

Effects of Ethanolic Fruit Extract of *Picralima nitida* (Stapf) on Conception and Estrogenicity in Female Albino Rats

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Abstract

In search for medicinal plants with renowned reputation of contraceptive and fertility-modulating ability, this work was designed to evaluate the effects of ethanolic fruit extract of *Picralima nitida* on conception and oestrogenicity using twenty-one sexually mature and twenty-four sexually immature female Sprague Dawley rats respectively. For the anti-conceptive study, 0 mg/kg, 50 mg/kg and 500 mg/kg of the extract was administered from day 1 to day 4 of gestation (7 rats per group). On the 20th day of gestation, percentage of pregnant females (PPF), live foetal number (LFN), corpora lutea number (CLN), resorbed embryo number (REN), foetal crown-rump length (FCRL) and fertility indices (FI) were evaluated. For the anti-estrogenic study, Groups 1 to 3 (ovariectomized immature female rats) received 0.1 mg/kg of Stilboestrol, 50 mg/kg of the extract, and 0.2 ml of paraffin respectively, once daily for four consecutive days. Group 4 comprised of sham-operated immature female rats with their ovaries intact. The extract elicited absolute conception failure in 42.86% of the treated animals. The extract significantly ($p < 0.05$) reduced the FCRL, LFN and FI, but increased the REN. Evaluation of the allometric weights of the uterine tissues, endometrial thickness and vaginal opening revealed that the extract at 50 mg/kg body weight was anti-oestrogenic in activity. Findings from this research strongly suggest that the ethanolic fruit extract of *Picralima nitida* possesses significant post-coital anti-fertility activity in rats, which could not be attributed to its oestrogenic activity.

Keywords: Albino rats, conception, fertility, oestrogenicity, *Picralima nitida*

Introduction

The use of plants by man to treat common ailments has existed from time immemorial (Henrich *et al.*, 2004). Globally, millions of people still depend on traditional medicines for their healthcare needs, especially in rural and peri-urban areas of the world (WHO, 2001; Kumar *et al.*, 2004). The relatively lower incidence of adverse reactions to plant preparations compared to modern conventional pharmaceuticals, coupled with their reduced cost, consequently encourage both the consuming public and health care institutions to consider plant medicines as alternatives to synthetic drugs (Nair *et al.*, 2004). Drugs that prevent conception and embryonic implantation are required to place under control the global population, which is estimated to reach 8.9 billion by the year 2050; far

higher than the 2004 estimate of 6.4 billion (Watcho *et al.*, 2011). Majority of the contraceptives exert their effects either by inducing myometrial contraction or by altering the endocrine synthesis and function. Pharmacological evaluation of medicinal plants with renowned reputation of contraceptive and fertility-modulating ability is greatly encouraged as this could precipitate possible replacement for hormonal or synthetic contraceptives which are not cost effective and predispose to some side effects. Scientific investigations have revealed that many medicinal plants are known to possess promising contraceptive properties (Farnsworth *et al.*, 1980).

Picralima nitida (Stapf) is a bonafide member of the family Apocynaceae, restricted in distribution to African rain forest regions. It is known as limeme (Congo), Eban or Obero (Gabon), Erin (Yoruba), Osuigwe (Igbo) and Bamborutuk or Eban (Cameroon). *Picralima nitida* is a medicinal plant with folkloric reputation of influencing

uterine muscle function. Our earlier *in vitro* studies revealed the ethanolic fruit extract of *Picralima nitida* as a potent spasmogen (Mbegbu *et al.*, 2014a). Another plant, *Plumeria rubra*, which is also a member of the family Apocynaceae, has a well-established anti-fertility activity (Dabhadkar *et al.*, 2012). *Picralima nitida* has also been shown to possess anti-diarrheic (Kouitchou *et al.*, 2006; Kouitchou, 2007), anti-diabetic (Aguwa *et al.*, 2001), analgesic (Duwiejua *et al.*, 2002), opioid (Menziez *et al.*, 1998), anti-plasmodial (Ezeamuzie *et al.*, 1994), anti-microbial (Fakeye *et al.*, 2004), anti-inflammatory (Obiri 1997; Duwiejua *et al.*, 2002), anti-pyretic (François *et al.*, 1996), trypanocidal (Wosu and Ibe 1989), as well as anti-leishmanial (Iwu *et al.*, 1992) activities. The bark is also used to prepare remedies for male sexual impotence (Adjanooun *et al.*, 1996). Phytochemical screening of the freshly prepared fruit extract of *Picralima nitida* revealed the presence of alkaloids, flavonoids, saponins, tannins and glycosides (Obasi *et al.*, 2012). The acute toxicity LD₅₀ was estimated at 14.5 and 12.5 g/kg body weight for male and female, respectively (Nyunaï and Njifutié, 2006).

Uterine contractions have been recognized as an important factor responsible for prevention of conception, implantation of newly formed zygote, as well as dislodgement of the already implanted ones. This is otherwise known as anti-conception. Drugs and plant extracts that precipitate myometrial contraction are known to cause anti-conception in animals. Our earlier *in vitro* studies (Mbegbu *et al.*, 2014a) proved that ethanolic fruit extract of *Picralima nitida* is a potent myometrial contractant, hence the need to investigate its potency *in vivo*. This study was therefore designed to evaluate the effects of ethanolic fruit extract of *Picralima nitida* on conception and oestrogenicity.

Materials and Methods

Plant material and its extraction

Mature unripe fruits of *Picralima nitida* (Stapf) were collected from the Tropical rain forest area of Anambra State of Nigeria. Botanical identification by the aid of freshly collected fruits and leaves was done at the International Centre for Ethnomedicine and Drug Discovery, Nsukka, where a voucher number INTERCEDD/32 has already been designated for *Picralima nitida* (Stapf). Fruits of *Picralima nitida* were cut into small pieces and dried under sunlight and subsequently pulverized with manual grinder into coarse powder. Three hundred and ninety five grams (395 g) of the ground plant materials were first extracted with 1.8 litres of absolute petroleum ether (60-80 °C) for 48 hours, followed by extraction with 2.5 litres of 30% aqueous ethanol for another 72 hours. In each case, the set ups were agitated every 2 hours. After filtering, the filtrates were poured into petri dishes, and solvents allowed evaporating at room temperature. The percentage yields were calculated using the following formula: yield = weight of extract divided by the weight of the starting material multiplied by 100. The extracts were preserved at 4 °C until usage.

Animals

Twenty-one female Sprague Dawley rats, 12-14 weeks of age, weighing between 150 and 200 grams were used to evaluate the anti-conceptive effects of the extract. For the determination of the oestrogenic activity of the extract, twenty-four immature female Sprague Dawley rats, 4-5 weeks of age, weighing between 35 and 45 grams were used. The animals were procured from Laboratory Animal Unit of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. Prior to the commencement of the experiments, the animals were acclimatized for a period of three weeks. They were housed in metal cages under room temperature, with 12-hour light and 12-hour dark cycle. Clean water and feed (Vital® Growers feed, GCOML, Jos, Nigeria) containing 14.5 % crude protein were supplied *ad libitum*. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care. The study was approved by the animal ethical committee of University of Nigeria, Nsukka, and the laboratory animals were handled humanely during the study.

Anti-conceptive activity

Each female rat was successively paired with a male rat. A second female rat was introduced into the cage only when mating with the first female rat had been confirmed. Successful mating was confirmed by the presence of a copulatory (vaginal) plug on the floor of the cage in the next morning and/or the presence of whitish flakes (remnants of the copulatory plug) in fresh vaginal smear made on a clean, grease-free microscope slide (Ochiogu *et al.*, 2006). Following mating, the female rats were separated from the males and the day copulatory plug and/or flakes were found designated day 1 of gestation. Thereafter, simple random sampling method was adopted to assign the twenty-one successfully mated female rats into three groups, comprising of seven rats each.

Groups 2 and 3 received intraperitoneal injection of 50 mg/kg and 500 mg/kg of the extract dissolved in distilled water respectively for four consecutive days starting from the first day of gestation. Rats in Group 1 were given 0.5 ml of distilled water and served as control. The choice of dose was based on the acute toxicity test (Nyunaï and Njifutié, 2006) and *in vitro* studies (Mbegbu *et al.*, 2014a).

On the 20th day of gestation, percentage of pregnant females per group (PPF), mean live foetal number (LFN), corpus luteum number (CLN), resorbed embryo number per pregnant female (REN) and foetal crown-rump lengths (FC-RL) were evaluated. The postcoitum Fertility Index (embryo score) was deduced from the equation (Wong *et al.*, 1987; Uchendu *et al.*, 2000):

$$FI (\text{Fertility Index}) = \text{LFN} \times \text{FC-RL} \times \text{PPF} / \text{CLN}.$$

Oestrogenic activity

Out of the twenty-four sexually immature female rats, eighteen were ovariectomized, whereas six were sham-operated, but had their ovaries intact. Fifteen days after ovariectomy, the 18 immature female rats were assigned to three groups of six rats each, using simple random sampling method. Animals in Group 1 were given subcutaneous

injection of 0.1 mg/kg body weight of Stilboestrol suspended in paraffin oil. Rats in Group 2 received subcutaneous injection of 50 mg/kg of the extract while rats in Group 3 were given 0.2 ml of paraffin oil only. All the treatments were given once daily and lasted for four consecutive days. Group 4 comprised of the six rats which were sham-operated but had their ovaries intact. These were not treated and were regarded as 'intact' rats.

Twenty-four hours after the last treatments, the body weights of the rats were recorded. The rats were subsequently anaesthetized and the uteri dissected out with removal of surrounding tissues, blotted on filter paper and immediately weighed on a sensitive digital weighing balance (Metler H₃O, Switzerland). The respective uterine allometric weights (UAW) were calculated in relation to the body weight of each rat. A portion of the uterine tissues from controls and treated groups were fixed for about 12 hours in Bouin's fixative, successively dehydrated in ascending grades of alcohol, cleared in xylene and embedded in a fresh molten paraffin wax in order to form a hard block. The blocks were then mounted on a microtome and sections of about 5-6 micrometer thick obtained. Finally the tissue sections were stained with haematoxylin and eosin (H&E) and examined under digital microscope.

Statistical analysis

The computer software, statistical package for social sciences (SPSS) version 16 for windows was used for the statistical analyses. The data obtained were subjected to one-way analysis of variance (ANOVA) and the means separated using Duncan's New Multiple Range Test. The results were presented as the mean with the standard error of the mean (SEM). Differences in the means were considered significant at the probability values less than 5% ($p < 0.05$).

Results

Percentage yield of the extracts

Percentage yield (w/w) was 9.44% w/w of the dry matter. The extract was dark brown in colour and semisolid in consistency.

Anti-conceptive activity

The extract at both 50 mg/kg and 500 mg/kg respectively elicited absolute conception failure (anti-conception) in 42.86% of the treated rats. Rats in the control group had 100% conception (Table 1). The mean body weight of the dams treated with 500 mg/kg body weight of the extract was significantly ($p < 0.05$) lower than the mean body weight of the rats in the control group, but did not significantly ($p > 0.05$) vary from the mean body weight of the rats treated with 50 mg/kg body weight of the extract. The mean live foetal number of the group treated with 500 mg/kg of the extract was significantly ($p < 0.05$) lower than those of the control group and the group treated with 50 mg/kg of the extract. There was no significant ($p > 0.05$) difference in the mean corpora lutea number across the groups (Table 2). The mean number of resorbed embryos in the rats treated with 500 mg/kg of the extract was significantly ($p < 0.05$) higher than the number of embryos resorbed in the control group. However, the mean number of resorbed embryos in the group treated with 50 mg/kg of the extract did not significantly ($p > 0.05$) differ from that of the control group (Table 2). The mean foetal crown-rump length of the foetuses harvested on day 20 of gestation from the rats treated with 50 mg/kg and 500 mg/kg of the extract were significantly ($p < 0.05$) lower than the mean foetal crown-rump length of the foetuses harvested from the control group (Table 2). The extract precipitated a dose-dependent decrease in the FI. The FI of the group treated with 500 mg/kg body weight of the extract (136.79) was significantly ($P < 0.05$) lower than the FI of the group treated with 50 mg/kg body weight of the extract (242.73), which was in turn lower than the FI of the control group (465.30) (Table 2).

Oestrogenic activity

The mean uterine allometric weight of the immature rats treated with 50 mg/kg body weight of the extract was significantly ($p < 0.05$) lower than the mean uterine allometric weight of the immature rats treated with paraffin oil and stilbesterol, but did not differ significantly ($p > 0.05$) from the mean uterine allometric weight of the 'intact' rats (Table 3).

Table 1. Effects of the extract on conception

| Group | Number Pregnant / Number Treated | % Conception | % Anti-conception |
|-----------|-------------------------------------|--------------|-------------------|
| Control | 7/7 | 100 | 0 |
| 50 mg/kg | 4/7 | 57.14 | 42.86 |
| 500 mg/kg | 4/7 | 57.14 | 42.86 |

Table 2. Anti-Fertility activity of the extract

| Groups | BW | LFN | CLN | REN | FCRL | FI |
|-----------|-----------------------------|--------------------------|---------------------------|---------------------------|--------------------------|----------------------------|
| Control | 260.12 ± 4.44 ^a | 7.20 ± 0.73 ^a | 8.00 ± 1.24 ^a | 0.80 ± 0.37 ^a | 5.17 ± 0.05 ^a | 465.30 ± 1.37 ^a |
| 50 mg/kg | 236.04 ± 9.77 ^{ab} | 8.00 ± 1.53 ^a | 9.67 ± 0.33 ^a | 1.67 ± 1.20 ^{ab} | 4.89 ± 0.10 ^b | 242.73 ± 3.66 ^b |
| 500 mg/kg | 221.38 ± 14.67 ^b | 5.00 ± 1.28 ^b | 10.33 ± 0.45 ^a | 5.33 ± 2.73 ^b | 4.71 ± 0.11 ^b | 136.79 ± 2.81 ^c |

*ab different superscripts in a column represents significant differences ($p < 0.05$) between groups.

NB: Body weight (BW), Live foetal number (LFN), Corpora lutea number (CLN), Resorbed embryo number (REN), Foetal crown-rump length (FCRL), Fertility index (FI).

The immature rats treated with stilbesterol showed significant ($p < 0.05$) increase in the mean uterine weight when compared to the rest of the groups (Table 3). The vagina of all the rats remained closed in the extract-treated and 'intact' groups whereas rats in the stilbesterol-treated as well as the paraffin-treated groups showed vaginal opening (Table 3).

The histoarchitecture (uterotrophic changes, such as thickness of the endometrium) revealed that the thickness of the endometrium in the stilbesterol-treated (Fig. 1) was markedly higher than the endometrial thickness of the paraffin-treated (Fig. 2), extract-treated and intact animals (Fig. 3). The extract-treated and 'intact' rats' uterine horns had the lowest endometrial thickness (Fig. 3).

Table 3. Estrogenic activity of the extract

| Group | BW (g) | UW (g) | UAW | VO |
|----------------------|----------------------------|---------------------------|---------------------------|------------|
| Extract-treated | 110.9 ± 7.60 ^a | 0.036 ± 0.01 ^a | 0.032 ± 0.01 ^a | Not open |
| Intact | 72.64 ± 8.49 ^b | 0.034 ± 0.00 ^a | 0.048 ± 0.00 ^a | Not open |
| Paraffin-treated | 104.44 ± 4.72 ^a | 0.084 ± 0.01 ^b | 0.081 ± 0.01 ^b | Open (++) |
| Stilbesterol-treated | 106.52 ± 4.62 ^a | 0.206 ± 0.02 ^c | 0.19 ± 0.02 ^c | Open (+++) |

*abc different superscripts in column represents significant differences ($p < 0.05$) between groups
NB: Body weight (BW), Uterine weight (UW), Uterine allometric weight (UAW), Vaginal opening (VO)

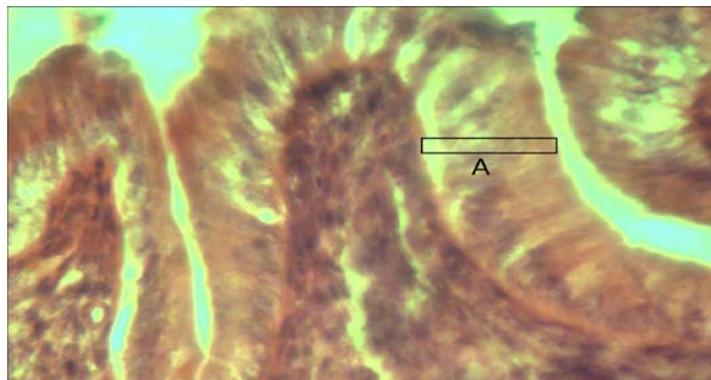


Fig. 1. Photomicrograph of endometrium from stilbesterol-treated rat, showing marked increase in endometrial thickness (A) and convolutions (transverse section, x400, H&E Stain)

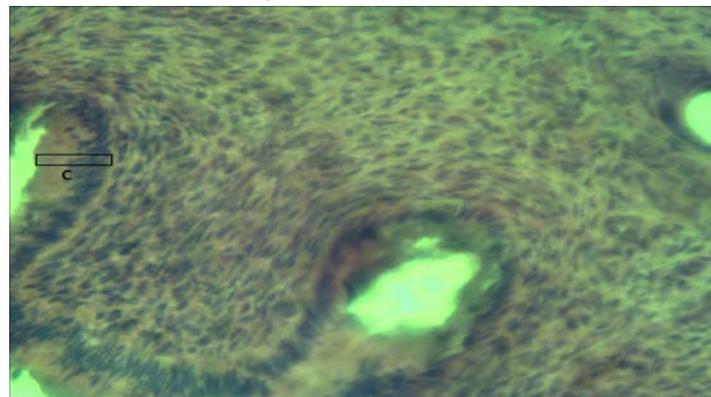


Fig. 2. Photomicrograph of endometrium from paraffin-treated rat, showing moderate increase in endometrial thickness (C) and convolutions (transverse section, x400, H&E Stain)

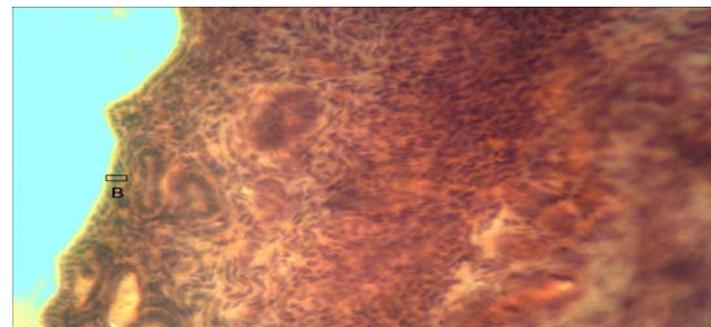


Fig. 3. Photomicrograph of endometrium from extract-treated and 'intact' rats, showing short endometrial thickness (B) and absence of convolutions (transverse section, x400, H&E Stain)

Discussion

Myometrial tonicity which characterizes the follicular phase of oestrous cycle must be abolished for conception and implantation to occur. The 42.86% absolute anti-conception recorded in the mated female rats treated with 50 mg/kg and 500 mg/kg body weight between days 1 and 4 could be attributed to the contractile effect of the extract, since the rats in the control group had 100% conception rate. This shows that the extract inhibits implantation when given at the zygotic stage (days 1-3) and the blastocyst stage (days 3-4) (Schmidt, 1995). The endometrial environment might not be conducive for implantation (Hazarika and Sarma, 2007) as revealed by the significant reduction in the mean live foetal number (LFN). In addition to the endometrial microenvironment, hyper motility of the myometrium can also prevent implantation, especially in view of the effect of the extract on the isolated uterine tissues (Mbegbu et al., 2014a). It is also possible that the newly implanted embryos that were exposed to the contractile effect of this extract were dislodged from the foeto-maternal units and consequently resorbed. The resorbed embryo number (REN) in the treated groups which was significantly ($p < 0.05$) higher than that of the control group further reveals the anti-conceptive effect of this extract. Embryo resorption which was more, especially at the higher treatment dose of the extract could also be responsible for the significant reduction in the mean live foetal number (LFN). Several reports indicate anti-fertility activity of crude extracts and active compounds in animal models (Badmi et al, 2003; Gebrie et al, 2005; Uchendu and Isek, 2008). The mechanism of anti-implantation activity of *Monecha ciliatum* has been proposed to be its strong uterotonic property (Uguru et al., 1998). Similarly, *Musanga cecropioides*, a common Nigerian medicinal plant used for its oxytocic effect has been reported to increase uterine contraction in a dose - dependent manner (Ayinde et al., 2006). The mean foetal crown-rump length (FCRL) of the fetuses harvested from the treated groups was found to be significantly lower than that of the control group, regardless of the fact that the decreased LFN supposed to have created more intra-uterine space for foetal growth. This suggests that the extract could have teratogenic or toxic effect. Nyunai and Njifutié (2006) had earlier associated this same plant with the toxicity of the intraperitoneal cavity. Toxicity of the kidney cells has also been recorded in an attempt to induce abortion on days 15 and 16 using 500 mg/kg body weight of ethanolic fruit extract of *Picralima nitida* (Mbegbu et al., 2014b). A reduction in FCRL in the treated pregnant rats as observed in the present study had also been reported when *Acanthus montanus* leaves extract (Nana et al., 2008) and *Hymenocardia acida* stem bark extract (Abu and Uchendu, 2011) were administered to pregnant rats. The significant difference in the body weights of the rats treated with 500 mg/kg body weight is simply a reflection of the significant reduction in the mean live foetal number (LFN) and foetal crown rump length (FCRL). Generally, the result of the study showed a dose-dependent decrease in the fertility index across the groups as the dose of the extract increased. The fertility index for the control group and groups that

received 50 mg/kg and 500 mg/kg of the extract were 465.30 ± 1.37 , 242.73 ± 3.66 , and 136.79 ± 2.81 respectively. This lowered fertility index could be attributed to the summation of the effects of the extract on the parameters analyzed, the contractile effect of the extract and the possible toxicity associated with the extract. Similar observations on reduced fertility indices were made when pregnant rats were treated with *Dalbergia axatilis* (Uchendu et al., 2000) and *Hymenocardia acida* (Abu and Uchendu, 2011).

Many antifertility plant extracts are known to exhibit oestrogenic activity in rats (Gebrie et al, 2005). Oestrogen causes an increase in protein synthesis, uterine weight, water uptake and retention of fluid leading to ballooning of the uterus (Rifai et al, 2001). In addition, oestrogen also induces uterotrophic changes such as increase in diameter of the uterus, thickness of endometrium, height of endometrial epithelium, providing non-receptive conditions for implantation (Dhar, 1995). Oestrogen causes vaginal opening, which is a qualitative measure of oestrogenic potency. Administration of the ethanolic fruit extract of *Picralima nitida* to immature rats at the dose of 50 mg/kg did not show any oestrogenic activity in that there were no changes in the average uterine weight and histoarchitecture (thickness of endometrium or height of endometrial epithelium) of the uterus when compared to the control 'intact' rats. The failure of vaginal opening further confirms that this extract is not oestrogenic in activity. Oestrogen is known as the predominant hormone in the follicular phase of oestrous cycle and elicits myometrial contractions. The failure of this extract to induce oestrogenicity suggests that the *in vitro* myometrial contraction elicited by this extract did not occur by the stimulation of the oestrogenic receptors. Mbegbu et al., (2014a) postulated that ethanolic fruit extract of *Picralima nitida* evoked myometrial contractions possibly by mobilization of the extracellular calcium and stimulation of the alpha adrenergic receptors.

Conclusions

In conclusion, this present study reveals that the ethanolic fruit extract of *Picralima nitida* possesses significant post-coital anti-conceptive activity in rats, which could not be attributed to oestrogenic activity of the plant extract.

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