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

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**1. Introduction**

*Senecio biafrae* (*S. biafrae*), a plant used in the African pharmacopeia, 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...
plant administered for 30 d to immature female rats showed the stimulating effects on the sexual maturation of the animal[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 extract. 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. biafra* 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. biafra*, additive parameters such as follicle count and serum reproductive hormone levels have also been included. Hence to assay the comparative effect of *S. biafra* 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. biafra* 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 chloride 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 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) FeCl₃, 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

A bout 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% H₂O₂. 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 (2880 r/min, 15 min) of the homogenates, their supernatants were collected and used for protein and cholesterol (for ovarian

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 M 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

| 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
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 ($P < 0.05$).
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...
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 their ovarian cell receptors. These later hormones fix to their receptors, leading to a reduction in the release of FSH and LH. This down regulating effect of 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 insulin-like growth factors linking proteins and increase in inhibin release. 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 preantral 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. Among polyphenolic compounds encountered in medicinal plants, phytoestrogens can stimulate the development of the ovary by acting as agonists of natural estrogens. 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 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).

Conflict of interest statement

We declare that we have no conflict of interest.

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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 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.

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