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Tetrapleura tetraptera Extract Inhibited Luteinizing Hormone and Estrogen Secretion in Clomiphene Citrate Treated Female Wistar Albino Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author EOA designed the study, performed the statistical analysis and wrote the protocol, the first draft of the manuscript and literature searches. Authors CON and CJN managed the analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Tetrapleura tetraptera stem bark has been reported to cause inhibition of luteinizing hormone release in cultured rat pituitary cells. Hence, we investigated the effects of *Tetraptera tetrapleura* pod extract on follicle stimulating hormone, luteinizing hormone, cortisol, progesterone and

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estrogen. Thirty non-pregnant female wistar albino rats were divided into group A - D. Group A rats were used as Control. Group B rats were administered 1 mg/kg/day of clomiphene citrate orally. Group C rats were administered 200 mg/kg of extract only, whereas group D rats were administered 1 mg/kg /day of Clomiphene citrate plus 200 mg/kg of extract. At the end of 14 days experiment, group A, B and D were found in proestrus phase and group C in diestrus phase. FSH and cortisol levels remained unchanged. Group C and D rats produced significant reduction ($P < 0.05$) in LH and estrogen levels in prolonged proestrus and normal diestrus respectively. Progesterone level was significantly high ($P < 0.05$) in the group C rats. The reduced LH level could be due to the anti-estrogenic effect of extract during proestrus when LH secretion is expected to surge. But co-administration resulted in high progesterone secretion, suggesting extract may have influenced progesterone secretion in group D rats simultaneously administered with clomiphene citrate and extract. The above findings indicated that *Tetrapleura tetraptera* pod extracts inhibited luteinizing hormone and estrogen even when co-administered with clomiphene citrate.

Keywords: Tetrapleura tetraptera; clomiphene citrate; estrous cycle.

1. INTRODUCTION

Tetrapleura tetraptera is a perennial plant that belongs to the family of Fabaceae. It is a single stemmed robust tree of about 30 m in height, generally found in the lowland forest of tropical Africa [1]. The fruit is locally known by several names including Oshosho, Ngala-ngala, or Osakrisa in Igbo, Aridan in Yoruba or Dawa in Hausa. It is very persistent, hanging at the ends of branches on stout stalks 25 cm long. It is shiny, glabrous, dark purple brown, usually slightly curved 15 -25 cm long by about 5 cm wide, with four longitudinal wing like ridges nearly 3 cm broad. The seeds which rattle in the pods are small, black, hard, flat, about 8 mm long embedded in the body of the pod, which does not split [2]. The dried fruit has an unpleasant aroma [1]. The fruits have low values for tannin, sterol, phenol and saponin, and high values for hydrogen cyanide, alkaloid and flavonoid [3]. The fruit is used as popular seasoning in the Southern and Eastern parts of Nigeria [4]. The fruit, leaf, and stem bark are used in traditional medicine. Previous study had shown that triterpenic saponin from stem bark extract exerted inhibitory effect on luteinizing hormone (LH) in cultured rat pituitary cell in a dose-dependent fashion [5]. The present study employed the use clomiphene citrate which is considered one of the most effective drug in the treatment of female infertility [6], in order to investigate the activities of methanolic extracts of *Tetrapleura Tetraptera* pod on LH, follicle stimulating hormone (FSH), progesterone, estrogen and cortisol with respect to the rats' estrous cycle since it regulates reproductive hormone secretion.

2. MATERIALS AND METHODS

Thirty non-pregnant adult female wistar albino rats (150-200 g) aged 120 days were obtained from the animal farm in Delta State University. The animals were subsequently transferred to the animal house of Madonna University for 30 days. They were then acclimatized for another 14 days in standard cages at a temperature of 25± 3℃, 12 hours light and 12 hours dark cycles. Rats were fed with standard pellets and tap water ad libitum. The drug Clomiphene citrate (Doppel, Italy) was purchased in the form of 20 mg/kg tablets. 200 tablets of Clomiphene citrate were crushed in a mortar into a fine powdered form and extracted with methanol (Sigma Aldrich, USA) to remove excipients. The active ingredient clomiphene citrate was oven dried and stored in a refrigerator at -4℃. Daily dose of clomiphene citrate of 1mg/kg/day as described by Boyar et al. [7], was administered orally to the rats using gavage. The experiment lasted for 14 days after acclimatization.

2.1 Drug and Extract Preparation

2.5 g of clomiphene citrate tablet (50 mg per tablet) was grounded into a powder form, suspended in methanol and sieved with Whatman paper (No 1) to remove excipients. The filtrates was then stored in a refrigerator (4°C).

Tetrapleura tetraptera fruits weighing 1 kg were purchased in a local market, Afor Ogbe in Ahiazu Local Government Area of Imo State, Nigeria.The fruits were identified in the herbarium of the Department of Pharmacognosy, Madonna University with voucher number

(MUE/PGSY/066). The fruits were then crushed using a local pestle to remove seeds from the pod. The seeds were separated from the pods. The 600 g of pods were sun dried for 7 days and ground in coarse from for Soxhlet method of extraction separately. In this method, Methanol (Sigma Aldrich, USA) was poured in soxhlet containing the pod in the form of Soxhlet per 250ml of methanol. At the end the methanol (solvent) is separated from the extract using Rotavapor device. The dose concentration of extract administered to the rats was within the range of safety according to acute toxicity studies performed by Effiong et al. [8].

2.2 Animal Ethics

All the animals received humane care according to the criteria outlined in the Guide for the Care and the Use of Laboratory Animals prepared by the National Academy Science and published by the National Institute of Health [9].

Twenty four non-pregnant wistar albino rats in metestrus phase were randomly divided into four groups (n=6) including: Group A (control) – normal without drug or extract. Group B received 1 mg/kg/day/day (body weight) of clomiphene citrate orally. Group C received 200 mg/kg/day (body weight) of methanol extract of *Tetrapleura Tetraptera* pod orally. Group D received 200 mg/kg/day (body weight) of methanol extract of *Tetrapleura Tetraptera* pod orally plus 1 mg/kg/day clomiphene citrate orally. The remaining six rats were not used because they were not in uniform phase as the others on the first day.

On days 1 and 14 of the study, between 7am – 9am (WAT), estrous cycle was determined using the method described by Marcondes et al. [10]. After 14 days experiment, animals were anaesthetized under chloroform and sacrificed in the morning between 9 am – 11 am (CAT). Blood was collected from the heart with a 5 ml syringe into EDTA bottles. FSH, LH, cortisol, oestrogen and progesterone were measured using enzyme-linked immunosorbent assay (ELISA) method.

2.3 Calculation and Statistics

Results were expressed as mean ± standard error of mean (SEM). Statistical significance of differences observed between control and experimental groups, ANOVA was used to analyze results. Any significant ANOVA was further analyzed by Tukey's post hoc test. P values < 0.05 were considered statistically significant.

3. RESULTS

The estrous cycle showed that group A and C recorded sustained prolonged duration of diestrus phase, whereas group B and D rats recorded prolonged estrus phases of the estrous cycle on day 11 - 13. Results showed that mean concentration of LH in group B (0.75 ± 0.05 mIU/ml) was significantly higher ($P < 0.05$) than in groups A, C and D (0.34 ± 0.08, 0.17 ± 0.030, and 0.21 ± 0.02) mIU/ml. The mean concentration of LH was also higher in group A than that of groups D and C respectively. The mean concentration of FSH and cortisol was not statistically significant ($P > 0.05$) in group A and B (0.022 ± 0.00 mIU/ml; 7.68 ± 0.18 ug/dl and 0.018 ± 0.00 mIU/ml; 5.46 ± 0.90 ug/dl) compared with groups C (0.022 ± 0.00 mIU/ml; 5.68 ± 2.30 ug/dl) and group D (0.017 ± 0.00 mIU/ml; 4.99 ± 2.34 ug/dl). Results also showed that mean concentration of progesterone in group A (2.90 ± 0.49 ng/ml) was significantly higher ($P < 0.05$) than in group B (0.26 ± 0.05 ng/ml). The mean concentration of progesterone was also significantly higher ($P < 0.05$) group C (3.68 ± 0.57) than in group D (1.33 ± 0.02 ng/ml). Progesterone level was significantly reduced ($P < 0.05$) in group B (0.26 ± 0.05 ng/ml) than in group A, C and D. There was statistically significant reduction ($P < 0.05$) in progesterone levels in group D compared to groups A and C.

The mean concentration of estrogen was significantly higher ($P < 0.05$) in group A and B (80.07 ± 4.68 and 79.57 ± 1.28 pg/ml) than in group D (70.33 ± 8.23pg/ml) and group C (58.49 ± 10.04 pg/ml). The mean concentration of estrogen was also significantly higher ($P < 0.05$) in group D than in group C.

4. DISCUSSION

The present study showed that methanolic extract of *tetrapleura tetraptera* pod caused significant reduction in LH and estrogen in the presence and absence of clomiphene citrate. Study in cultured pituitary cells have shown that saponin from the stem bark extract of *tetrapleura tetraptera* inhibited LH release from the gonadotropes in a dose-dependent fashion in female rats [5]. Therefore, the present study investigated the possible mechanism responsible for LH inhibition by monitoring the estrous cycle of rats and some reproductive hormones that

regulate LH secretion. The estrous cycle is important in reproductive hormone regulation [11], and because drugs that interfere with reproductive functions do so by changing the morphology of the reproductive tract or by a disturbance in the duration of particular phases of the estrous cycle [12]. The group A, B and D rats had 3 quick successive estrous cycles in 14 days, characterized by a short diestrus phase. Although the rats were in the proestrus on the last day, the 3 quick successive cycles appeared to have been triggered by clomiphene citrate, in contrast to non-clomiphene citrate treated group C. This therefore, suggested that clomiphene citrate influenced the estrous cycle in the study.

In order to determine whether the estrous changes could influence hormone production, the last phases of the respective groups were taken into account, because according to Spornitz et al. [13], LH usually increases in proestrus phase, whereas estrogen begins to rise in metestrus and peaks in late proestrus phase. Based on the estrous cycle in Table 1b, group B recorded prolonged estrus phases with short diestrus. LH increase in group B could be clomiphene citrate effect as expected to elicit significant increase in LH. Clomiphene citrate has been shown to augment estrogen-induced preovulatory LH surge in anovulation [14].

Group C recorded the longest duration of diestrus evidenced with reduction in LH and estrogen, whilst group D recorded one-half of estrus and one-half of disetrus (as shown in Day 11 - 14), and ended in prostrus on Day 14. The LH level significantly reduced, therefore suggesting that ovulation could be altered (possibly pseudo-ovulation) because of the importance of LH surge prior to ovulation.

Normally, FSH steadily increases estrogen level by producing new ovum containing follicles in the ovary. The new follicles in turn steadily produces estrogen that stimulates the anterior pituitary to secrete LH responsible for triggering ovulation in intact adult female. As observed, extract did not affect FSH but caused inhibition of estrogen production in the follicles leading to the significant reduction in the secretion of LH, although several studies have shown that not only estradiol but progesterone regulate the pituitary secretion of LH via hypothalamic gonadotropin releasing hormone [15,16,17,18].

In addition, dry fruit extract of *Tetrapleura tetraptera* has been shown to contain saponin [19]. Studies have also reported that saponin inhibits aromatase enzyme [20,21]. This aromatase enzyme is responsible for estrogen production [22]. Therefore, it seemed the mechanism responsible for LH reduction could be saponin inhibition on aromatase enzyme corroborating the findings by el Izzi et al. [5].

Table 1a. Hormonal changes following administration of 200 mg/kg/day of *tetrapleura tetraptera* methanolic extract with and/or without 1 mg/kg/day of clomiphene citrate

	LH (mIU/ml)	FSH (mIU/ml)	Progesterone (ng/ml)	Estrogen (pg/ml)	Cortisol (ug/dl)
Group A (Control)	0.34 ± 0.80*	0.022 ± 0.00	2.90 ± 0.49*	80.07 ± 4.68	7.68 ± 0.18
Group B	0.75 ± 0.50	0.018 ± 0.00	0.26 ± 0.05	79.57 ± 1.28	5.46 ± 0.90
Group C	0.17 ± 0.30*	0.022 ± 0.00	3.68 ± 0.57*	58.49 ± 10.04*	5.68 ± 2.30
Group D	0.21 ± 0.02*	0.017 ± 0.00	1.33 ± 0.02*	70.33 ± 8.23*	4.99 ± 2.34

** P < 0.05 denotes significant. All values are expressed as mean ± SEM; n = 6 in each group*

Table 1b. Estrous cycle changes following administration of 200 mg/kg/day of *tetrapleura tetraptera* methanolic extract with and/or without 1 mg/kg/day of clomiphene citrate

	Day 1-2	Day 10-11	Day 11-12	Day 12-13	Day 13-14
Group A (Control)	Metestrus-proestrus	Estrus-metestrus	Diestrus	Diestrus-diestrus	Diestrus-proestrus
Group B	Metestrus-proestrus	Proestrus-estrus	Estrus	Estrus-diestrus	Diestrus-proestrus
Group C	Metestrus-proestrus	Estrus-metestrus	Diestrus	Diestrus	Diestrus
Group D	Metestrus-proestrus	Proestrus-estrus	Estrus	Estrus-diestrus	Diestrus-proestrus

In the other hand, inhibition of saponin on aromatase will impair estrogen synthesis, consequently resulting in the failure of FSH-LH-estrogen surge, because estradiol enhances maximal value of gonadotropin-releasing hormone dose-LH secretory response [23].

Progesterone increases during metestrus, diestrus, and peaks in proestrus phase of the estrus cycle [13]. The result showed that progesterone level was elevated in group C. This could be due to permissive effect of the extract in the face of synergy between drug and extract, since reduction in progesterone was clearly evidenced in proestrus group B treated with clomiphene citrate only and group D treated with extract only.

Lamfon and Al-matrafi [24] showed that clomiphene citrate significantly reduced progesterone level in female rats. The group C had successive changes of phases that could as well be attributed to the permissive effect causing formation of corpus luteum that is responsible for the biosynthesis and secretion of progesterone. Furthermore, stress had been found to interfere with gonadotropin secretion in women [25]. Thus we studied the effects of extracts on cortisol as a stress marker. Cortisol has been found to inhibit estradiol-induced elevation of LH in intact female rats without altering FSH level [26]. The cortisol level remained unchanged suggesting that extract caused reduction in LH and estrogen independent of cortisol.

5. CONCLUSION

It can be concluded that the methanolic pod extract of *Tetrapleura tetraptera* caused prolonged diestrus phase characterized by low LH and estrogen. This reduction in serum LH could be due to low estrogen production by the extract. Moreso, the rise in progesterone in the face of clomiphene citrate administration remains unclear but appeared to be independent of gonadotropin regulation. Therefore, *Tetraptera tetrapleura* can be used in family planning since plant inhibited ovulation in mature female rats.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all the experiments followed standard protocol and was approved by the Research Committee, Department of Human Physiology, Professors A. Nwafor (Adjunct Professor to the Department) and A. C. Ugwu (Sir), External Examiner to the Department. All experiments have therefore been performed in accordance with the ethical standards laid down in 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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