Original Research Article

Disruption of Ovarian Utilisation of Proteins by *Tetrapleura tetraptera* Fruit Extract Impairs Oestrous Cycle and Ovarian Functions in Female Rats

ABSTRACT

Aim: Reports that some phytochemicals interfere with reproductive functions, in both humans and animals necessitated this study which is aimed at determining the effects of fruit extract of *tetrapleura tetraptera* on oestrous cycle and ovarian functions in females. **Methods**: Adult female *wistar* rats weighing 160-180g with regular 4-5 days oestrus cycle were selected into 4 groups of 6 animals each. Group 1 (control) administered 1ml distilled water; groups II, III and lV were daily treated with *the* extract at doses 75mg/kg, 150mg/kg and 300 mg/kg body weight respectively, orally for 21 days. Microscopic evaluation of vaginal smear was done daily to determine the various stages of the estrous cycle, their duration, as well as the estrous cycle length. After 24 hours of last administration, each rat was weighed, sacrificed, and right ovary was homogenised and the homogenate used for analyses of total protein content, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) concentration, according to standard protocols. **Results:** There was significant ($P < 0.05$) increase in duration of diestrus phase and estrous cycle length in all the extract-treated groups, compared to control animals. Also, there was relative reductions in the duration for proestrus ($p<0.05$), estrus ($p<0.05$) and metestrus ($p<0$) phases of the cycle, with a relative increase in duration for diestrus phase ($p<0.05$) in animals treated with 150mg/kg and 300mg/kg body weight respectively. In addition, a significant ($P < 0.05$) increase was observed in ovarian Protein, and Superoxide dismutase (SOD) enzyme activity; as well as significant ($P < 0.05$) reduction in malondialdehyde (MDA) level and in weight gain in the test animals, compared to the control. **Conclusion:** *Tetrapleura tetraptera* fruit extract disrupts ovarian utilisation of proteins in the ovaries, thereby impairing oestrous cyclicity, and body weight. These could result to infertility.

Key words: *Tetrapleura tetraptera,* estrous cycle, ovarian function, protein, infertility.

1. INTRODUCTION

Mankind, since from the early existence, have depended on medicinal plants for various health needs including controlling physiological activities and pathological conditions. Some of these phytochemicals interfere with reproductive functions especially, the reproductive cycle [1-3] and

ovarian secretory functions in both humans and animals. Such effects may be at different levels of the hypothalamic-pituitary axis, or at the ovarian level, resulting in alterations in the gonadotrophic hormones (FSH and LH) and female sex hormones (Oestrogen and Progesterone) levels.These effects could alter ovulation and therefore fertility, which are of pubic health concern, hence contributing to the increasing global attention about the use of phytomedicines[4].

Tetrapleura tetraptera plant is found in tropical Africa, especially West Africa low lands forests. The fruits are made up of a fleshy pulp with small, brownish-black seeds. They are green when tender, but dark brown when fully ripe, with high nutritional value [5]. When dry, they have a pleasant aroma, and therefore are used as spice in Central and West Africa [6,7]. In Nigeria, it is called "Aridan or aidan" by the Yurobas, "Dawo" among the Hausas, "Oshosho" by the Igbos, Efik call it "Edeminang", the Ibobios call it "Uyayak" [8], while the Ijaws call it Pakipaki [personal interviews].

The fruits are used in managing convulsions, inflammation, rheumatism, flatulence, jaundice and fevers, as well as adult onset type 2 diabetes mellitus [9,10]. The leaf extract has antioxidant effect [11], while the aqueous fruit extract possess hypoglycemic properties [12]. The fruits are also used in preparing soups for mothers after delivery to prevent contraction of the uterus [13], as well as for stimulation of lactation [14], thus suggesting its potential in altering female reproductive functions. Literature search revealed paucity of studies on effects of the fruits of *T. Tetraptera* on female reproductive functions. Therefore, this study was aimed at determining the effects of the fruit extract of *T. Tetraptera* on estrous cycle and ovarian functions in females, using *wistar* rats as animal models.

2. MATERIALS AND METHODS

2.1 Plant materials and extraction: Dry fruits of *Tetrapleura Tetraptera* purchased from a market (oil mill market) in Port Harcourt, Rivers State Nigeria, were identified and authenticated by taxonomist in the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria, (herbarium number, UPH/C/104). The fruits were washed clean, dried, sliced into small pieces and then machine-grounded into fine, powdery particles.

Using the cold extraction method, 3000g of the finely crushed powder was stocked in 9L hydro-methanol solvent (20:80) in an aspirator jar. The mixture was shaken vigorously for 30 minutes and then left to stand for 72 hours at room temperature (23-28 ^{0}C). The resulting solution was then filtered using a wire gauze and a sieve with tiny pores (0.25mm). The filtrate was

concentrated to dryness at 40 ^{0}C by vaporizing in an oven, and the brownish gel-like extract then stored in a refrigerator.

2.2 Animal model: Experimental studies on the changes occurring during reproductive cycles are often done using the female rats as suitable laboratory animal models because of their short oestrous cycle length [15,16]. Healthy, non-pregnant adult female *wistar* rats (160-180g) obtained from the animal facility of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Port Harcourt, were used for this study. The rats were housed in wire-meshed wooden cages at natural environmental conditions of temperature (25±2°C) and natural light: dark cycle; and had free access to standard rat chow and water *ad libitum.* They were allowed to acclimatise for 10 days. The initial body weight of all the animals were measured at commencement of the experiment. All animals used in this study were handled in accordance with the international, national and institutional guidelines for care and use of laboratory animals in Biomedical Research as promulgated by the Canadian Council of Animal Care (2009).

2.3 Determination of oestrous cycle: Prior to commencement of administration of the extract, the estrous cycle of each rat was evaluated for a period of 21 days, with modifications of previously reported procedures [15,17]. Briefly, vaginal smear was collected from each rat once daily (8:00 - 9:00 am) by gently inserting the tip of a plastic micropipette containing 10µl saline solution into the vagina of the rat, flushed two to three times, and the fluid aspirated and smeared on a clean glass slide. Light microscopic observation of the cell types obtained in each smear was done. The proportion of the different types of cells was used in evaluating the phase and the duration of the oestrous cycle. Animals with regular normal oestrous cycle were selected for the experiment.

2.4 Experimental design: A total of twenty-four (24) female *wistar* rats showing regular 4-5 days oestrus cycle were selected and assigned into four groups of six animals each. Group 1 (control) was administered 1ml distilled water; group II, III and lV were daily treated with the extract at doses of 75, 150 and 300 mg/kg body weight respectively. The above doses were administered by oral gavage for 21 days (4-5 regular oestrous cycles), since the mean estrous cycle length of rats is 4-5 days [18], starting when animals were in the proestrous phase of the oestrus cycle. The various stages of the estrous cycle and their duration, as well as the estrous cycle length were determined. The percentage of the duration of Oestrous cycle that the animals spent in each phase of the entire oestrous cycle within the 21 days duration of the study was

calculated, from where the relative differences in each of the test groups, compared to the control group, were determined.

2.5 Sample collection: After the last administration, and while each animal was in oestrous phase, each rat was sacrificed using chloroform anaesthesia, after measuring the body weight. The ovaries from each rat were dissected out, cleared of adherent tissues and weighed. One ovary from each rat was homogenised and the ovarian tissue homogenate was used for biochemical analyses: Total Protein content was evaluated using Biuret reagent and as described [19. The activity of Superoxide dismutase (SOD) was evaluated using previous description [20]. Malondialdehyde (MDA) concentration was also determined by measuring the level of formation of thiobarbituric acid reactive substances (TBARS) as reported [21].

Ethical approval for this study was sought for and granted by the Research and Ethical Committee of the University of Port Harcourt.

2.6 Statistical Analysis: The data were analysed using statistical package for social sciences (SPSS) software (version 25.0). Differences in mean values between groups were evaluated using Analysis of variance (one-way ANOVA) followed by Dunnett multiple comparison test. Results were considered statistically significant at $P < 0.05$ and 95% confidence level. The results are presented as mean and standard error of mean (M±S.E.M), as well as in percentages.

3. RESULTS

3.1 Effects of *T. tetraptera* fruit extract on estrous cycle in female *wistar* rats

Effects of *T. tetraptera* fruit extract on the different phases of estrous cycle is as presented in figure1. The durations for the proestrus, estrus and metestrus phases of the estrous cycle in all the test groups showed no significant difference ($p>0.05$), when compared to the durations in their respective control group animals. On the other hand, there was a dose-dependent significant ($P < 0.05$) increase in duration of the diestrus phase in all the extract-treated groups, when compared to the control group animals. Also, the length of the estrous cycle (figure 2) was observed to significantly ($P < 0.05$) increase in a dose-dependent manner in all the extract treated test groups, compared to the control group animals. Further analysis (figure 3) shows that in animals treated with 150mg/kg and 300mg/kg body weight respectively, there was relative reductions in the duration for proestrus ($p<0.05$), estrus ($p<0.05$) and metestrus ($p<0$) phases of the estrous cycle, with a corresponding relative increase in that of the diestrus phase ($p<0.05$) of the estrous cycle, when compared to that of the respective control group animals.

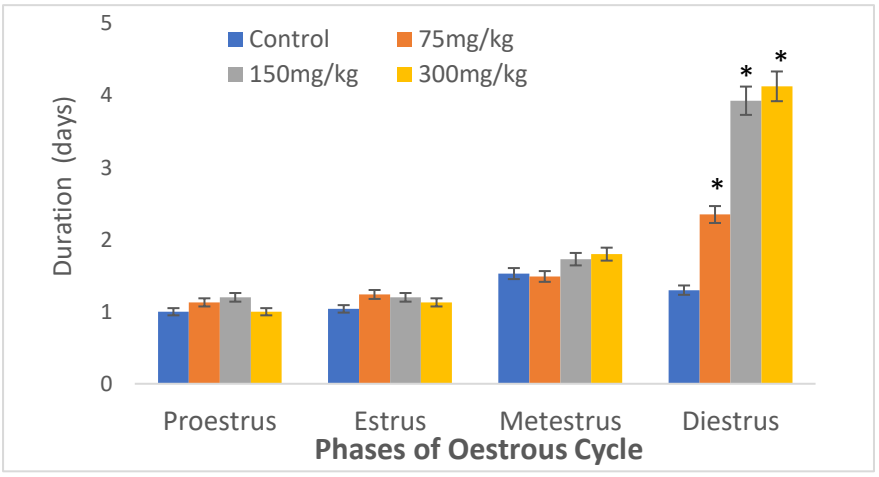

Figure 1: Effect of *Tetrapleura tetraptera* fruit extract on the different Phases of oestrous cycle in rats. *($p < 0.05$).

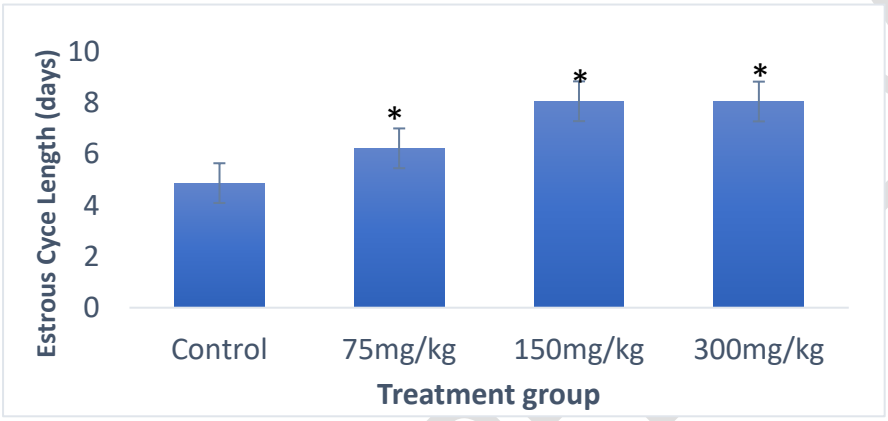

Figure 2: Effect of *Tetrapleura tetraptera* fruit extract on the duration (days) of oestrous cycle in rats. *($p < 0.05$)

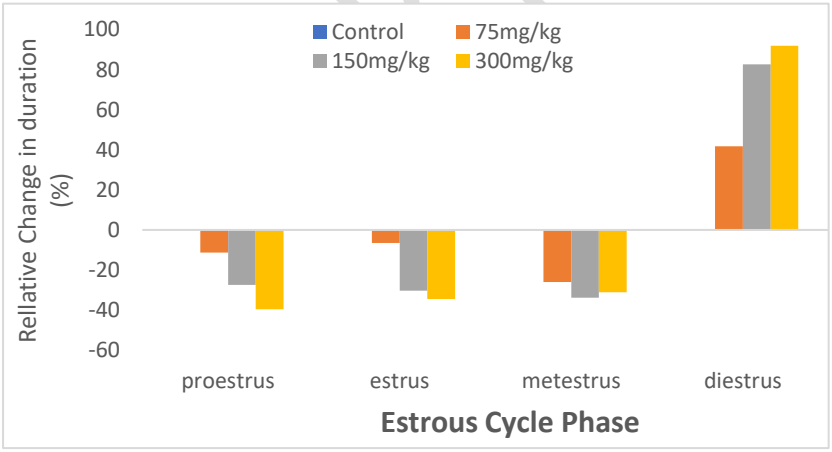

Figure 3: Relative Change in duration (%) of each phase of the oestrous cycle in female treated rats, compared to the control group animals.

3.2 Effects of *T. tetraptera* fruit extract on ovarian tissue oxidative stress markers and proteins

The results (Table 1), show a dose-dependent significant ($P < 0.05$) increase in ovarian tissue Superoxide dismutase (SOD) enzyme activity, and in protein level; with a significant ($P < 0.05$) decrease in malondialdehyde (MDA) level, in the extract-treated test animals, compared to those in the control group. Further analyses show the percentage relative difference in levels of oxidative stress markers and proteins in the ovarian tissues, when compared to the control group animals (figure 4). A relative increase of 44%, 142%, and 360% in Superoxide dismutase (SOD) was observed in the 75mg/kg, 150mg/kg and 300mg/kg extract treated test groups respectively. Malondialdehyde (MDA), on the other hand, showed a relative decreased by 53%, 42% and 68% in the 75mg/kg, 150mg/kg and 300mg/kg extract treated groups respectively. Testicular Protein level also shows a dose-dependent relative increase by 97%, 215% and 221% in the 75mg/kg, 150mg/kg and 300mg/kg extract treated groups respectively, compared to the control group animals.

Table 1: Effects of fruit extract of *Tetrapleura tetraptera* on ovarian tissue proteins and Oxidative Stress markers in female *wistar* rats.

Parameter	**Treatment Groups**			
	Control	**75mg/kg**	**150mg/kg**	**300mg/kg**
SOD (milliU/ml)	11.03±0.99	15.89±0.38	26.71±2.28*	50.76 ± 7.60**
MDA (mmol/L)	21.32 ±0.51	10.06 ± 0.58**	12.41±0.00**	13.09±2.49**
Protein (mg/ml)	0.65 ± 0.01	1.28 ± 0.06*	2.05 ± 0.48*	2.09 ± 0.22*

Data is represented as mean ± SEM (n = 5) where *p ≤ 0.05 or **p ≤ 0.01 is statistically significant compared to control group.

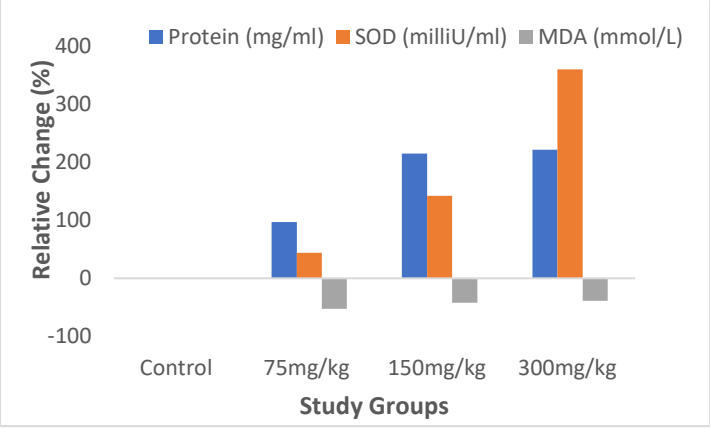

FIgure 4: Relative Change (%) in levels of ovarian tissue proteins and Oxidative Stress markers in female treated rats, compared to control.

3.3 Effects of *T. Tetraptera* on body weight: *Tetrapleura tetraptera* was also observed to affect body weight in female *wistar* rats (Table 2). To demonstrate this, we calculated the difference in weight from the initial and final body weights in both the control and test groups. We also calculated the relative difference in weight in the test groups. The results show that animals in

both the control and test groups increased in weight during the study period. However, there was a significant ($P < 0.05$) relative reduction in weight gain in all the animals in the test groups, compared to those in the control group; with a relative reduction of 82.86% (in 75mg/kg b.w.), 88.57% (150mg/kg b.w.) and 64.29% (300mg/kg b.w.) respectively, compared to that of the control group.

Table 2: Effects of *Tetrapleura* ***teraptera*** fruit extract on body weight of female *wistar* rats.

Treatment group	Initial body Weight (g)	Final Body Weight (g)	Weight difference (g)	Relative Reduction (%)
Control	168.40	182.40	14.00 ± 3.33	0.00
75mg/kg	171.40	173.80	2.40 ± 0.19*	-82.86*
150mg/kg	176.20	177.80	1.60 ± 0.69 *	-88.57*
300mg/kg	153.4	158.4	5.00 ± 0.21*	-64.29*

Data is presented as Mean ± SEM; N = 6 in each group; *P< 0.05 when compared to the control group.

4 Discussion

This study evaluates the effect of fruit extract of *T. tetraptera* on oestrous cyclicity and ovarian functions in *wistar* rats. Evaluation of the estrous cycle in laboratory rodents can be a useful measure of the intergrity of the hypothalamic-pituitary-ovarian reproductive axis. In this study, there was relative reduction in the durations that the extract-treated animals spent in proestrus, estrus and metestrus phases of the cycle respectively, while prolonging the duration of the diestrus phase and the entire estrous cycle. The relative reductions in the duration of the phases implies reduction in the time needed for development of the follicles in the preovulatory phase of the estrous cycle.The relative reduction in the metestrus phase in the extract-treated animals indicates a reduction in the number of matured graafian follicles, implying that the extract possibly impaired ovulation, thereby reducing the number of ova eventually released and fertilised. On the other hand, the prolongation in the diestrus phase observed in this study indicates that the luteal phase of the cycle was prolonged, with the corpora lutea retained for longer duration than normal, thereby prolonging the cycle length, and consequently increasing secretion of progesterone. This could hinder or impair ovulation. These results are consistent with earlier reports of the ability of plant extracts to reduce the duration of proestrus, estrus, and

metestrus phases[22,23], while prolonging the diestrus phase and the oestrous cycle length [24,25].

Biochemical and physiological changes occur in ovaries during the estrus cycle [26]. From this study, a significant increase in protein, superoxide dismutase (SOD) with a decrease in malondialdehyde (MDA) was observed in the ovarian tissues. Protein, considered to be the body's building material is involved in alterations of almost every physiological function [27]. The increase in total protein in the ovary of extract-treated rats suggests an increased synthesis and secretion, but, that critical steps or enzymes activities needed for utilisation of the proteins in the body, such as for body building, synthesis of enzymes and glycoprotein hormones, etc, may have been impaired by the extract, hence the accumulation. The Physiological regulation of the estrus cycle, ovulation and follicular growth involve neuroendocrine, and endocrine factors such as gonadotrophic (LH and FSH) and ovarian hormones (androgen, estrogen and progesterone) [28]. Also, ovarian hormones are produced by different cells of ovary: thecal cells, corpus luteum and granulosa cells of mature graafian follicles [29]. Therefore, the impaired ovarian utilisation of protein observed in this study, possibly resulted to imbalances or alterations in the gonadotrophic and hence, the sex hormones [30], and eventually resulted to the observed impairments in the durations of estrus cycle and their phases [31,30].

Superoxide dismutase is an antioxidant enzyme that provides protection within the cell by removing reactive oxygen species from cellular environment through catalysing the dismutation of two superoxide radicals to hydrogen peroxide for detoxification by the enzyme catalase [32]. Malondialdehyde, on the other hand, is a marker for lipid peroxidation and is considered an indication of cell membrane destruction by free radicals. Malondialdehyde conjugates with amino groups of proteins to form intra- and inter-molecular cross links that inactivates the membrane bound enzymes and receptors [33]. Therefore, the non availability of protein impairs the conjugative activity of MDA, thereby protecting the membrane bound enzymes and receptors from inactivation. Therefore, the observed increase in SOD, with associated reduction in the level of MDA in the animals treated with the extract, agrees with the reported anti-oxidant properties of *Tetrapleura tetraptera* fruit extract [34], and it's capacity to prevent oxidative stress-induced impairments in ovarian functions. These results also show that the observed alterations in oestrous cyclicity by the extract was not due to oxidative damage in the ovary. but possibly due to pre-ovulatory endocrine factors such as impairments in the secretion of gonadotropic hormones and estrogen which are needed for follicular growth, maturation and ovulation.

Therefore, the alteration of oestrous cyclicity, absence of lipid peroxidation, as well as the relative reduction in body weight observed in all the extract treated animals, could be attributed to the disruptive effects of *Tetrapleura tetraptera* fruit extract on protein utilisation by the ovaries for the synthesis and secretion of biomolecules required for ovarian functions.

5 Conclusion: The findings from this study show that *Tetrapleura tetraptera* fruit extract disrupts protein utilisation by the ovaries thereby impairing oestrous cyclicity, and body weight gain. The alterations in oestrous cyclicity indicates an impairment in follicular development and ovulation, and eventually could result to infertility.

Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

References

1. Amah CI, Yama OE, Noronha CC. Infecund evaluation of cycling female Sprague-Dawley rats: An aftermath treatment with Momordica charantia seed extract. *Middle East Fertil Soc J* 2012; 17(1): 37-41.

2. Sheeja EJ, Ajeet P, Papiya B, Shilpi S. Antifertility activity of Momordica charantia descourt pulp and seed hydroalcohol extract. *J Appl Pharm* 2012; 3(4): 682-696.

3. Dicken CL, Israel D, Davis JB, et al. Peripubertal vitamin D3 deficiency delays puberty and disrupts the estrous cycle in adult female mice. *Biol Reprod* 2012; 87: 51.

4. Gamache PH, and Acworth IN. Analysis of phytoestrogens and polyphenols in plasma, tissue and urine using HPLC with coulometric array detection. *Proc Soc Exp Biol Med* 1998; 217: 274-280.

5. Irondi A.E., Anokam K.K., Chukwuma P.C. Phenological variation in the *in-vitro* antioxidant properties and alpha-amylase inhibitory activity of *Tetrapleura tetraptera* Pod, *Int.J. Pharm. Sci. Drug Res.* 2013; 5: 108–112.

6. Moukette B.M., Pieme A.C., Biapa P.C.N., et al.. In vitro ion chelating, antioxidative mechanism of extracts from fruits and barks of *Tetrapleura tetraptera* and their protective effects against fenton mediated toxicity of metal ions on liver homogenates, *Evid.-Based Complement. Altern. Med.* 2015; (2015) 1–15.

7. Okwu, D.E.. The potential of Ocimum gratissimum, Pergularia extensa and Tetrapleura tetraptera as a spice and flavouring agents. *Nigerian Agricultural Journal* 2003; 35: 143-148.

8. Armstrong WP. But Pollinated TetrapleuraTetraptera Fruits. The Source of Tropical sea beans.2010.

9. Aladesanmi JA. Tetrapleura tetraptera: molluscicidal activity and chemical constituents. *Afr J Tradit Complement Altern Med* 2007; 4(1): 23-36.

10. Odesanmi OS, Lawal RA, Ojokuku SA. Effects of ethanolic extract of Tetrapleura tetraptera fruit on serum lipid profile and kidney function in male Dutch-white rabbits. *Nig Q J Hosp Med* 2011; 21(4): 299-302.

11. Atawodi SE, Adekunle OO, Bala I. Antioxidant, organ protective and ameliorative properties of methanol extract of Anogeissus leiocarpus stem bark against carbon tetrachloride-induced liver injury. *Int J Pharm Sci Res* 2011; 2(6): 1443-1448.

12. Lekana-Douki JB, Liabagui SL, Bongui JB, et al.. In vitro antiplasmodial activity of crude extracts of Tetrapleura tetraptera and Copaifera religiosa. *BMC Res Notes* 2011; 4: 506.

13. Nwawu, J. I. and Akali, P.A. Anticonvulsant activity of the volatile oil from the fruit of Tetrapleura tetraptera. *Journal of Ethnopharmacology* 1986.; 18: 193-107.

14. Enwere, N. J. Foods of plant origin. *International Journal of Microbiology* 1998; 9 (94) : 329-334.

15. Marcondes, F. K; Bianchi, F.J. and Tanno, A.P. Determination of the estrous cycle phases of rats: Some helpful considerations. Brazilian Journal of Biology 2002; 62: 609-614.

16. Spornitz, U.M., Socin C.D., David A.A. Estrous stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium. *The Anatomical Research*. 1999; 254: 116-126.

17. Shrestha J, Shanbhag T, Smita S, et al.. Antiovulatory and abortifacient effects of Areca catechu (betel nut) in female rats. *Indian J pharmacol* 2010; 42: 306-311.

18. Long, J. A. and Evans, H. M. (1922). The Estrous cycle in the rat and its associated phenomena. Memories of University of Carlifonia. 6: 1- 148.

19. Gornall, A.G; Bardawill, C.J. and David, M.M. Determination of serum proteins by means of the buireto reaction. *Journal of Biological chemistry*1949.; 177, 751-766.

20. Kakkar, P; Das, P. and Viswanathan, P.N . A modified spectrophotometric assay of superoxide dimutase. *Indian Journal of Biochemistry Biophysics*. 1984; 21: 130-132.

21. Ohkawa, H; Ohishi, N. and Yagi, K.. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Annals of Biochemistry*. 1979; 95: 351-358.

22. Oluyemi, K.A ; Omutuyi, I.O; Jimoh, R.O; et al. Erythropoietic and anti-obesity effects of Garcinia Cambogia in wistar rats. *Biotechnology and applied Biochemistry* 2007; 46: 69-72

23. Uchendu, C.N; Kamalu, T.N. and Asuzu, I.U. A preliminary evaluation of antifertility activity of a triterpenoid glycoside from Dalbergia Saxatilis in female wistar rats. *Pharmaceutical Research* 2000; 41 (5): 521-525.

24. Shibeshi W, Makonnen E, Debella A, Zerihu L, Phytochemical, contraceptive efficacy and safety evaluations of the methanolic leaves extract of Achyranthes aspera in rats. Pharmacologyonline.,2006; 3: 217–224.

25. Kage DN, Malashetty VB, Seetharam YN, Suresh P, Patil SB. Effect of ethanol extract of whole plant of Trichosanthes cucumerina var. cucumerina L.on gonadotropins, ovarian follicular kinetics and oestrous cycle for screening of antifertility activity in albino rats. *Intern. J. Morphol.*, 2009; 27(1): 173 – 182.

26. Marshall,W.J.; Bangert, S.K. Clinical Biochemistry: Metabolic and Clinical Aspects. Churchill Livingstone/Elsevier, 2008.

27. Duncan, J.R; Prasse, K.W. and Mahaffey, E.A. Veterinary Laboratory Medicine, Iowa State University Press, Ames. 1994; 102-107.

28. Mahesh, V.B.; Dhindsa, D.S.; Anderson, E.; Kalra, S.P. Regulation of Ovarian and Testicular Function. *Springer US*, 2012.

29. Ciaccio, L.A. Estrogen and Progesterone: Factors Involved in the Regulation of Estrous Behavior in the Female Hamster (Mesocricetus auratus), 1970.

30. Shivalingappa H, Satyanaranyan ND, Purohit MG, et al. Effect of ethanol extract of *Rivea hypocraterifomis* on the estrous cycle of the rat. *J Ethnopharmacol* 2002; 82: 11-17.

31. Circosta, C.; Sanogo, R.; Occhiuto, F. Effects of Calotropis procera on oestrous cycle and on oestrogenic functionality in rats. *Farmaco*., 2001, 56, 373-8.

32. Rzeuski, R; Chiubek, D. and Machoy, Z. Interactions between fluroide and biological free radical reactions. *Fluroide* 1998; 31: 43-45

33. Sen S, Chakraborty R, Sridhar C, et al.. Free radicals antioxidants diseases and phytomedicines: current status and future prospects. *Int J pharm sciRes* 2010;3 (1) 91-100.

34. Nwozo SO, Orojobi BF. Hypolipidemic and Anti-oxidant effects of *Tetrapleura tetraptera* fruit including seeds in hypercholesterolaemic rats. *Seed Sci. and Biotech*. 2010; 4(1):73-78.