EFFECTS OF METHANOL EXTRACT OF *Piper umbellatum* LEAVES ON CONTRACEPTIVE AND SEXUAL BEHAVIOUR IN RODENTS

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ABSTRACT

Effects of methanol leaves extract of *Piper umbellatum* on conception and sexual behaviour were investigated in rodents. The leaves of *Piper umbellatum,* a rapidly growing shrub of Piperaceae family has a reputation as a fertility regulating plant among the Ibibio tribe of South-South region of Nigeria. This study was designed to investigate if the leaves possess any true contraceptive properties. Adult female rats and mice, having regular estrus cycle confirmed by daily smear analysis were used. The selection of animals for use in the study was determined by the presence of at least two consecutive 4-day estrus cycle. The animals were administered with 192 - 576 mg/kg body weight intraperitoneally (i.p) of extract in divided doses for 4 days. On the 5th day, fertile males were introduced using 3:1 (F/M) ratio and were allowed to remain with females until the experiment was terminated. Estrogenic and antiestrogenic activities of the extract were assessed in bilaterally ovariectomized immature rats, estrus and ovulatory effects were determined on sexually matured female rats while mount frequency, lordosis frequency, lordosis latency and lordosis quotient were the indices of female sexual behaviour determined. The extract protected the rodents from conception from one to three gestational periods. There were no fetal abnormalities observed in the pups. It also caused a significant ($p<0.001$) dose-dependent increase in uterine wet weight and vaginal opening; however, its vaginal cornification was not progressive in rats. It inhibited in a dose-related fashion regular estrus cycle and ovulation respectively. The extract increased both lordosis quotient and its frequency while the latency was decreased indicating involvement of phytoestrogen in the extract. The exhibited contraceptive effects which confirm its folkloric use may in part be due to its secondary metabolites which included phenols and saponins among others.

Key words: *Piper umbellatum, rodents, anticonceptive, methanol extract, sexual behaviour*

Introduction

Successful establishment of pregnancy requires profound synchronization of finely tuned processes at different levels of the reproductive system (Telleria *et al.,* 1997). Therefore, disruption of pregnancy results due to interference on one or more of these processes as a result of use of drugs, chemicals or disease state among others.

Plants and their metabolites have shown profound effects in this regard. Among which are *Asparagus pubescens, Cassia nigricans* and *Ricinus communis* (Okwuasaba *et al ;* 1991; Nwafor *et al;* 1988; Nwafor and Okwuasaba *et al;* 2001). *Piper umbellatum,* a rapidly growing shrub of Piperaceae family, has many ethnobotanical uses among the Ibibio tribe of South-South region of Nigeria. The leaves are used as mosquito repellant, treatment of stomachache, gonorrhoea and internal heat (Inyang, 2003). It is also used as a fertility regulating plant (Effiong, Department of Pharmacognosy and Natural Medicine, University of Uyo, Personal Communication, 2009). However, no reference in literature revealed its possible contraceptive efficacy either in animal or human studies. The present investigation was to establish if the leaves possess any true contraceptive properties, possible estrogenic/antiestrogenic effects using the indices such as the uterine wet weight ratio, premature vaginal opening and degree of vaginal cornification as well as its effects on estrus cycle, ovulation and sexual behaviour with a view to elucidating its mechanism of action.

Materials and methods

Collection and identification of plant material

The plant material used in this study was collected from Itak Ikot Akap village in Ikono Local Government Area of Akwa Ibom State, Nigeria in the month of June, 2009. The plant was identified and authenticated by Dr. (Mrs) Margaret Bassey of Department of Botany and Ecological Studies, University of Uyo. A specimen voucher has been made and deposited at the Department of Pharmacognosy and Natural Medicine, Faculty of Pharmacy, University of Uyo. The dried leaves were pulverized by grinding using pestle and mortar. Thereafter, 320 g of the ground leaves were successively macerated in n-hexane, chloroform, ethylacetate and methanol based on their polarity for 72 h each, they were filtered and evaporated in rotary evaporator and stored at -4^{0}C until required for use. The choice of methanol extract was borne out of earlier pilot tests on the different extracts.

Animal Stock

Adult and young immature albino female mice (weighing 25 – 30 g and 13 -18 g, respectively) and albino female rats (weighing 165 – 200 g) were used in the study. All the animals were housed in a cross-ventilated room (temperature 22 $\pm$ 2.5^{0}C, 12 h light /12 h dark cycle) and were fed with standard growers mash (Bendel Feeds, Edo State, Nigeria) and water *ad-libitum.*

All animals experiments were conducted in accordance with the Internationally accepted laboratory animal use and care (based on Helsinki convention) and guidelines and rules of Faculty of Pharmacy, University of Uyo, for animal experimentation.

Phytochemical screening

Phytochemical screening of the methanol extract was performed according to the method of Harbone, (1984). Tests for alkaloids, saponins, tannins, terpenes, simple sugars, flavonoid, anthraquinones and cardiac glycosides were carried out.

Acute toxicity study

Median lethal dose (LD_{50})

For the determination of median lethal dose (LD_{50}), mice were divided into ten groups each containing seven (7) animals. The extract was administered intraperitoneally (i.p) in a dose range of 400 – 2600 mg/kg body weight. A maximum of 0.15ml was given. All animals were observed for physical signs of toxicity for 24h. The LD_{50} was calculated using the method of Miller and Tainter, (1944).

Antifertility activity

For this study, adult female mice and rats, having regular estrus cycle confirmed by daily smear analysis were used. The selection of animals for use in the study was determined by the presence of at least two consecutive 4-day estrus cycles. Starting at proestrus, the animals were administered with 192 – 576 mg/kg (i.p;) body weight of extract dissolved in saline in the presence of dimethyl sulfoxide (0.1ml) in divided doses for 4 days. On the 5th day, fertile males were introduced using 3:1 (F/M) ratio, the presence of clumps of spermatozoa in the vaginal smear the next morning confirmed that mating had occurred. The males were allowed to remain with females until the experiment was terminated. The control group was injected with saline (5 ml/kg, i.p;) (Nwafor *et al.,* 1998).

Estrogenic and antiestrogenic activities

Estrogenic and antiestrogenic activities of the methanol extract of *Piper umbellatum* were assessed in bilaterally ovariectomized immature rats using the method of Edgren and Calhoun, (1957). The end points used to determine the estrogenic effect of the extract include: uterine wet weight ratio, degree of vaginal cornification and quantal vaginal opening.

Exactly 1 week after bilateral overiectomy, the rats were randomized into the various experimental groups. Then, 17-β-estradiol was dissolved in corn oil and administered subcutaneously (s.c.) at a dose of 0.1 μg rat per day for 4 consecutive days as a reference standard.

For evaluating estrogenic activity, different groups of animals received only the plant extract (i.p.) at various doses(192 – 576 mg/kg) whereas for the anti-estrogenic activity, various doses of the extract were administered conjointly with 17-β-estradiol (0.1μg/rat per day) for 4 consecutive days.

Controls were simultaneously maintained and received vehicle only. The animals were sacrificed 24 h after the last treatment. The estrogenic potency of the extract was also evaluated using immature female mice. The mouse uterine weight bioassay of Rubin, (1951) was employed. Albino female mice (21-23 days old) were treated with 192 – 576 mg/kg of extract (i.p.) for 4 days consecutively, and autopsied 24h after the last injection. The body weight and the wet uterine weight were recorded. Suitable controls receiving either saline or 17-β - estradiol (0.1 μg/rat per day) as reference hormone was maintained.

Effect on estrous cycle

Vaginal smears were checked daily between 09:00 and 10:00 h. The vaginal epithelium cells observed under the light microscope were classified in a 4-day cycling rat, the vagina smear predominantly shows leukocytes on metaestrus and diestrus. On proestrus, the smear is characterized by mostly nucleated epithelial cells, which change to predominantly cornified, squamous epithelial cells by the morning of estrus. (Kilen and Schwartz, 1999).

To determine the effect of extract on estrus cycle, twenty four cycling female rats were used for this experiment. The phases of their estrus cycle were determined and only those that show regular estrus cycle for more than once were used. They were divided into four groups of six animals each. Groups 2- 4 were administered with 192, 384 and 576 mg/kg of extract (i.p) respectively. The vaginal smear of the rats were examined daily for eight days (two consecutive estrus phases) while the control group received saline (5 ml/kg; i.p). The changes were observed and recorded (Gebrie *et al.,* 2005).

Effect on ovulation

To determine the effect of the extract on ovulation the method of Telleria *et al.,* (1997) was adopted. Twenty four cycling female rats were used. Group 1 served as a control and received 5 ml/kg of normal saline (i.p). Groups 2-4 were given 192- 576 mg/kg of extract respectively. The rats were injected with the extract at the late proestrous phase and at the end of estrus the next day, the animals were laparatomized, and the presence of ovary in the uterine horn was determined with the aid of hand lens.

Effect of extract on female sexual behaviour

Animals were treated at 12:00h proestrous with different doses of extract (192 – 576 mg/kg and sexual behavior was evaluated the same day in the dark phase at 20:00h. The mating session started when a male mounted a female and was terminated at the end of 15 min or when the male partner ejaculated, whichever came first. Only mounts in which the male showed pelvic thrusting were scored. The female's response to a mount was categorized as a lordosis response when it displayed a rigid posture with arching of the back, elevation of the hind quarters, and deviation of the tail to facilitate male mounting and intromission. Failure to achieve lordosis was typified by a

lack of positional response or by other responses, such as vocalization and active attempts by the female to escape, or by complete immobility with the hind quarters down. Female sexual behavior was expressed as the ratio of the number of lordosis responses to mounts (that is, mount alone, mounts with intromissions, and mounts with intromissions and ejaculations) by the male sexual partner. The Lordosis quotient (LQ) was calculated as a percentage of the total number of lordosis responses divided by the total number of mounts (Telleria *et al.,* 1997)

Statistical Analysis: Multiple comparisons of Mean ± SEM were carried out by one way analysis of variance (ANOVA), followed by Tukey-Krammar multiple comparisons tests. A probability level of less than 5% was considered significant.

Results

Anticonceptive activity

The extract (192 – 576 mg/kg protected the rats from conception from one to three gestational periods. Similar results were obtained from the mice (Table 1). The effect of the extract was reversible. Though there were some significant changes in both the weight and length of the pups, there were no foetal abnormalities observed in the pups (liters) of both the rats and mice over three months (Tables 2 and 3).

Table 1: Anticonceptive effect of *Piper umbellatum* leaves extract in adult female rats and mice

Dose (mg/kg)	Number of Pups	Degree of protection over n-gestational period
Rat (Control)	3.33 ± 1.25	Nil
192	2.33 ± 1.22	1 (3/6)*
384	1.67 ± 0.83	3 (3/6)*
576	0.67 ± 0.73	3(3/6)*
Mice (control)	3.50 ± 0.93	Nil
192	2.00 ± 0.93	1 (3/6)*
384	4.20 ± 1.18	3(3/6)*
576	2.20 ± 1.58	2 (4/6)*

Values represent Mean ± SEM

* Numerator indicates the number of rats that were protected.

Table 2: Effect of *Piper umbellatum* extract on weight and length of pups (rats)

Day 1	**Day 1**	**Day 1**	**Day 10**	**Day 10**	**Day20**	**Day 20**	**Day 30**	**Day 30**
Treat-ment Mg/kg	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)
Control	5.27±0.08	6.14±0.04	20.14±0.02	11.24±0.08	30.67±0.20	17.80±0.15	51.20±0.20	21.90±0.16
192	4.79±0.14*	5.90±0.09**	18.65±9.14*	10.70±0.10*	29.70±0.02*	14.60±0.01*	49.70±0.02*	21.50±0.36
384	4.60±0.08*	5.75±0.07*	18..60±0.01*	10.60±0.02*	28.70±0.01*	16.70±0.13*	49.10±0.20*	21.10±0.29**
576	4.80±0.01*	5.35±0.14*	19.25±0.09*	10.90±0.10*	26.25±0.44*	15.30±0.20*	47.50±0.40*	20.10±0.12*

Values represent Mean ± S.E.M
Significance relative to control *p<0.01, **p<0.01; (n = 6)

Table 3: Effect of *Piper umbellatum* extract on weight and length of pups (mice)

	Day 1		Day 10		Day 20		Day 30	
Treatment (mg/kg)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)	Weight(g)	Length(cm)
Control	1.69±0.03	4.60±0.05	6.02±0.12	8.17±0.04	10.08±0.20	12.00±0.11	18.35±0.48	15.10±0.28
192	1.65±0.04	4.10±0.05*	5.53±0.07*	7.60±0.09*	7.96±0.10*	10.1±0.08*	13.10±0.20*	14.60±0.09*
384	1.46±0.02*	4.10±0.04*	5.10±0.10*	7.70±0.08*	8.01±0.05*	10.95±0.05*	13.30±0.28*	14.60±0.13*
576	1.47±0.03*	3.58±0.07*	4.34±0.08*	7.40±0.06*	7.60±0.08*	10.40±0.07*	13.25±0.34*	14.20±0.09*

Values represent Mean ± S.E.M
Significance relative to control *p<0.001; n=6

Estrogenic and antiestogenic effect

The extract showed a significant (p< 0.001) dose-dependent increase in uterine wet weight, vaginal opening in both rats and mice. However, its vaginal cornification in rats, was not progressive. In the presence of reference drug (17-β-estradiol), this effect (vaginal cornification) was antagonized in rats (Tables 4 & 5).

Table 4: Estrogenic and anti- estrogenic effects of *Piper umbellatum* extract in bilaterally overiectomized young immature female rats.

Treatment /Route of Administration	Dose (mg/kg)	Mean body weight	Relative uterine Weight(mg/100mgb.w)	Vaginal Opening	Vaginal Conrnification
Control (i.p)	3m1/kg	80.80±4.65	62.44± 1.60	-	-
Extract (i.p)	192	81.80± 4.52	67. 51± 1.51*	-	±
”	384	92.00± 4.22	73.72±0.80*	+	±
”	576	98.00±7.29	156.71±2.05*	+++	±
17-β-estradiol (S. c)	0.1μg	98.00± 3.50	306.74±1. 04*	++	+++
17-β estradiol (S.c) + Extract (i.p)	0.1μg + 192	80.50± 4.35	200.37±1.03*	+	++
”	0.1μg + 384	82.00±3.38	254 .59±1. 02*	++	++
”	0.1μg + 576	82.8± 2.71	286.61±1.12*	++	++

Values represent Mean ± SEM; Significance relative to control *p<0.001
i.p = intraperitoneal
s.c = subcutaneous

Effects on estrus cycle

The result is as shown in figure 1. The extract (192 mg/kg) caused 33.3 % and 66.7 % inhibitions of first and second phases of estrus cycle in the rats respectively. Similarly, higher doses of the extract also inhibited estrus cycle in a dose-dependent fashion.

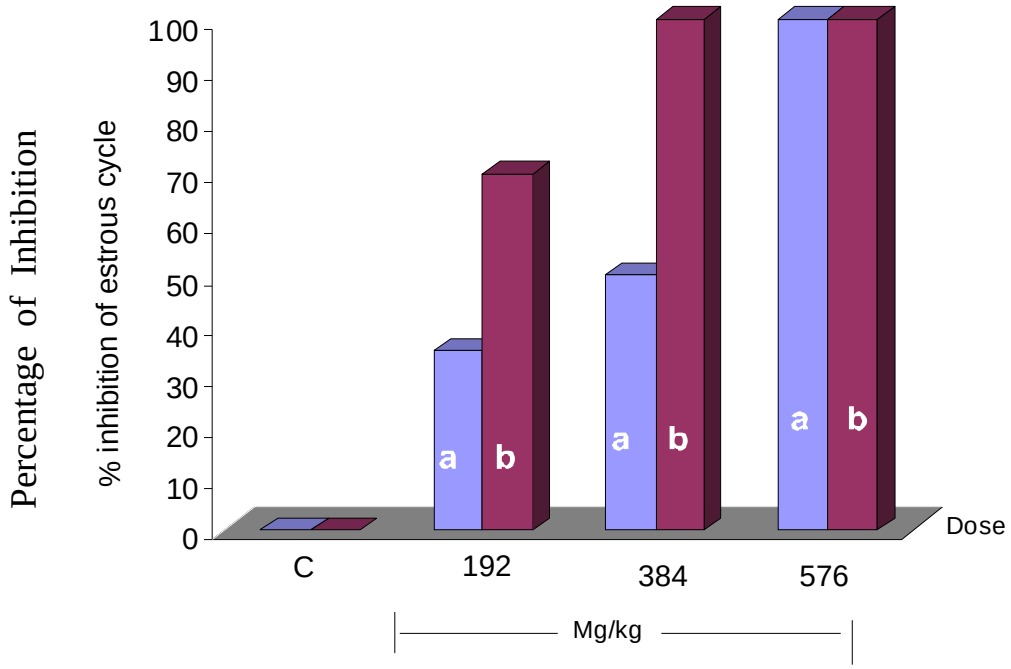
Effect on Ovulation

The effect of extract on ovulation is as shown in figure 2. The extract exhibited inhibitory effect on ovulation. This inhibition was in a dose-dependent manner which ranged from 66.6 % to 100 % respectively.

Table 5: Determination of estrogenic and anti-estrogenic effects of *Piper umbellatum* extract in young immature female mice.

Treatment /Route of Administration	Dose (mg/kg)	Mean body weight	Relative uterine Weight(mg/100gb.w)	Vaginal Opening	Vaginal Conrnification
Control (i.p)	3m1/kg	15.50± 0.70	65.74± 0.21	-	-
Extract (i.p)	192	81.20± 2.12	65. 59± 0.30*	±	±
”	576	19.50± 1.12	93.99±1.30*	++	++
17-β-estradiol (S. c)	0.1μg	19.00± 1.06	624.53±2. 06*	++	+++
17-β estradiol (S.c)	0.1μg + 576	17.50± 1.08	604.08±1.04*	+	++
+ Extract (i.p)				++	

Values represent Mean ± S. E. M
Significance relative to *p<0.001; i.p.= Interperitioneal
S.c=Subcutaneous



a = first estrus cycle
b = second estrus cycle
c = Control (Normal Saline)

Figure 1: shows the inhibitory effect of extract on estrus cycle

Effect on female sexual behaviour

The effect of the extract on female sexual behaviour is as shown in Table 6. The extract caused a significant increase in Lordosis frequency with a concomitant decrease in latency relative to control ($p < 0.001$). It also increased the Lordosis quotient from 47.06 to 74.64.

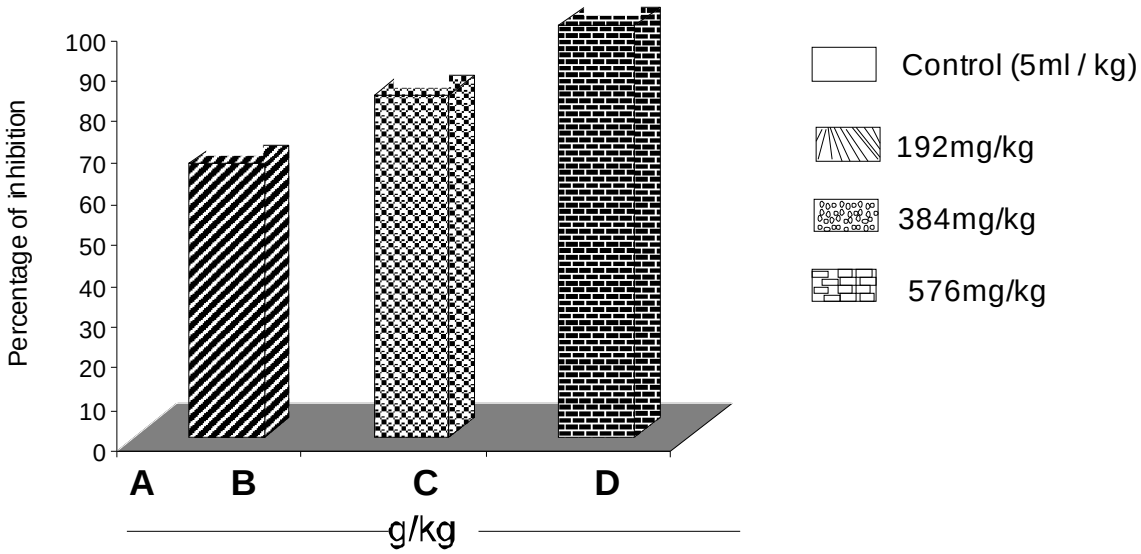


Figure 2: Effect of extract on ovulation

Acute Toxicity

The median lethal dose (LD_{50}) was calculated to be 1920 ± 70 mg/kg body weight. The physical signs of toxicity included jumping, increased locomotive activity, circuit movement and self-probing. Others were restlessness, gasping and death.

Table 6: Effect of Extract on female sexual behaviour

Drug/Chemical mg/kg	Mount frequency (mf)	Lordosis frequency (LF)	Lordosis Latency	Lordosis quotient (LQ) (%)
Control(Normal saline 5mg/kg)	8.50 ± 0.33	4.00 ± 0.11	25.83 ± 0.32	47.06
192	11.16± 0.26[a]	8.33 ± 0.23a	20.00±0.03[a]	74.64
384	10.00 ±0.51	7.16 ± 0.41[a]	15.00± 0.89[a]	71.60
576	8.83± 0.42[a]	6.33± 0.24[a]	20.50± 0.22[a]	71.69
17-β-estradiol (0.1μg/kg)	8.50 ±0.33	16.15 ± 0.34[a]	26.00±0.15	190.12

Values represent Mean ± SEM
Significance relative to control: [a]$p < 0.001$
17-β-E = 7-β-estradiol (n =6)

Phytochemical Screening

Phytochemical screening revealed the presences of alkaloids, cardiac glycosides, saponins, tannins and flavonoid.

Discussion

Conception is the product of fusion of male and female gamates. The failure of this fusion is dependent on a number of factors that ranged from reproductive organ pathology to interference with reproductive processes by chemicals or drugs. These chemicals or drugs interfere with one or multiple sites of reproductive processes or on developing embryo. These processes include hormonal, changes in normal physiological processes including biochemical derangement and tissue/organ pathology (Soejarto *et al.,* 978).

The effect of methanol extract of *Piper umbellatum* leaves on conception and sexual behaviour was investigated in rodents. The extract protected the rats and mice from conception from one to three gestational periods. The pups showed no foetal abnormalities. The extract also showed a significant dose-dependent increase in uterine wet weight and vaginal opening. The results indicate that the extract possesses anticonceptive and estrogenic effects. This conclusion is supported by the following observations:

i. pretreated animals that were left with fertile males were protected from conception over varied gestational periods.

ii. a dose-dependent increase in uterine wet weight and a premature vaginal opening were seen in young ovariectomized rats and immature mice. It is known that administration of estrogen has uterotrophic effects in several animal species including rats and mice (Edgren and Calhoun, 1957), such effects are associated with growth and proliferation of the endometrial cell numbers, vaginal opening and cornification. Estrogencity in premature animals is characterized by increase in uterine wet weight, premature vaginal opening and cornification (Turner and Bagnara, 1970).

The estrous cycle is a cascade of hormonal and behaviour events which are progressive, highly synchronized, and repetitive. The cycle is divided into four stages, centred around the period called estrous when mating behaviour is displayed. The period preceding estrus is *proestrus which signified the period of follicular growth in the ovary* while the period succeeding estrus is termed metaestrus. *Metaestrus is a recovery period following ovulation, while diestrus* is a period when the ovarian secretions from the corpus luteum *prepare the uterus for implantation.* If fertilization does not occur, the cycle is repeated and another set of follicles is prepared for ovulation.

That the extract showed dose- dependent inhibitory effect in both estrus cycle and ovulation, portrayed interference in the sequence of hormonal and neural changes that are associated with ovulation fertilization. Phytochemical screening of the extract revealed that it contained saponin, tannis, alkaloid, cardiac glycosides and flavonoid.

Administration of estrogen or phytoestrogen causes elevated serum estrogen at proestrus leading to serum fall in progesterone with concomitant rise in inhibin (fall in FSH surge) causing a resultant abortive estrus phase (Kilen and Schwartz, 1999). It therefore means that the presence of phenol and saponin in the extract may in part have contributed to the observed effects seen in estrus cycle and in ovulation.

Estrogen affects all the components of estrus, including receptivity, proreceptivity and non behavioural components. Sexual receptivity (sexual behaviour) can be induced with two discontinous pulses of estrogen, each of which lasts about an hour: one at the beginning of estrogen treatment and another during the later part of estrogen exposure. In rats, estrogen alone can produce high levels of lordosis behaviour in ovariectomized females, but the combination of estrogen followed by progesterone produces more complete display of female reproductive behaviour. (Thornton and Finn, 1999). In a number of animal species, including rats, it is common to calculate the lordosis quotient (LQ) as a numerical measure of receptivity. The LQ is the ratio of the number of times lordosis is shown in response to a fixed number of mounts (usually 10) multiple by 100. Hence, a female which shows lordosis to 8 of 10 mounts would have an LQ of 80 indicating high receptivity while animal showing 20 has low receptivity (Thornton and Finn, 1999). The extract induced increase in lordosis quotient with a corresponding decrease in lordosis latency indicating involvement of phytoestrogen in the extract.

In conclusion therefore, though the work is not exhaustive, the observed effects of the extract could be due to the presence of the active ingredients (secondary metabolites). These effects therefore support the local folkloric use of the plant in fertility regulation among the Ibibios of South-South region of Nigeria.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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