stem extracts of *S. biafrae* on immature female rat reproductive function. the duration of 30 d of treatment used during previous work has been shortened to 20 d.

Related reports

Many therapeutic solutions exist for its treatment, and one of them is the utilization of natural substances from medicinal plants. Literature shows that *S. biafrae* contains many secondary metabolites of different polarities such as dihydroisocoumarins, terpenoids, sesquiterpenes, amino acids *etc*. that can play many therapeutic roles.

Innovations & breakthroughs

Data regarding reduction in the duration of the treatment would obviously lead to a better evaluation of the biological effect of the plant extract and fractions (from 30 d of treatment to 20 d for immature female rats). For a better view of the mode of action of the active compounds in the plant (by using two solvents as methanol/methylene chloride extracts. was shown to have a better extraction power on the active secondary metabolites of plants at cellular level).

Applications

It may be important to use a natural substances from medicinal plant such as *S. biafrae* to try to overcome the infertility disease. The results of the present study suggest that methanol/methylene

chloride extracts was shown to have a better extraction power on the active secondary metabolites of plants at cellular level on immature female rats. Reduction of the duration of 30 d of treatment used during previous work has been shortened to 20 d.

Peer review

This is a good study in which the authors using the leaf and stem extracts of *S. biafrae* of methanol/methylene chloride, and obtained good results of the two extract; due to the polarity of the solvent used for extraction, could indicate the type of secondary metabolite that is active in the plant.

References

[1] Telefo PB, Lienou LL, Yemele MD, Lemfack MC, Mouokeu C, Goka CS, et al. Ethnopharmacological survey of plants used for the treatment of female infertility in Baham, Cameroon. *J Ethnopharmacol* 2011; **136**(1): 178-87.

[2] Awodoyin RO, Akinyemi CY, Bolanle OO, Antiabong IC. Spatial distribution and abundance of *Solanecio biafrae* (Olive & Heirne) C. Jeffrey and structure of weed communities in some cocoa plots in Ekiti, Oyo and cross river states, Nigeria. *Ife J Sci* 2013; **15**(3): 661-76.

[3] Iwu MM. *Handbook of Africa medicinal plants*. Boca Raton: CRC Press; 1993.

[4] Focho DA, Nkeng EAP, Lucha CF, Ndam WT, Afegenul A. Ethnobotanical survey of plants used to treat diseases of the reproductive system and preliminary phytochemical screening of some species of malvaceae in Ndop Central Subdivision, Cameroon. *J Med Plants Res* 2009; **3**(4): 301-14.

[5] Lienou LL, Telefo PB, Bayala B, Yemele DM, Lemfack MC, Mouokeu C, et al. Effect of ethanolic extract of *Senecio biafrae* on puberty onset and fertility in immature female rat. *Cameroon J Exp Biol* 2010; doi: 10.4314/cajeb.v6i2.68528.

[6] Lienou LL, Telefo BP, Bale B, Yemele D, Tagne RS, Goka SC, et al. Effect of the aqueous extract of *Senecio biafrae* (Oliv. & Hiern) J. Moore on sexual maturation of immature female rat. *BMC Complement Altern Med* 2012; doi:10.1186/1472-6882-12-36.

[7] Koller M, Pletscher C, Jost M. Solvents. Luzern: Work Medicine Division, SUVA; 2013. [Online] Available from: http://www.suva.ch/Fr/factsheet-loesungsmittel.pdf [Accessed on 15th September, 2014]

[8] Tabopda TK, Fotso GW, Ngoupayo J, Mitaine-Offer AC, Ngadjui BT, Lacaille-Dubois MA. Antimicrobial dihydroisocoumarins from *Crassocephalum biafrae*. *Planta Med* 2009; **75**(11): 1258-61.

[9] Dairo FAS, Adanlawo IG. Nutritional quality of *Crassocephalum crepidioides* and *Senecio biafrae*. *Pak J Nutr* 2007; **6**(1): 35-9.

[10] Adebooye OC. *Solanecio biafrae* (Oliv. & Hiern) C. Jeffrey. PROTA 2. Vegetables/Légumes [CD-Rom]. Wageningen: Wageningen University; 2004.

[11] Leibowitz SF, Akabayashi A, Alexander J, Karatayev O, Chang GQ. Puberty onset in female rats: relationship with fat intake, ovarian steroids and the peptides, galanin and enkephalin, in the paraventricular and medial preoptic nuclei. *J Neuroendocrinol* 2009; **21**(6): 538–49.

[12] Aiyegoro OA, Okoh AI. Phytochemical screening and polyphenolic antioxidant activity of aqueous crude leaf extract of *Helichrysum pedunculatum*. *Int J Mol Sci* 2009; **10**(11): 4990-5001.

[13] Harborne JB. *Phytochemical methods: a guide to modern techniques of plant analysis.* 2nd ed. London: Chapman and Hall; 1998, p. 54-84.

[14] Aiyegoro OA, Okoh AI. Preliminary phytochemical screening and *in vitro* antioxidant activities of the aqueous extract of *Helichrysum* longifolium DC. *BMC Complement Altern Med* 2010; doi:10.1186/1472-6882-10-21.

[15] Trease GE, Evans WC. *Pharmacognosy.* 15th ed. London: Saunders Publishers; 2002, p. 42-4, 221-9, 246-9, 304-6, 331-2, 391-3.

[16] The Counsil of the European Communities. Council Directive of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Brussels: The Counsil of the European Communities; 1986. [Online] Available from: http://ec.europa.eu/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf [Accessed on 8th September, 2014]

[17] Noble JE. Quantification of protein concentration using UV absorbance and Coomassie dyes. *Methods Enzymol* 2014; **536:** 17-26.

[18] Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. *Ann Clin Biochem* 1969; **6**: 24-7.

[19] Richmond W. Preparation and properties of cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin Chem* 1973; **19**(12): 1350-6.

[20] Roeschlau P, Bernt E, Gruber W. Enzymatic determination of total cholesterol in serum. *Z Klin Chem Klin Biochem* 1974; **12**(2): 226.

[21] Mackenzie G, Peng D. *Statistical modelling in biostatistics and bioinformatics*. Berlin: Springer; 2014.

[22] Malairajan P, Geetha Gopalakrishnan, Narasimhan S, Jessi Kala Veni K. Analgesic activity of some Indian medicinal plants. *J Ethnopharmacol* 2006; **106**(3): 425-8.

[23] Bussmann RW, Gilbreath GG, Solio J, Lutura M, Lutuluo R, Kunguru K, et al. Plants use of the Maasai of Sekenani Valley, Maasai Mara, Kenya. *J Ethnobiol Ethnomed* 2006; **2**: 22.

[24] Hu J, Zhang Z, Shen WJ, Azhar S. Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutr Metab (Lond)* 2010; doi: 10.1186/1743-7075-7-47.

[25] Burrows H. *Biological action of sex hormones.* Cambridge: Cambridge University Press; 2013, p. 526.

[26] Müller JC, Botelho GG, Befalo AC, Boareto AC, Rattmann YD, Martins ES, et al. *Morinda citrifolia* Linn (Noni): *in vivo* and *in vitro* reproductive toxicology. *J Ethnopharmacol* 2009; **121**(2): 229–33.

[27] Souza FA, Osorio JP, D'Oliveira-Sousa A, do Vale Filho VR, Marc H, Liliana CL, et al. [Folliculogenesis and ovulation in equine species]. *Rev Med Vet* 2011; **22**: 43-50. Spanish.

[28] Counis R, Combarnous Y, Chabot V, Taragnat C. Regulation of the synthesis and release of pituitary gonadotrophins. In: [*Reproduction in mammals and man*]. Thibault C, Levasseur MC, editors. Paris: INRA; 1993, p. 65-84. French.

[29] Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nat Neurosci* 2011; **14**(6): 704–10.

[30] Wink M. Molecular modes of action of drugs used in phytomedicine. In: Bagetta G, Cosentino M, Corasaniti MT, Sakurada S, editors. *Herbal medicines. Development and validation of plant-derived medicines for human health.* Boba Raton: CRC Press; 2011.

[31] Miller AE, Heyland A. Endocrine interactions between plants and animals: implications of exogenous hormone sources for the evolution of hormone signaling. *Gen Comp Endocrinol* 2010; **166**(3): 455–61.

[32] Liu MM, Huang Y, Wang J. Developing phytoestrogens for breast cancer prevention. *Anticancer Agents Med Chem* 2012; **12**(10): 1306-13.

[33] Vaya J, Tamir S. The relation between the chemical structure of flavonoids and their estrogen-like activities. *Curr Med Chem* 2004; **11**(10): 1333-43.