TOXICITY STUDY OF ETHANOL EXTRACT OF SCLEROTIA OF *Pleurotus tuberregium* ON REPRODUCTIVE PARAMETERS OF NON-PREGNANT WISTER RATS

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Abstract: The Sclerotia of *Pleurotus tuberregium* are used in the South-south region of Nigeria to treat infertility in women. The effect of the ethanol crude extract of *P. tuberregium* on some fertility parameters was evaluated on non-pregnant female albino Wister rats to determine the biosafety of the sclerotia of *P. tuberregium*. In the course of the experiment the ethanol crude extract of *P. tuberregium* was administered to the animals orally at the doses of 100, 500, and 1000 mg/kg per day for 28 days. The result showed that weight of various organs especially those of the reproductive parameters (the ovary and uterus) of tested animals significantly increased within the tested period when compared with the control. The histological results revealed that the extract is better as a fertility drug at lower doses although there were no alteration in the cellular structure of tested animal organs even at high dose. This study therefore suggests that the sclerotia of *P. tuberregium* is useful in maintaining reproductive indices and can be recommended for medical use.

Keywords: Body weight, fertility, histology, non-pregnant rats, *Pleurotus tuberregium*

Introduction

Around 25 species of mushrooms are commercially cultivated and considered as edible out of more than 2,000 which are distributed round the world (Guillamón *et al*., 2010). Nevertheless, wild mushrooms are attracting more significance due to their nutritional and pharmacological activities (Aida *et al*., 2009).

The folkloric herbal mushroom preparations used as anti-fertility treatments have been described in several literatures. Using recent experimental methods, reasonable amount of mushrooms originating from Indian have been studied for their anti-fertility effects. Patel and Goyal (2012) enclosed a review on mushrooms providing reports on studies conducted to verify their fertility regulation. About 14 species have been evaluated for their anti-fertility properties out of which 8 have been established to be efficient (Heleno *et al*., 2010). Varieties of researches carried out on female rodents have been reported to posses underlying reproductive and physiological changes. Traditional medicinal practitioner in the south- south region of Nigeria uses Sclerotia of *Pleurotus tuberregium* as a fertility remedy in women that are desirous to have children.

Pleurotus popularly referred to as "oyster mushrooms", is a genus with roughly 40 species which are generally available and edible. Despiteits nutritional value it possesses medicinal and health-promoting properties (Sarikurkcu *et al*., 2008). All over the world, *Pleurotus* species have been used by people of diverse cultures for several decades owing to the fact that they contain several compounds with important nutraceutical and pharmacological properties. Some of these substances include lectins which has been found to have antitumor antiproliferative and immunomodulatory effects (Synytsya *et al*., 2011). Different *Pleurotus* spp. have also been studied and established to have anti-inflammatory, antiviral, hematological, antitumor, immunomodulatory, antibacterial, hypocholesterolic and antioxidant activities.

Synytsya *et al*. (2011) carried out an experiment on human leukemia cells and induced apoptosis in HL-60 cells using extracted Water-soluble polysaccharides obtained from *P. tuberregium* (a notable non-poisonous mushroom) and discovered that it had antiproliferative effect. In the treatment of various disorders, medicines would be used over a period of time, it is therefore important to ensure the biosafety of the users of such medicines. Most scientists are researching on a widely available, relatively cheap, effective and easily accepted medicine of plant origin that is also noninvasive, non-hormonal in action and nontoxic even when used over a long period of time. Medicinal mushroom are important rudiments of native medical method in many countries. Nowadays, traditional medicines have received substantial attention and a great number of mushrooms have been studied for their biological activities (Alves *et al*., 2012). The wide distribution of this desert mushroom and its common use in folk medicine and as animal fodder stimulated also the performance of this investigation. Thus, the present research was birthed to explore the biosafety of sclerotia of *P. tuberregium* on wistar rats.

Materials and Methods

Location of study

This research was embarked upon in the animal house of the Department of Animal and Environmental Science, Faculty of Life Sciences, University of Benin, Benin city, Edo State. Nigeria.

Preparation of sample

The sclerotia of *Pleurotus tuberregium* used in this study were obtained from Oliha market, located in Siloku road, in Oredo local government area, Benin City, Edo State, Nigeria. The sclerotia of *P. tuberregium* was identified and confirmed by Dr. Abbot Oghenekaroa mycologist in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin. Benin City.

Experimental animals

Forty female virgin wistar albino rats of average weight 170-200 g, were obtained from the Animal house in the Department of Anatomy, College of Medical Sciences, University of Benin. These rats were housed in The Department of Animal and Environmental Biology, University of Benin and kept in cages with ambient temperature and maintained under standard laboratory conditions, which included 12-h light and 12-h dark cycle. The female rats were separated into five groups of eight rats each and were fed with standard diet and clean water *ad libitum* for three weeks as acclimatization period prior to the experiment. Experimental animals were kept fasted overnight before the study and were handled in accordance with the organization for economic and co-operation and development (OECD) 420 guideline.

Preparation of extract

Fresh Sclerotia were air driedfor five days after which the brownish rough back was peeled off with a knife to expose the whitish inner part and thereafter ground into powdery form. Five hundred grams (500 g) of sclerotia powder was extracted by cold extraction in 95% ethanol for 72 h and concentrated

using rotary evaporator and dried completely in an oven at 40°C.

Experimental design

The female rats were divided into five groups of 8 rats per group and were administered distilled water, 100, 500, and 1000 mg/kg of crude ethanol extract of sclerotia of *P. tuberregium* daily for 28 days.

Group 1 (normal control)- received distilled water
Group 2- received 100 mg/kg of extract
Group 3- received 500 mg/kg of extract
Group 4- received 1000 mg/kg of extract

Animal sacrifices

The animals were sacrificed after the period of treatment. The bloods of animals were collected by vain puncture with the use of sterilized syringe and were transferred into Specimen bottles containing anti-coagulant and plasma separated by centrifuging the blood at 3000 revolutions/min. and stored at -20^{0}C.

Collection of tissues

All the animals were sacrificed 24 h after the last administration. The uterus, ovaries, liver and kidney, were carefully isolated and freed from mesenteric fat placed on filter paper to get rid of surplus fluid. Ovaries, uterus, liver and kidney on the left side were used for histological analysis. The organs were collected and placed in 10^{0}C formalin to prevent decay.

Routine histological preparation

According to Owolabi and Ogunnaike (2014) bouin's fluid was used to fix the dissected ovary, uterus, liver and kidney over night at room temperature thenthe tissues was transferred to 90% ethanol for dehydration. These tissues were then cleaned with xylene for two consecutive times at one and half hours each, after which they were placed in molten paraffin wax I and II at one and half hour each. Infiltration was finally in wax- III in an oven at 65^{0}C overnight. Molten paraffin wax contained in metal moulds was used to embed the tissues after which orientation was carried out to allow transverse section for the uterus, liver and kidney and coronal section for ovaries.

A thickness of 6 µm was sectioned for each tissue block using Leica rotary microtome (Leica R3425, Leica Microsystems, England, UK) and sectioned chips were softly placed to float on top of a temperate water bath at 40^{0}C. These floated chips were fixed on egg albumin encrusted microscopic slides, and placed in an oven at a constant temperature of 40^{0}C for 45 min for firm fixing of tissues on slides. Dewaxing was carried out on slides with xylene which was changed twice and afterwards hydrated using 50% ethanol and then soaked in distilled water for 6 min. This was then followed by staining with Ehrlich's hematoxylin and counter staining with Eosin. After staining, tissues were washed with water and dehydrated using 70% ethanol followed by rinsing with xylene-I and II. A drop of Distyrene, Plasticizer, and Xylene mixed together (mountant DPX) was finally placed on each section before cover slips were applied. Histopathological examinations were then carried out on these animal tissues using microscope to photograph every tenth section of each tissue.

Statistical analysis

All results are expressed as Mean ± Standard Error of Mean (SEM). Comparison of data was carried out using one-way analysis of variance (ANOVA). Graph pad prism 6 version software (UK) was used for all data analysis. $P < 0.05$ was regarded as indicating significant difference.

Results and Discussions

In Table 1, statistical evaluation revealed that from the first week of administration of crude ethanol extract of sclerotia of *P. tuberregium* to wistar rats for four weeks before sacrificing, there were significant changes in the weight of t rats when compared with Groups 2, 3 and 4.

Table 1: Effect of crude ethanol extract of the sclerotia of *P. tuberregium* on weight indices

Dose (mg/kg)	Weight of rats (g) Weight of rats (g)				
	Day 0	Day 7	Day 14	Day 21	Day 28
Control	172 ±0.82	173 ±0.77	174 ±0.78	176 ±0.80	179 ±0.63
100	175 ±1.19	176 ±1.37	178 ±1.14	182 ±1.21^b	184±1.14^c
500	175 ±1.17	178±1.17	180±1.16^c	182±0.76^c	184±1.07^c
1000	175 ±1.04	179 ±1.04	181 ±1.16^c	187 ±0.84^c	190 ±1.16^d

Values are in Mean ± SEM, n=8. ^{b}= p<0.01,^{c}= p<0.001, ^{d}=p<0.0001 compared to day 0, same letters in both rows and columns are not significantly different, but are significantly different from figures without letters in but rows and columns

The ethanol extract of *P. tuberregium* caused no toxic signs such as reduced appetite, sleepiness, excessive urination and shivering at graded doses of 100, 500 and 1000 mg/kg body weight. Therefore, it is not toxic at these doses. According to the toxicity rating chart, this extract is classified as nontoxic per kilogram body weight in humans (Chang and Wasser, 2012).

The body weight of the rats showed increase at various doses of administered extract throughout the four weeks, but the increase in weights were highly significant from day 14 to 28 at 0.01, 0.001 and 0.0001 respectively when compared with day 0. The increase in body weight may be due to the fact that the mushroom is composed of vitamins. Captivity is an attribute which minimizes expulsion of energy; this might also have contributed to the increase in body weight recorded. This is in line with the findings of Ergönül *et al*. (2013) who also recorded increase in body weight of rats.

Organ weight index

In Table 2, the weight index revealed that there was increase in the ovary, uterus, liver and kidney in the treatment groups (Groups 2, 3 and 4) when compared with the control group and were significant at p<0.05, p<0.01 and p<0.0001.

Table 2: Effect of crude ethanol extract of the sclerotia of *P. tuberregium* on organ weight indices

Parameters	Weight of Organs (g)			
	Control	100 mg/kg	500 mg/kg	1000 mg/kg
Ovary	0.11±0.004	0.22±0.005 ^{d}	0.25±0.007 ^{d}	0.28±0.007^d
Uterus	0.17±0.014	0.23±0.009^b	0.26±0.009 ^{d}	0.29±0.009 ^{d}
Liver	4.13±0.06	4.58±0.10 ^{b}	4.60±0.08 ^{b}	4.50±0.13 ^{a}
Kidney	0.33±0.01	0.39±0.01 ^{b}	0.38±0.02 ^{b}	0.38±0.007 ^{b}

Values are in Mean ± SEM, n=8. ^{a}= P <0.05,^{b}= P < 0.01 and ^{d} = P < 0.0001, compared to control, same letters in both rows and columns are not significantly different, but are significantly different from figures in control.

The weight of the ovary in all treated group increased significantly at P < 0.0001 when compared with the control group. At 100 mg/kg, the weight of the uterus increased significantly at P < 0.01 but at 500 and 1000 mg/kg, the uterus increased significantly at P < 0.0001, when compared with the control group. Research carried out by Owes and Ashby (2002) illustrated that compounds which cause elevation in reproductive parameters like the uterus and ovary, are possible estrogen agonist.

The weight of the liver increased significantly at P < 0.01 in rats treated with 100 and 500 mg/kg of the extract when compared with the control group but at 1000 mg/kg the weight of the liver, when compared with the control group was significant at P <0.005 at 100, 500 and 1000 mg/kg, the weight of the kidney when compared with the control group were all significant at P < 0.01. Compounds which increased the organ weight index of the liver and kidney may be potent hepatic enzyme –inducing substances and thus may be useful as immune booster (Ajayi *et al*., 2016).

Histological study

The effect of crude ethanol extract of sclerotia of *Pleurotus tuberregium* on the histology of the ovary (Plates A – D), uterus (Plates E – H), liver (Plates I – L) and kidney (Plates M – P). In all the plates of the four sensitive organs observed there was no visible disease or lesion.

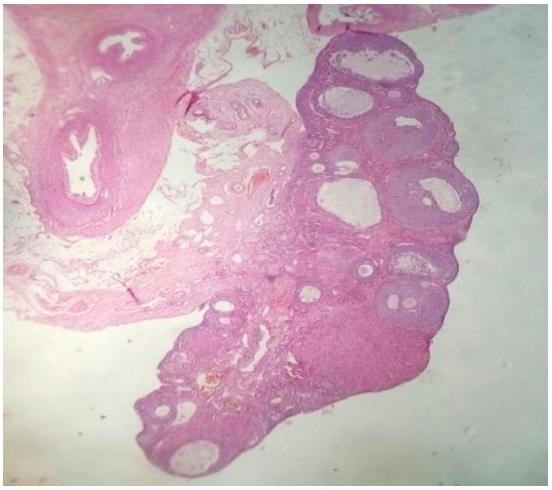

Plate A: The section of the ovary of rat administered distilled water (control): shows normal ovary with numerous follicles and corpus luteum at different stages of development

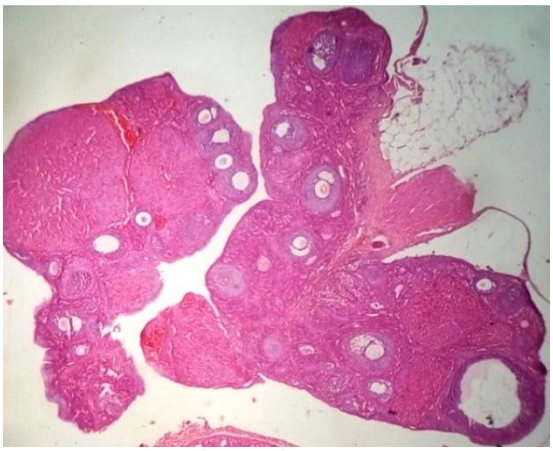

Plate B: The section of ovary of rat administered 100 mg/kg extract: shows several primary and secondary ovarian follicles and Corpora lutea

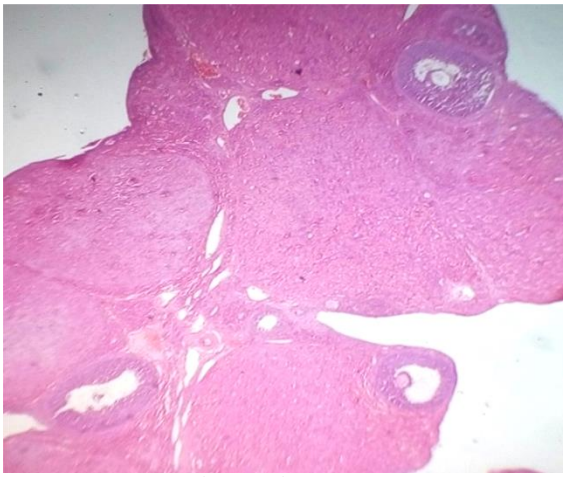

Plate C: The section of ovary of rat administered 500 mg/kg extract: shows Graafian follicle seen amongst other developing follicles and Corpora lutea

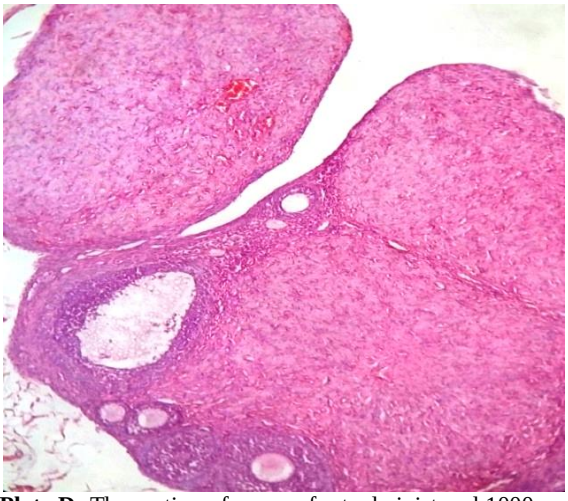

Plate D: The section of ovary of rat administered 1000 mg/kg extract: shows Graafian follicle seen amongst other developing follicles and Corpora lutea

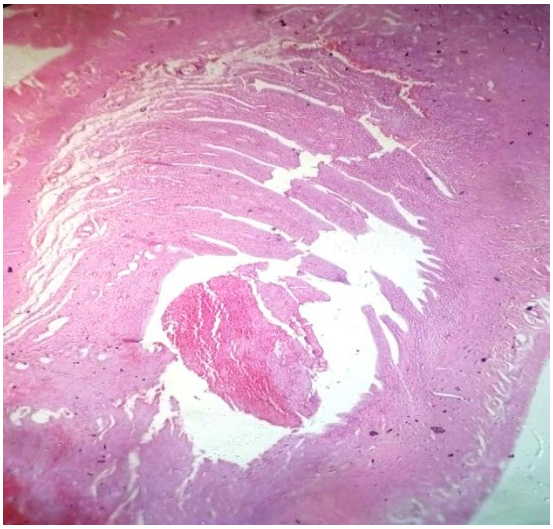

Plate E: The section of the uterus of control rat administered distilled water: shows endometrial gland with enclosed endometrial cavity containing haemorrhage

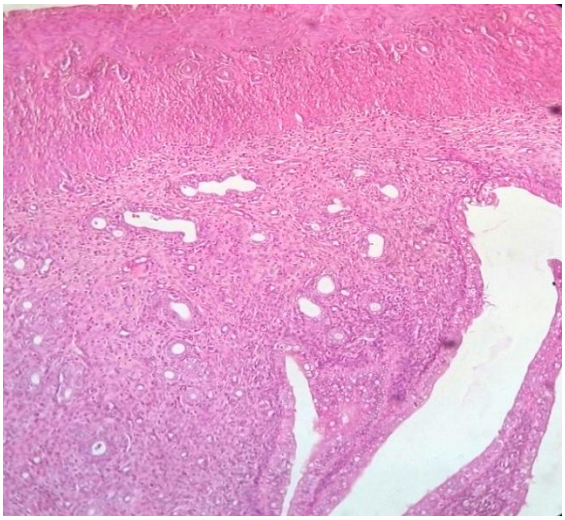

Plate F: The section of uterus of rat administered 100 mg/kg extract shows normal myometrium field. The endometrial stroma is infiltrated by inflammatory cells. Eosinophils are highly present but the uterus still holds normal endometrial glands with part of the endometrial cavity shown

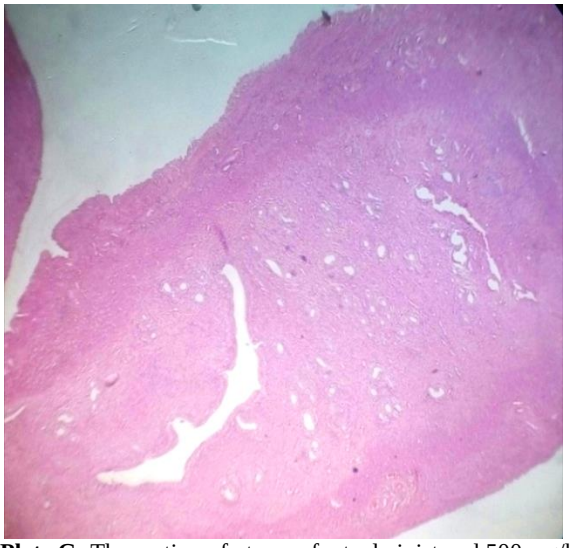

Plate G: The section of uterus of rat administered 500 mg/kg extract shows normal myometrium and endometrial with glands and stroma as well as endometrial cavity

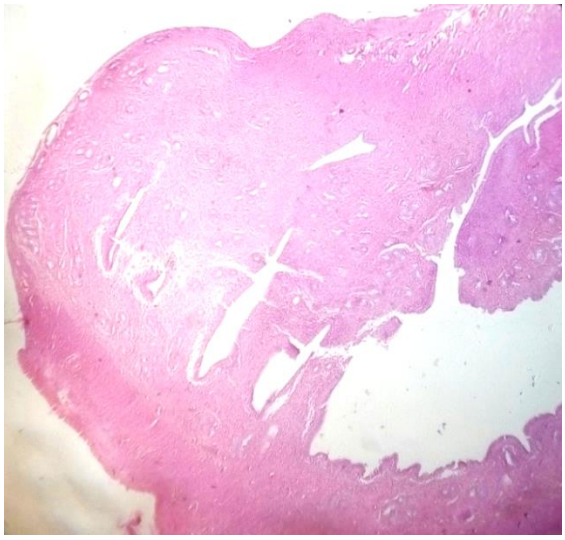

Plate H: The section of uterus of rat administered 1000 mg/kg extract shows uterus of rat a normal myometrium and endometrial as well as glands and uterine cavity

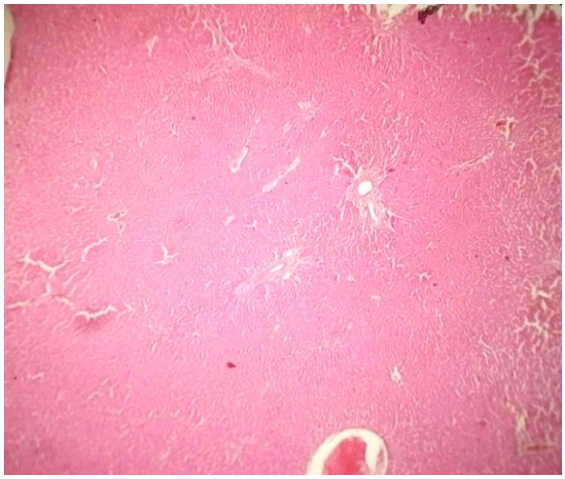

Plate I: The section of the liver of control rat administered distilled water: shows a normal architecture with normal hepatocyte radically distributed about the central veins. Portal tracts are also seen

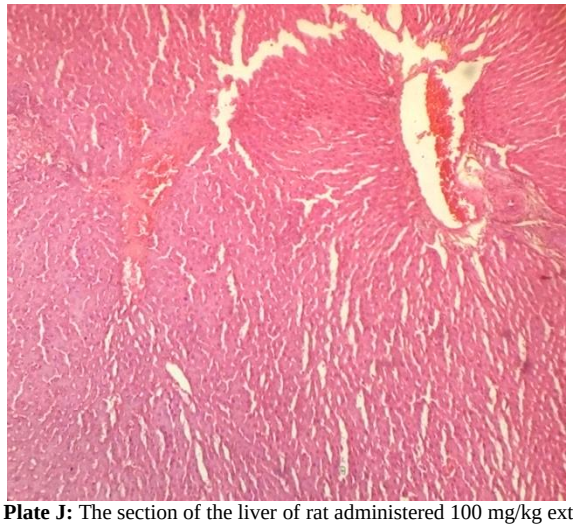

Plate J: The section of the liver of rat administered 100 mg/kg extract shows a normal architecture with normal hepatocyte radically distributed about central veins and portal tracts

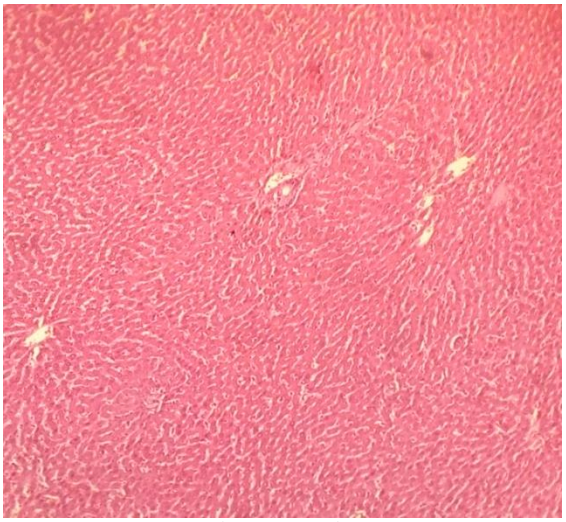

Plate K: The section of the liver of rat administered 500 mg/kg extract shows a normal architecture with normal hepatocyte radically distributed about central veins and portal tracts

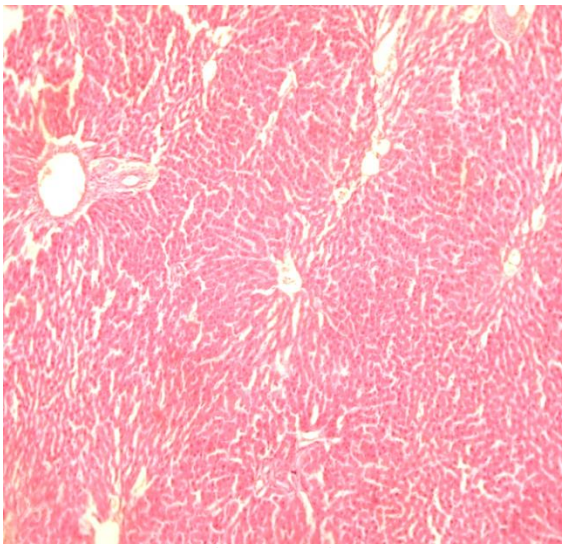

Plate L: The section of the liver of rat administered 1000 mg/kg extract shows a normal architecture with normal hepatocyte radically distributed about central veins and portal tracts

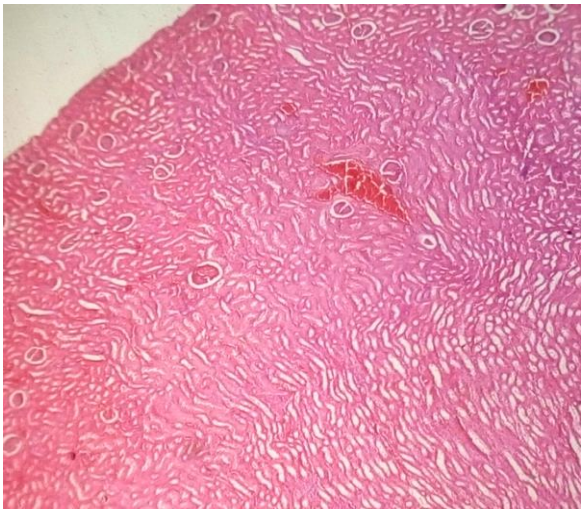

Plate M: The section of the kidney of control rat administered distilled water: shows a normal architecture with numerous normal glomeruli and tubulointerstitial appearance

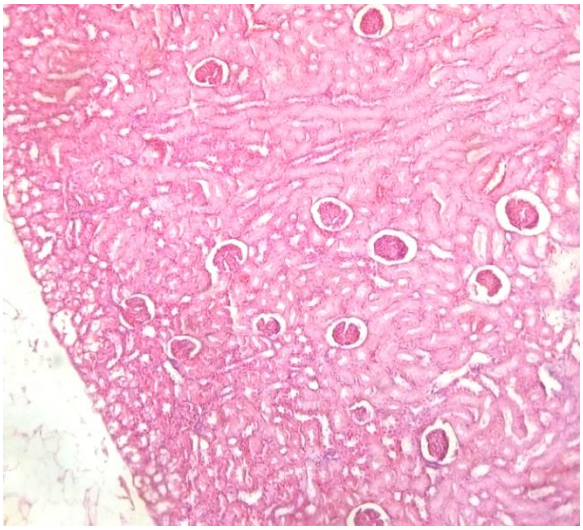

Plate P: The section of the liverof rat administered 1000 mg/kg extract shows a normal architecture of glomeruli, tubules and interstitial

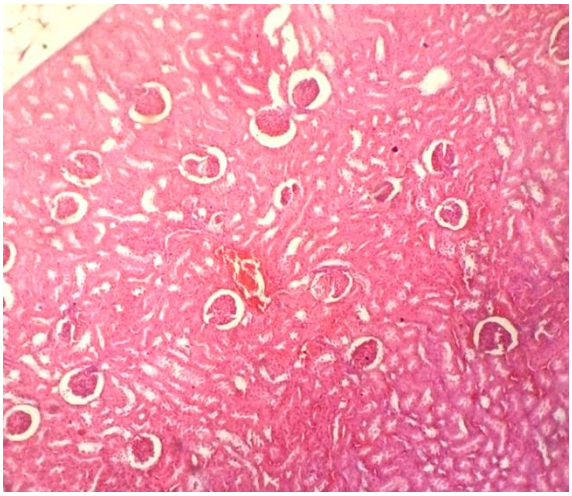

Plate N: The section of the kidney of rat administered with 100 mg/kg extract shows a normal architecture of glomeruli, tubules and interstitial

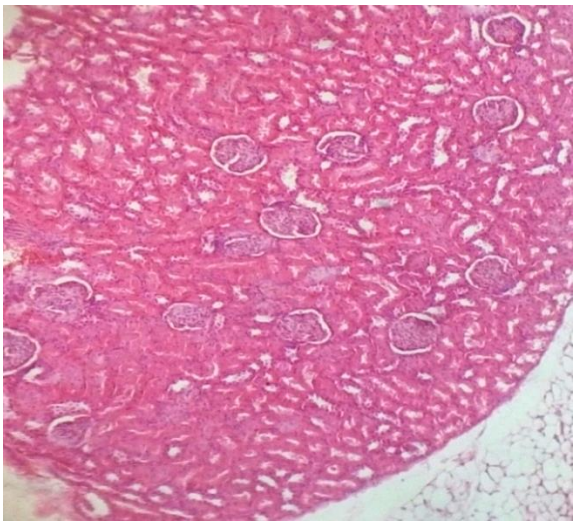

Plate O: The section of the kidneyof rat administered 500 mg/kg extract shows anormal architecture of glomeruli, tubules and interstitial

Numerous histological, morphological, biochemical and physiological changes take place in the ovary during the estrous cycle. Throughout the maturation of preovulatory follicles, ovulation occurs under the influence of extra ovarian and ovarian hormones. Discrepancy in these hormones leads to abnormality in ovarian duration and functions of the estrous cycle (Kinsky, 2001). The citation of estrous cycle in rats administered extracts (100.500 and 1000 mg/kg) orallyfor 28 days, showed decrease in the duration of metestrous and estrous phases. Thiswas also characterized by a discontinuation of the pro-estrous phase. The discontinuation of the pro-estrous phase is an indication that there will not be maturation of the follicle which will further lead to immaturities of graffian follicle. Presence of immature graffian follicle is characterized by decrease in metastrous and estrous phases. Thus, ovulation willbe subdued. This finding was further substantiated by our histopathological study where the transverse section of the ovary revealed the availability of developing andprimary follicles with no matured follicle and graffian follicle.

Ovary is well thought-out to be a collection of three endocrine tissues, the follicle, the corpus luteum and the stroma. The net weight of the ovary is constituted from the collective weight of these tissues (Mattila *et al*., 2001). All through the estrous cycle the weight of the ovarian tissue increases under the influence of gonadotrophic and steroidal hormones. Thus the increase in the weight of ovaries of the rats administered extract (100, 500 and 1000 mg/kg) indicate increase in the action of the follicle, the corpus luteum and the stroma of the ovary.

The uterus is a vital reproductive organ in vertibrates and other animals. It is used in the housing of feotuses. The sections of uterus of rat administered (100, 500 and 1000 mg/kg) extract showed normal myometrium field, endometrial stroma and glands. The section of uterus of rat administered 100 mg/kg extract shows normal myometrium field with the endometrial stroma infiltrated by inflammatory cells an indication that at this dose the extract is capable of combating any form of inflammation occurring in the uterus. Eosinophils were also highly present at this lower dose an indication that the extract will be more effective as a pro-fertility drug at lower doses. The organ weight of the uterus of rats in the treated groups showed that the uterus of the treated groups increased compared with the uterus of rats in the control group.

The kidney and liver are very important in the homeostatic and urninary function aswellas detoxification and elimination of harmful wastes from the body (Elias and Bengelsdorf, 2002). The histology results for the kidney and liver in rats treated with 100, 500 and 1000 mg/kg extract did not show any visible damage or disease on these organs.

Conclusion

The histological result of the sclerotia of *Pleurotus tuberregium* administered at various doses on the vital organs have pro-fertility potential and did not reveal any disease or lesion on any of the organs implying the safety of the sclerotia of *P. tuberregium* on animals at low and high doses.

Conflict of Interest

Authors declare that there is no conflict of interest reported on this work.

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