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Effect of Ethanol Extract of Leaves of *Chrysophyllum albidum* on the Reproductive Hormones of Lead-Exposed Female Wistar Albino Rats

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ABSTRACT: This study investigated the ameliorative effect of the ethanol extract of leaves of *Chrysophyllum albidum* on the ovaries and reproductive hormones of lead-exposed female Wistar albino rats. Lead has no physiological role in the body but exerts many deleterious effects on various organs and systems in humans including the reproductive system; this it achieves mainly by the production of free radicals leading to oxidative stress. A total number of forty-two rats (160-180g) were divided into seven groups; group 1 served as the normal control group, group 2 rats were administered 200mgkg^{-1} of lead and left untreated (negative control group) while groups 3,4,5,6 and 7 were administered lead (200mgkg^{-1}) and 250, 500, 750, 1000 and 1250mgkg^{-1} body weight of the ethanol extract of the studied plant respectively. All groups received feed and water *ad libitum* for 30days. A significant ($p \leq 0.05$) increase was observed in FSH, LH, estrogen and progesterone levels in *C. albidum* treated groups compared to the negative control group. The histology of the negative control group showed the presence of cysts with no ovarian follicle whereas treated groups showed normal histology of the ovaries with oocytes except for groups 6 and 7.This ameliorative ability of *C. albidum* observed in the present study may be attributed to its high antioxidant properties. Further studies on *C. albidum* could lead to the development of new and highly affordable drugs for the treatment of female infertility.

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Reproductive systems are regulated by hormones, and hormonal disorders such as inadequate or excessive production of hormones are the most common causes of infertility. Lead affects the central nervous system as well as hematopoietic, reproductive, hepatic and renal systems and the risk is greater in foetuses and children than in adults as they are still in their developmental stages (Dumitrescu *et al*., 2015). Rajesh *et al*. (2015) reported that lead has serious adverse effects on the female reproductive system of mice even at very low doses. Dumitrescu *et al*. (2015) reported that long term exposure of female rats to low lead levels resulted in structural changes in their ovaries including diffuse oedema, necrosis in the ovarian follicles, optical empty spaces and denudation of the ovarian follicles at various stages of follicle evolution. They concluded that these structural alterations indicate infertility in female rats. *Chrysophyllum albidum,* a forest fruit tree usually found in tropical Africa and commonly known as African star apple or white star apple, like many medicinal plants in Nigeria has been employed in folklore medicine for the treatment of diseases. Various parts of the plant have been shown to have different therapeutic effects in diseases and health conditions. Study by Jayeoba *et al*. (2010) showed that *C. albidum* contains many minerals with lead (Pb) being totally absent. *Chrysophyllum albidum* has hypoglycemic and hypolipidemic effects on alloxan-induced diabetic rats as reported by Olorunnisola *et al*. (2008). Onyeka *et al.* (2012) reported that the root bark of ethanol extract of *C. albidum* lowered serum testosterone and gonadotrophins concentration. In another study on the ethanol leaf extract of *C. albidum* by Anna *et al.* (2013), they reported that *C. albidum* decreased hormonal profile but did not suppress spermatogenesis. In this present study, ethanol leaves extract of *C. albidum* was employed to investigate its effect on the reproductive hormones of lead-exposed female wistar albino rats.

MATERIALS AND METHODS

Procurement of plant materials: The leaves of *C. albidum* were obtained in March, 2016 from Orji Village in Owerri North Local Government Area of Imo State, Nigeria.

Experimental Animals: A total number of fifty-seven matured female Wistar albino rats was used for this study. They were purchased and housed in the Animal House of Pharmacology Department, University of

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Port Harcourt, Choba Campus, Rivers State. The rats weighed between 160 and 180g. They were allowed to acclimatize for 1week and were fed normal rat feed (Top feeds- grower's mash) and clean water *ad libitum* during the acclimatization period and throughout the experimental period. Fifteen of the rats were used for the acute toxicity test to ascertain that the highest dose of the extract used (1250mg/kg) was not lethal to the rats using the Limit Test Method described by Walum (1998); while the remaining forty-two rats were used for the study.

Preparation of plant extract: The leaves of *C. albidum* were rinsed, allowed to dry under room temperature for 3weeks, then ground into powder using manual home grinder. The extraction was done following the method used by Anna *et al*. (2013). The ground leaves were soaked in 90% ethanol for 24 hours, sieved with muslin cloth and filtered using Whatmann No3 filter paper. The extract obtained was allowed to dry completely over a water bath (40^0C), stored in clean dry containers, and kept in the refrigerator until usage. When needed, the extract was dissolved in normal saline (0.90% NaCl) at a concentration of 1g/ml, and preserved in the refrigerator throughout the study duration.

Experimental design: Forty-two female Wistar albino rats weighing between 160 and 180g were divided into seven groups of six rats in each group. The experimental design of Azab *et al*.(2015) was adopted for the study, and the rats were divided as follows:
Group I: Normal Control; received feed and water only *ad libitum* for 30days.
Group II: Negative Control; received feed, 200mg/kg b.w of lead acetate and water *ad libitum* daily for 30days.
Group III: received feed, 200mg/kg b.w of lead acetate, 250mg/kg b.w *C. albidum* ethanol leaf extract and water *ad libitum* daily for 30days.
Group IV: received feed, 200mg/kg b.w of lead acetate, 500mg/kg b.w*C. albidum* ethanol leaf extract and water *ad libitum* daily for 30days.
Group V: received feed, 200mg/kg b.w of lead acetate, 750mg/kg b.w *C. albidum* ethanol leaf extract and water *ad libitum* daily for 30days.
Group VI: received feed, 200mg/kg b.w of lead acetate, 1000mg/kg b.w *C. albidum* ethanol leaf extract and water *ad libitum* daily for 30days.
Group VII: received feed, 200mg/kg b.w of lead acetate, 1250mg/kg b.w *C. albidum* ethanol leaf extract and water *ad libitum* daily for 30days.
Administration of extract was done orally using a cannula for 30days.

*Specimen collection***:** Twenty four hours after the last administration of extract, the rats were anaesthetised using cotton wool soaked in chloroform. The anaesthetised rats were placed on a dissecting slab and the blood samples were collected from the jugular-vein with lithium-heparin bottles for biochemical analysis. The ovaries of the experimental rats were excised, placed in sample holders containing 10% normal saline and then taken to the laboratory for histopathology examination.

Estimation of Follicle Stimulating Hormone (FSH) concentration: The FSH concentration of the samples were carried out following the method described by Mboso *et al.*, (2013) using AccuBind Enzyme-Linked Immunosorbent Assay (ELISA) Microwells test kits (Monobind Incorporation, USA). The microplate wells were formatted to assay serum reference, control and test specimens in duplicates. A 50µl of the serum reference, control and specimen were pipetted into the assigned wells and 100µl of FSH-Enzyme reagent solution was pipetted into each well. The microplate was gently swirled for 30sec, incubated for 60min at room temperature and the contents discarded by decantation. The decant was washed three times with 350µl of wash buffer, then 100µl of the substrate solution was added to the wells, incubated for 15min at room temperature and 50µl of stop solution finally added to all the wells and mixed gently for 20sec. The absorbance in each well was read at 450nm using a microplate reader. A curve of absorbance for each duplicate serum reference versus the corresponding FSH concentration was plotted and the best-fit curve through the plotted points was drawn. To determine the FSH concentration for the unknown, the average absorbance of the duplicates was located on the vertical axis, the intersecting point on the curve located and the FSH concentration was then read from the horizontal axis of the graph.

Estimation of Luteinizing Hormone (LH) concentration: The LH concentration of the samples were carried out following the method described by Mboso *et al.*, (2013) using AccuBind Enzyme-Linked Immunosorbent Assay (ELISA) Microwells test kits (Monobind Incorporation, USA). The microplate wells were formatted to assay serum reference, control and test specimens in duplicates. A 50µl of the appropriate serum reference, control and specimen were pipetted into the assigned wells and 100µl of LH-Enzyme reagent solution was also pipetted into the wells. The microplate was gently swirled for 30sec, incubated for 60min at room temperature and the contents discarded by decantation. The decant was washed three times with 350µl of wash buffer, then 100µl of the substrate solution was added to all wells, incubated for 15min at

room temperature and 50µl of stop solution finally added to all wells and mixed gently for 20sec. The absorbance in each well was read at 450nm using a microplate reader. A curve of absorbance for each duplicate serum reference versus the corresponding LH concentration was plotted and the best-fit curve through the plotted points was drawn. To determine the LH concentration for the unknown, the average absorbance of the duplicate was located on the vertical axis, the intersecting point on the curve located and the LH concentration was then read from the horizontal axis of the graph.

Estimation of progesterone concentration: The progesterone concentration of the samples were carried out following the method described by Mboso *et al.*, (2013) using AccuBind Enzyme-Linked Immunosorbent Assay (ELISA) Microwells test kits (Monobind Incorporation, USA). The microplate wells were formatted to assay serum reference, control and test specimens in duplicates. A 25µl of the appropriate serum reference, control and specimen were pipetted into the assigned wells and 50µl of Progesterone Enzyme reagent solution was pipetted into all wells. The microplate was gently swirled for 20sec, incubated for 60min at room temperature and the contents discarded by decantation. The decant was washed three times with 350µl of wash buffer, then 100µl of the substrate solution was added to all wells, incubated for 20min at room temperature and 50µl of stop solution finally added to all wells and mixed gently for 20sec. The absorbance in each well was read at 450nm using a microplate reader. A curve of absorbance for each duplicate serum reference versus the corresponding progesterone concentration was plotted and the best-fit curve through the plotted points was drawn. To determine the progesterone concentration for the unknown, the average absorbance of the duplicate was located on the vertical axis, the intersecting point on the curve located and the progesterone concentration was then read from the horizontal axis of the graph.

Estimation of estrogen concentration: **T**he estrogen concentration of the samples were carried out following the method described by Mboso *et al.*, (2013) using AccuBind Enzyme-Linked Immunosorbent Assay (ELISA) Microwells test kits (Monobind Incorporation, USA). The microplate wells were formatted to assay serum reference, control and test specimens in duplicates. A 25µl of the appropriate serum reference, control and specimen were pipetted into the assigned wells and 50µl of estradiol (estrogen) biotin reagent was pipetted into all wells. The microplate was gently swirled for 30sec, incubated for 90min at room temperature and the contents discarded by decantation. The decant was washed three times with 350µl of wash buffer, then 100µl of the substrate solution was added to all wells, incubated for 20min at room temperature and 50µl of stop solution finally added to all wells and mixed gently for 20sec. The absorbance in each well was read at 450nm using a microplate reader. A curve of absorbance for each duplicate serum reference versus the corresponding estrogen concentration was plotted and the best-fit curve through the plotted points was drawn. To determine the estrogen concentration for the unknown, the average absorbance of the duplicate was located on the vertical axis, the intersecting point on the curve located and the estrogen concentration was then read from the horizontal axis of the graph.

Histological examination: Histological examinations were carried out following the method described by Awvioro (2002). The ovaries were excised immediately after the rats were sacrificed and placed in sample holders containing 10% normal saline. Water was removed from the specimen using graded percentage of alcohol in ascending order from a lower concentration to the absolute, agitation was done using Junior Orbit Shaker and the alcohol from the blocks or sections of tissue were cleared by immersing them in xylene. Thin uniform sections for histological examination were produced using a rotary Microtome. A grease-free slide was placed on a warm plate and was flooded with distilled water. A section was laid on the surface of the slide and every major grease was removed by stretching the surrounding wax carefully with mounted needles. As the water warmed, the section flattened out. The slide was then removed from the hot plate, labelled and dried. The slides were stained using haematoxylin and eosin stains. After staining, the sections were prepared for microscopic examination by mounting them in a suitable medium under a glass cover slip using a mountant. The slides were then viewed under a microscope.

Statistical analysis: All data were subjected to statistical analysis. Values were reported as mean ± standard error of mean (SEM). The statistical package used was SPSS, while one way ANOVA was used to test for differences between treatment groups. The results were considered significant at p-values of less than 0.05, that is, at 95% confidence level ($p \leq 0.05$

RESULTS AND DISCUSSION

The results of the female reproductive hormones assayed are shown on Table1. The negative control group showed a significant ($p \leq 0.05$) decrease in the concentrations of FSH, progesterone and estrogen compared to the normal control group. Though there was a marked decrease also in the LH concentration of

the negative control group compared to the normal control, this decrease was not significant ($p \leq 0.05$). There were significant ($p \leq 0.05$) increase in the FSH, LH, progesterone and estrogen concentrations of *C. albidum* treated groups compared to the negative control group in a dose-dependent manner up till group5 (750mg/kg B.W extract) except for progesterone where group7 had the highest value. Among the *C. albidum* treated groups, significant ($p \leq 0.05$) increase in the FSH concentration of group5 compared to groups 3,6 and 7; significant ($p \leq 0.05$) increases in LH concentrations of groups 5 and 6 compared to group3, and significant ($p \leq 0.05$) decrease in LH concentration group7 compared to groups 5 and 6 were observed.Group7 showed a significant ($p \leq 0.05$) increase in progesterone concentration compared to group6; while the estrogen concentration of group5 significantly ($p \leq 0.05$) increased compared to groups 3,6 and 7.

The results of the histological examination of ovaries of the control and test rats are presented in Plates 1 to 7. The histology of the negative control group showed the presence of cysts with no ovarian follicle but treatment with *C. albidum* returned the ovaries to their normal histological states and restored the production of ovarian follicles. There was absence of oocytes in groups 6 and 7.

Table 1: Serum female reproductive hormone concentrations of the experimental rats

GROUPS	FSH (mlu/ml)	LH (mlu/ml)	Prog. (ng/ml)	Estro. (pg/ml)
Group 1	2.47 ± 0.09^{b}	2.23 ± 0.30	27.20 ± 0.80^{b}	64.33 ± 4.18^{b}
Group 2	1.53 ± 0.22^{a}	1.30 ± 0.15	16.67 ± 1.16^{a}	42.00 ± 1.15^{a}
Group 3	2.13 ± 0.15^{x}	$3.00\pm0.06^{b,x}$	31.13 ± 2.55^{b}	$43.33\pm1.76^{a,x}$
Group 4	2.47 ± 0.19^{b}	$3.40\pm0.12^{a,b}$	28.07 ± 1.33^{b}	53.00 ± 4.51
Group 5	$2.87\pm0.09^{b,c}$	$4.13\pm0.32^{a,b,c}$	30.60 ± 1.25^{b}	$62.00\pm1.53^{b,c}$
Group 6	2.00 ± 0.10^{x}	$3.83\pm0.09^{a,b,c}$	$27.57\pm1.23^{b,c}$	$41.00\pm3.79^{a,x}$
Group 7	1.83 ± 0.09^{c}	$2.50\pm0.17^{b,x}$	$36.67\pm3.32^{a,b,x}$	$38.67\pm5.70^{a,x}$

Values are expressed as mean ± SEM; n= 6; Statistical package used is SPSS.Key: Group 1= Normal control; Group 2= Negative control; Group 3= Treated with 250mg/kg Extract; Group 4= Treated with 500mg/kg Extract; Group 5= Treated with 750mg/kg Extract; Group 6= Treated with 1,000mg/kg Extract; Group 7 = Treated with 1,250mg/kg Extract.
Values with superscript "a" are significantly different from the normal control, Group1, ($p<0.05$)
Values with superscript "b" are significantly different from the negative control, Group2, ($p<0.05$)
Values without superscript "a" and "b" are not significantly different from groups 1 and 2, ($p>0.05$)
Values with superscript "c" are significantly different from values with superscript "x" across the treated groups, ($p<0.05$)
Values with superscript "x" are not significantly different from each other across the treated groups, ($p>0.05$)

The findings in this present study corroborate that of Ayinde *et al*., (2012) who observed a non-significant ($p \leq 0.05$) decrease in LH and a significant ($p \leq 0.05$) decrease in FSH concentrations in lead-exposed rats compared to the control group. Waseem and Rehman (2015) reported significant ($p \leq 0.05$) reduction in progesterone and estrogen concentrations in lead-exposed mice compared to the control group. A contrary result in progesterone level was reported by Nakade *et al*., (2015) who observed no significant ($p \leq 0.05$) change in the progesterone level of rats treated with different concentrations of lead compared to the control group. The histological study of the ovaries in this study revealed that lead induced ovarian cysts in the ovaries of the negative control rats with no formation of oocytes.

However, the treated groups showed that the lead-induced abnormalities in the ovaries were mitigated by *C. albidum* leaves extract, though the highest doses (1000 and 1250mg/kg b.w) showed evidences of toxicity as no oocyte was seen indicating possible maturation arrest. The group treated with 750mg/kg b.w (group5) *C. albidum* leaves extract had the most ameliorative effect on the reproductive system.

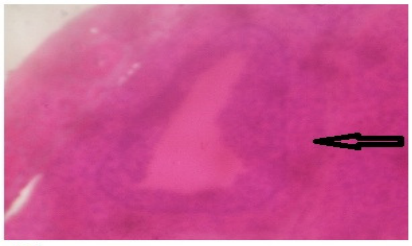
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Plate1: Ovary histology of group 1 (Normal control rats).H&E X200.Micrograph of ovary showing corpus luteum (arrowed structure). This implies that this ovary has been able to produce a mature ovarian follicle which led to production of an ovum at the time of ovulation.

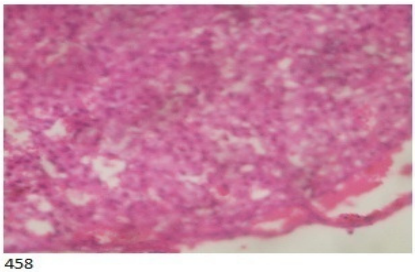
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Plate2:Ovary histology of group 2 (Negative control rats),H&E X200.Micrograph of ovary showing the cortical area of the ovary

without any ovarian follicle. Some cysts are seen which represents distortion of ovarian histology.

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Plate3 Ovary histology of group 3 (Treated rats- 250mg/kg b.w extract)H&E X200. Micrograph of ovarian tissue showing the cortical region. Some ovarian follicles were seen. The arrowed follicle is a secondary ovarian follicle. This ovary reveals normal histologic features.

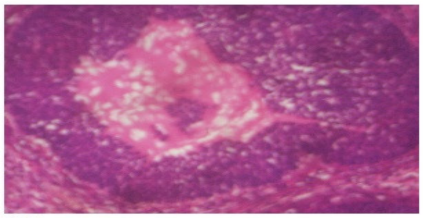

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Plate4: Ovary histology of group 4 (Treated rats- 500mg/kg b.w extract)H&E X200.Micrograph of ovarian follicle showing a primary oocyte with normal histological features.

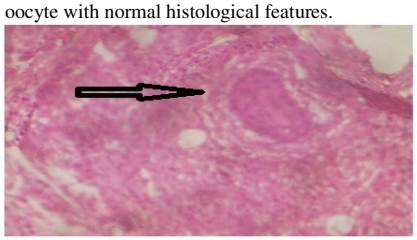

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Plate5: Ovary histology of group 5 (Treated rats- 750mg/kg b.w extract)H&E X200.Micrograph of ovarian tissue showing corpus luteum. This is a normal finding in the histology of the ovary.

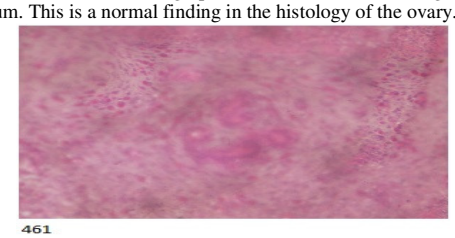

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Plate6: Ovary histology of group 6 (Treated rats- 1000mg/kg b.w extract).H&E X200.Micrograph of ovarian tissue with no oocytes seen. It is possible that there was maturation arrest.

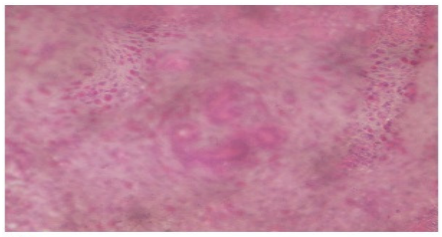

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Plate7: Ovary histology of group 7 (Treated rats- 1250mg/kg b.w extract).H&E X200.Micrograph of ovarian tissue without any oocytes seen. It is possible that there was maturation arrest.

The results of the reproductive hormonal assays of the negative control group compared to the normal control group suggest that serum levels of FSH, LH, estrogen and progesterone were suppressed by treatment with lead. Treatment with *C. albidum* however conferred ameliorative effects on the experimental rats as observed by the increases in FSH, LH and estrogen concentrations among treated groups in a dose-dependent manner up till group 5. Although significant increases in progesterone concentrations were observed among treated groups compared to the negative control group, these increases were not dose-dependent. Suppression of female reproductive hormones has been shown to cause alteration and subsequent seizure of the *estrous* cycle (known as amenorrhoea) of female rats at di*estrous* stage. *Estrous* cycle is the reproductive cycle which takes place in sexually matured female animals and gives an indication of the functionality of the ovary and uterus (Kumar *et* al., 1997; Ugwoke *et al.,* 2005; Andersson *et al.*, 2013; Rajesh *et al.*, 2015). This hormonal suppression observed in the present study can be attributed to lead-induced stress which causes the hypothalamus to release glucocorticoid hormones in response to the stress. Continuous increase in serum glucocorticoid concentration leads to production of free radicals, and also decline in the concentration of GnRH leading to reduced synthesis and secretion of LH and FSH. Progesterone is produced majorly by the corpus luteum whose formation is triggered by a surge of LH; hence significant decrease in the progesterone level of the negative control group compared to the normal control group in this present study can be attributed to the reduced synthesis of LH. Estrogen has antioxidant properties, but with suppressed generation of FSH and elevation of glucocorticoids, its synthesis and secretion is highly reduced. This leads to excessive oxidative stress which can subdue or hinder folliculogenesis and the entire *estrous* cycle (Sapolsky *et al.,* 2000; Nepomnaschy *et al.*, 2004; Chatterjee and Chatterjee, 2009). Treatment with *C. albidum* was able to confer protection against lead-induced toxicity as evidenced in the significant ($p \leq 0.05$) increases in FSH, LH, progesterone and estrogen in a dose-dependent manner up till the group treated with 750mg/kg b.w. of the extract. This positive effect on the reproductive hormones observed in this study can be attributed to *C. albidum*'s reactive oxygen species scavenging ability since it is rich in antioxidants. Phytochemical analysis of *C. albidum* showed that it contains vitamin C, vitamin E, vitamin A, selenium, iron, copper, zinc, manganese, and a lot of other vitamins, minerals and alkaloids. Its vitamin C content was found to be 100

times that of oranges and 10 times that of guava and cashew (Amusa *et al.*, 2003; Jayeoba *et al*., 2010; Thomas and Olufunke, 2012). Vitamin A can quench O_2, prevent and stop lipid peroxidation; on the other hand, vitamin C can eliminate peroxyl radicals in the aqueous phase of cells hence inhibiting peroxidation initiation. Furthermore, vitamin C reduces circulating glucocorticoids produced during stress (Rifici and Khachadurian, 1993); hence reduction in serum glucocorticoids increases the secretion of GnRH by the hypothalamus while vitamin E neutralizes free radicals thereby preventing lipid oxidation inside membranes (McDowell, 2000; McDowell *et al.*, 2007). With increased serum GnRH concentration, syntheses and secretions of LH, FSH (and subsequently estrogen and progesterone) are also elevated. This eventually restores the folliculogenesis and estrous cycle back to normal (Sapolsky *et al.,* 2000; Nepomnaschy *et al.*, 2004; Chatterjee and Chatterjee, 2009). However, except for progesterone assay where the highest concentration was observed in group7 rats; FSH, LH and estrogen assays revealed significant decreases in the concentrations of groups 6 and 7 (1000 and 1250 mg/kg b.w) compared to group5 (750mg/kg b.w). Absence of oocytes, suggesting possible maturation arrest were equally observed from the histology of groups6 and 7 rats. These observations could suggest that *C. albidum* possibly has toxic effects on the reproductive hormones and ovaries of the experimental rats at concentrations of 1000mg/kg B.W and above.

The elevation in female hormonal levels in this present study probably explains why in the Eastern part of Nigeria, *C. albidum* is linked to female fertility and as such used as one of the ingredients in preparing herbal medicines for women trying to conceive. Also pregnant women are often seen eating (and advised to eat) the fruits especially in the early stage of pregnancy; and even cutting down *C. albidum* trees was forbidden in the olden days.

*Conclusion***:** Based on findings in this study, it can be concluded that *C. albidum* effectively ameliorated the deleterious effects of lead on the reproductive hormones and ovaries of experimental rats especially at the concentration of 750mg/kg b.w. This study has therefore been able to establish that *C. albidum* can be employed in the treatment of female infertility; and lead toxicity. Further investigations should be carried out to ascertain the actual active agent that conferred this protection and to possibly develop easily available drugs that can boost fertility in females.

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