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Effects of Ethanol *Terminalia catappa* Leaf Extract on Serum Reproductive Hormones in Poloxamer-induced Hypercholesterolemia in Wister Rat

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Abstract

This research work was carried out to evaluate the effects of ethanol extract of *Terminalia catappa*, on sexual hormones following poloxamer-induced hypercholesterolemia in Wistar rat. Thirty- five (35) Wistar female rats were used for the study following an acclimatization period of 7 days. The rats were randomly divided into 5 groups of 7 rats each. Group A: Was fed with normal chow and distilled water and served as Normal Control. Group B: Was induced with 1.0g/kg dose of P-407 without treatment negative control. Group C: Were induced with 1.0g/kg dose of P-407 and treated with atorvastatin (ATV) at 20mg/kg as served as positive control, Group D: Was induced with 1.0g/kg dose of P-407 and treated with leaves extract (HLE) 100mg/kg for 14days. Group E: Was induced with 1.0g/kg dose of P-407 and treated with leaf extract (HLE) at 200mg/kg for 14days. The dose regimens were administered once daily for the period of the study. The rats were monitored for clinical signs and death. The results reveal that there was a significant ($P<0.05$) increase in serum oestrogen, progesterone, follicle stimulating hormone (FSH), and luteinizing hormone (LH) when compared with the normal, standard and hyperlipidemic control. Contrastingly, there was a significant decrease ($P< 0.05$) in serum testosterone and prolactin (PRL) when compared to the normal and standard control. It can be inferred from this present research work that the extract may stimulate or regulate ovulation and promote sexual health and drive possibly due to the presence of phytonutrient or phytoandrogens.

Keywords: *Terminalia Catappa*, Reproductive Hormones, Poloxamer, Hypercholesterolemia

Introduction

Cardiovascular diseases (CVDs) are the main cause of death worldwide. According to the World Health Organisation (WHO), (2011), about 17 million people die of CVDs annually (49% of all deaths due to non-communicable diseases) [1]. This rate is almost 2 times higher than the mortality rate for cancer, which is the second cause of death in the world. In addition, CVDs are the main cause of premature deaths. Data on the European population in 2012 showed that CVDs caused 38% and 35% of deaths among women and men < 75 years of age, respectively [2].

Hypercholesterolemia, also called high cholesterol, is the presence of high levels of cholesterol in the blood. It is a form of hypercholesterolemia (high levels of lipids in the blood), hyperlipoproteinemia (high levels of lipoproteins in the blood), and dyslipidemia (any abnormalities of lipid and lipoprotein levels in the blood) [3].

Hypercholesterolemia in general can be classified as either primary (familial) or secondary (acquired) hypercholesterolemia. It is also called familial due to a genetic defect, it may be monogenic: A single gene defect or polygenic: Multiple gene defects. Secondary is acquired because it is caused by another disorder like diabetes, nephritic syndrome, chronic alcoholism, hypothyroidism and with use of drugs like corticosteroids, beta blockers and oral contraceptives.

Secondary hypercholesterolemia together with significant hy pertriglyceridemia can cause pancreatitis [4]. The main cause of Hypercholesterolemia includes changes in lifestyle habits in which risk factor is mainly poor diet in which fat intake form saturated fat and cholesterol exceeds 40 percent of the total calories uptake [4].

Genetic contributions are usually due to the additive effects of multiple genes ("polygenic"), though occasionally may be due to a single gene defect such as in the case of familial hypercholesterolaemia [5]. In familial hypercholesterolemia, mutations may be present in the APOB gene (autosomal dominant), the autosomal recessive LDLRAP1 gene, autosomal dominant familial hypercholesterolemia (HCHOLA3) variant of the PCSK9 gene, or the LDL receptor gene. Familial hypercholesterolemia affects about one in 250 individuals [6].

Generally hypercholesterolemia does not have any obvious symptoms but they are usually discovered during routine examination or until it reaches the danger stage of a stroke or heart attack. Patients with high blood cholesterol level or patients with the familial forms of the disorder can develop xanthomas which are deposits of cholesterol may form under the skin, especially under the eyes.At the same time, patients with elevated levels of triglycerides may develop numerous pimple-like lesions at different sites in their body [7].

Hypercholesterolemia is the most important risk factor for atherosclerosis, which is the major cause of cardiovascular disease. Atherosclerosis is a pathologic process characterized by the accumulation of lipids, cholesterol and calcium and the development of fibrous plaques within the walls of large and medium arteries [8].

Atherosclerosis, the major cause of coronary artery disease, characterized by the accumulation of lipid and the formation of fibrous plaques within the wall of the arteries resulting in narrowing of the arteries that supply blood to the myocardium, and results in limiting blood flow and insufficient amounts of oxygen to meet the needs of the heart. Elevated lipid profile has been connected to the development of coronary atherosclerosis [9].

Although hypercholesterolemia itself is asymptomatic, longstanding elevation of serum cholesterol can lead to atherosclerosis (hardening of arteries) [5]. Over a period of decades, elevated serum cholesterol contributes to formation of atheromatous plaques in the arteries. This can lead to progressive narrowing of the involved arteries. Alternatively smaller plaques may rupture and cause a clot to form and obstruct blood flow [10]. This present research work is designed to determine the effect of *T. cattapa* on reproductive hormones following poloxamer induced hypercholesterolemia in Wister rats.

Medicinal plants have been effective in the treatment and management and treatment of several ailments. Coronary heart diseases such as atherosclerosis result from the buildup of cholesterol and other substances in the arterial walls. Astorvastatin and some other oral medication can be used to prevent cardiovascular disease at high doses and to treat abnormal lipid level. Cardiovascular diseases (CVD) have been named among the leading cause of death globally and it is common in people over 50years and above. Hence, the paucity of this research work. The aim of this study is to determine the effect of ethanol extracts of *Terminalia catappa* on sexual hormones following poloxamer-induced hypercholesterolemia in female Wister rats.

Materials and Methods

Materials

Plant materials

Fresh leaves of *Terminalia catappa* was collected from UNICROSS environment, Okuku, University of Cross River State, Nigeria. The leaves were taken to the University of Calabar, Department of Botany for identification and authentication. The voucher number of 206 has been deposited for future reference at the department's herbarium.

Experimental animals

Thirty- five (35) Wistar male rats were obtained from the animal holding unit of the Department of Medical Biochemistry, University of Cross River State (UNICROSS). The animals were allowed to acclimatize for a period of 7 days, in a well-ventilated room at room temperature and relative humidity of 29°C and 70% respectively with 12 hours natural light-dark cycle. They were allowed food and water *ad libitum*. Good hygiene was maintained by daily cleaning and removal of faeces and spills from their cages.

Assay kits:

All assays kits for Total cholesterol (TC), Triacylglycerol (TAG), High Density Lipoprotein (HDL) and Low Density Lipoprotein (LDL) were purchased from Randox laboratories Ltd® (Northern Ireland, UK), Ardmore, Co. Antrum UK.

Method

Preparation of extract of *Terminalia catappa* leaves

The leaves of *Terminalia catappa* was collected around University of Cross River State (UNICROSS) and air dried at room temperature for a period of 21days until constant weight was obtained. The dried leaves were then pulverized to powdered form by a machine blender and sieved. Thereafter, 400g of the pulverized plant material (*Terminalia catappa*) was dissolved in 1200ml of 70% petroleum ether for 72 hours. This was followed with vacuum filtration and extracts was concentrated using an evaporator water bath at 40°C to obtain a solvent free extract, and stored in a refrigerator at 4°C. Preparation of standard drug: Atorvastatin (Pfizer Ireland Pharmaceuticals, Ireland) was purchased in a tablet form at strength 20mg. Tablets were crushed into powder, dissolved in distilled water and administered orally.

Induction of hyperlipidemia
Poloxamer 407 (Lutrol F127; BASF, Ludwigshafen, Germany) was used as the inducing agent. Hyperlipidemia was induced as described by Megalli (2005). Briefly; 1.0g/kg dose of P-407 was introduced intraperitoneally. All syringes were placed on ice prior to P-407 administration to maintain the polymer in a mobile viscous state during the injection, since P407 solutions at concentrations greater than about 23% w/w exhibit reverse thermal gelatin properties.

Experimental design
A total of 35 healthy Wistars rats were used. The rats were randomly divided into 5 groups of 7 rats each. Group A: were fed with normal chow and distilled water only for 14 days (normal control). Group B: Were induced with 1.0 g/kg dose of P-407 according to Mansurah [11], without treatment (negative control). Group C: Were induced with 1.0 g/kg dose of P-407 and treated with Atorvastatin (ATV) at 20 mg/kg body weight/day for 14 days Group D: Were induced with 1.0 g/kg dose of P-407 and treated with leaves extract (HLE) at 100mg/kg body weight/day for 14 days Group E: Were induced with 1.0 g/kg dose of P-407 and treated with leaf extract (HLE) at 200mg/kg body weight/ day for 14 days. The dose regimens were administered once daily for the period of the study. The rats were monitored for clinical signs and death.

Collection and preparation of sera samples:
At the end of the 14-day experimental period, the anesthesia was performed on all experimental animals. The anesthetized animals were bled by cardiac puncture. The blood samples were collected and centrifuged at a speed of 3000 rpm for 15 minutes and serum collected into plain sample bottles for sexual hormones haematological parameters

Effect on female sexual hormones
Determination of testosterone concentration
Serum testosterone concentration was determined by the method of Horton and Tait [12], using test kits procured from Monobind Inc., U.S.A.

Procedure
Each serum reference, control and sample specimen micro plate's wells to be assayed was formatted in duplicate. Then 0.01 ml (10 µl) of serum reference, control or specimen was pipetted into the appropriately assigned well. The working testosterone-enzyme reagent (0.05 ml) was added to all wells. The micro plate was swirled gently for 20-30 seconds to mix. Then 0.050 ml (50 µl) of testosterone-biotin reagent was added to all wells. The micro plate was swirled gently for 20-30 seconds to mix. It was covered and incubated for 60 minutes at room temperature. The contents of the micro plate were discarded by aspiration. Wash buffer (350 µl) was added and aspirated. This was done three times using automatic plate washer. Then 0.10 ml (100 µl) of working substrate solution was added to all wells and gently mixed for 15-20 seconds. The absorbance was read at 450 nm within 30 minutes in a micro plate reader.

Determination of serum follicle stimulating hormone concentration
Serum follicle stimulating hormone (FSH) concentration was determined by the method of Odell *et al.* [13] using test kits procured from monobind inc., U.S.A.

Procedure
Each serum reference, control and sample specimen microplates' well to be assayed was formatted in duplicate. Then 0.005 ml (50 µl) of serum reference, control or specimen was pipette into the appropriate assigned well. Thereafter, 0.10 ml (100 µl) of FSH-enzyme reagent solution was added to all wells the microplate was swirled gently for 20-30 seconds to mix and then covered. It was incubated for 60 minutes at room temperature. The contents of the microplate were discarded by aspiration. Wash buffer (350 µl) was added and aspirated. This was done three times using automatic plate washer. Then 0.10 ml (100 µl) of working substrate solution was added to all wells and incubated at room temperature for 15 minutes. Stop solution (0.05 ml) was added to each well and mixed gently for 15-20 seconds. The absorbance in each well was read at 450 nm within 30 minutes in microplate reader.

Determination of serum luteinizing hormone concentration
Serum luteinizing hormone (LH) concentration was determined by the method of Kosasa [14], using test kits procured from Monobid Inc., U.S.A.

Procedure
Each serum reference, control and sample specimen microplates' well to be assayed was formatted in duplicate. Then 0.05 ml (50 µl) of serum reference, control or specimen was pipetted into the appropriate well. LH-Enzyme reagent (0.01 ml) was added to all wells. The microplate was swirled gently for 20-30 seconds to mix and then covered. It was incubated for 60 minutes at room temperature. The contents of the microplate were discarded by aspiration. Then, 350 µl of wash buffer was added and aspirated. This was done three times using automatic plate washer. Then 0.01 ml (100 µl) of working substrate solution was added to all wells and incubated at room temperature for 15 minutes. The stop solution (0.05 ml) was added to each well and mixed gently for 15-20 seconds. The absorbance in each well was read at 45 nm test kits 30 minutes in a micro plate reader.

Result
The effect of petroleum ether extract of *T. catappa* on serum sexual hormones in poloxamer induced hypercholesterolemic female Wistar rat following the administration of extract of *T. catappa* leaf was found to significantly ($P< 0.05$) decrease serum prolactin when compared with the normal, standard and hypercholesterolemic control (Fig 1).
More so, following the administration of the extract, the extract significantly ($p< 0.05$) increase serum progesterone, oestrogen, FSH and LH when compared with the normal, standard and hyperlipidaemic control (Fig. 2-5). Alternatively, the extract of *T. catappa* significantly reduced at 100 and 200mg/dl when compared with the normal, standard and hypercholesterolemic control (Fig 6).

A. Effect of ether extract of *T.catappa* leaf on serum sexual hormones in poloxamer induced hypercholestrolemia in female Wistar rats

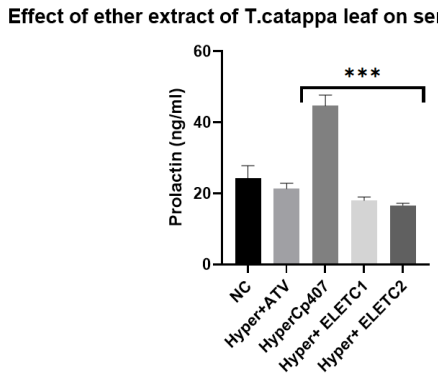

Fig 1: Effect of extract of *T.catappa* leaf on serum prolactin hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

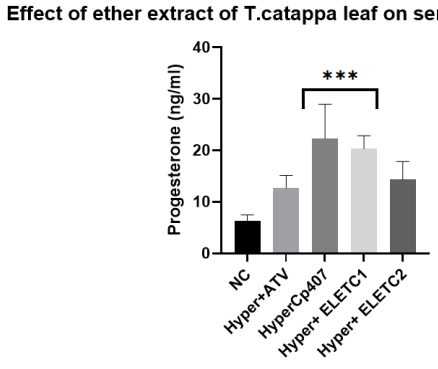

Fig 2: Effect of extract of *T.catappa* leaf on serum progesterone hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

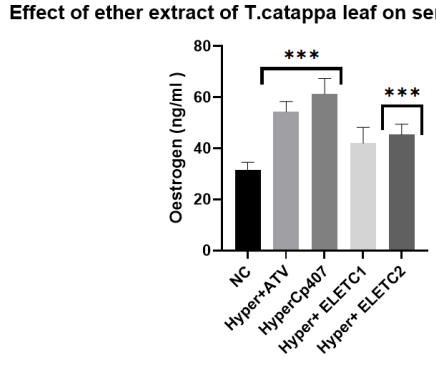

Fig 3: Effect of extract of *T.catappa* leaf on serum oestrogen hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

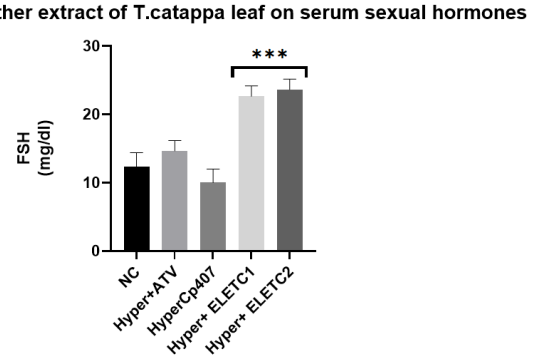

Fig 4: Effect of extract of *T.catappa* leaf on serum FSH hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

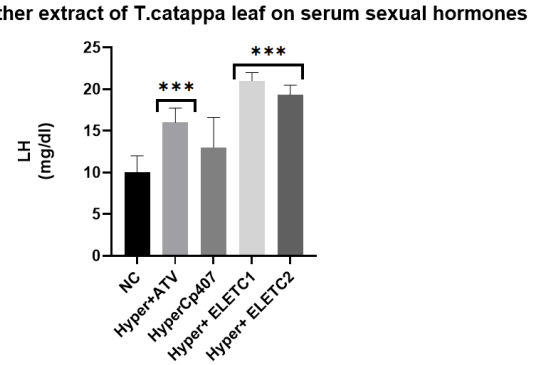

Fig 5: Effect of extract of *T.catappa* leaf on serum LH hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

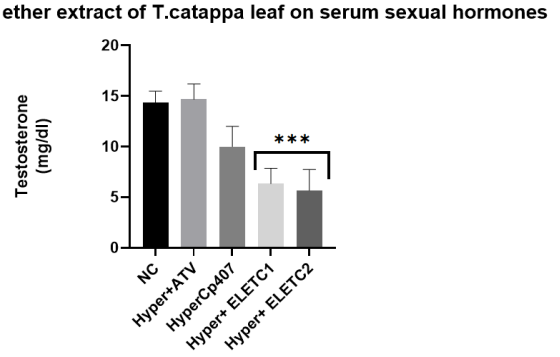

Fig 6: Effect of extract of *T.catappa* leaf on serum testosterone hormones in poloxamer induced hypercholestrolemia in female Wistar rats

Key: NC: Normal Control, Hyper + ATV: Hyperlipidemic rats + atorvastatin (20mg/Kgbwt), HyperCp407: Hyperlipidemic rats control, induced with poloxamer 407, Hyper + ELETC1: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (100mg/kg) a Hyper + ELETC2: Hyperlipidemic rats + ethanol leaf extract of *Termilania catappa* (200mg/kg).

Discussion

Women have a reduced cardiovascular disease (CVD) risk compared to men which could be partially driven by sex hormones influencing lipid levels post-puberty. Prior to menopause, it is known that women have a lower risk of cardiovascular disease (CVD) and coronary heart disease compared to age matched men; it is reported that women have around half the CVD risk and almost a 10-year delay in first myocardial infarction event compared to men [15-17]. Sex hormones have been proposed to drive these differences mechanistically [18] and there is evidence that early versus late menarche may result in differential long term cardiovascular traits, including altered lipoprotein levels, in women [19]. In support of this observation, following menopause a reduction in circulating oestrogen levels increases susceptibility to developing metabolic diseases including metabolic syndrome, non-alcoholic fatty liver disease, and diabetes in women [20].

Oestrogen is a primary female sex hormone produced mainly by the ovarian follicles and corpus luteum, and also by the placenta. The three major types of oestrogens are oestrone, oestradiol and oestriol, of which, oestradiol is the most potent oestrogen [21]. Oestrogen production from the ovaries declines around and after menopause. oestrogen is a cardio protective hormone for women. But in postmenopausal women due to lack of the oestrogen, cardio protective function is lost and increased the coronary artery diseases [22]. However, several other physiological changes which develop during menopause may also influence the risk of cardiovascular disease, such as aging effect, decreasing resting metabolic rate and physical activity [23]. Again, following menopause due to lacking of estrogen, women have increased risk for central obesity, hyperlipidemia, glucose intolerance and hypertension. Among these factors the hyperlipidemia seems to be the major issue [24]. Oestrogen also has anti-inflammatory properties. In postmenopausal women due to lacking of Oestrogen there is increased cytokines level including tumour necrosis factor alpha, and IL- 6, It has been reported that cholesterol elimination through bile acid synthesis and export-is strongly inhibited by increased serum levels of tumour necrosis factor alpha, which also favors fatty acid synthesis rather than fatty acid oxidation [24]. The observed significant increase in oestrogen suggest that the extract may possibly improve the reproductive function and reduce predisposition to the atherosclerosis or coronary diseases by inducing a vascular effect in hyperlipidemic condition.

Prolactin (PRL) is an anterior pituitary hormone, and its receptor is expressed in most peripheral organs. Its most well-known physiological role is to support lactation, but it has broad functions in metabolic, osmoregulatory, and immunoregulatory pathways [25]. More recently, it has been discovered that prolactin is produced in adipose tissue and that the prolactin receptor is expressed in adipose tissue [26]. PRL stimulates and maintains lactation in women. During the menstrual cycle, its serum levels are variable and exhibit slight elevation during mid-cycle. PRL levels are also elevated in sleep, exercise, nipple stimulation, sexual intercourse, hypoglycemia, pregnancy as well as surgical stress. It is also raised in post-partum females and newborns [27]. A subsequent study from the same cohort later found that prolactin was associated with higher cardiovascular and all-cause mortality over a period of 10 years [28]. Similarly, the decrease in prolactin level as seen in this present research following the administration of *T. catappa* extract, suggest that the decrease may improve metabolic osmoregulatory and immunoregulatory role in hyperlidemic rat possibly due to the presence of phytonutrients or phytoandrogens.

FSH plays a vital role in ovulation by stimulating follicular growth and oestrogen secretion in synchrony with LH, while LH plays a vital role in follicular maturation, rupture and ovulation. A new hormonal pattern is established at menopause, which is characterized by high levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and low level of oestrogen [29]. Menopause has a wide starting age range, but usually be expected in the range of 42-58 years [30]. After menopause, the morbidity and mortality from cardiovascular diseases (CVD) are increased. Postmenopausal women are 4- 8 times more likely to die of coronary artery disease than premenopausal women [31]. It has been suggested that the rate of morbidity from coronary artery diseases accelerate more quickly in postmenopausal women than do those of males after the age of 45 years. It can be inferred from this present research since the experimental animal have not attained menopause that the extract may synergistically enhance sexual drive in hyperlipidemic rats.

Progesterone is a female sex hormone similar to the oestrogens, is an endogenous steroid released by the ovaries and the adrenal glands. Progesterone, in association with oestrogen, helps to regulate the accessory organs during the menstrual cycle [32]. It stimulates and regulates ovulation and plays a major role in maintaining pregnancy. Progesterone is the principal cause of the decline in cholesterol in the luteal phase and the early first trimester. Progesterone remains at a relatively low level throughout the follicular phase and during ovulation, but increases sharply during the luteal phase. In the event of conception and implantation, progesterone continues to climb across the first trimester [33]. The significant increases in progesterone concentration from this present research work suggest that the extract helps to stimulate or regulate ovulation or maintain sustainable pregnancy.

Conclusion

The inferences from this present research work suggest that the extract may stimulate or regulate ovulation and promote sexual health and drive possibly due to the presence of phytonutrient or phytohydrogens.

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