Original Article

Antifibrotic Potential of *Tetracarpidium conophorum* (African Walnut) Leaves Extract on Diethylstilbestrol-Induced Rat Model of Uterine Fibroid

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ABSTRACT

Background: The increased prevalence of uterine fibroid (UF) and its life-threatening impact among women of reproductive age led to the development of this study. The study investigated the antifibrotic potential of *Tetracarpidium conophorum* aqueous extract on UF-induced rats. **Materials and Methods:** Sixty-four female Wistar rats, with an average weight of 200 g, were used for the study. The rats were randomly divided into eight groups of eight animals each. UF was induced by oral administration of diethylstilbestrol (DES) and intramuscular injection of progesterone at dosages 1.35 and 1.0 mg/kg body weight, respectively. Group 1 was administered normal saline orally for 8 weeks. Groups 2 and 3 were treated with progesterone and a combination of DES and progesterone, respectively. Groups 4 and 5 were pretreated with 200 and 400 mg/kg *T. conophorum* extract, respectively, for 3 weeks before the administration of DES and progesterone for 5 weeks. Groups 6 and 7 were administered DES and progesterone for 5 weeks before being treated with 200 and 400 mg/kg *T. conophorum* extract, respectively, for 3 weeks. Group 8 was the self-recovery group-administered DES and progesterone for 5 weeks after which they were given normal saline orally for 3 weeks. **Results:** After the treatment period, the rats were euthanized, and blood was collected, while the uteruses were harvested. Co-administration of DES and progesterone produces UF conditions. However, pre- and post-treatment with 200 mg/kg of extract mitigated the effects that were induced by DES and progesterone, but no remarkable preventive and curative effects were observed with the higher dosage (400 mg/kg). There were a reduction of the serum prolactin level in the treatment groups and an increased serum progesterone level in the posttreatment group. **Conclusion:** The study has shown that *T. conophorum* has both preventive and curative effects on UF at low dosage (200 mg/kg).

KEYWORDS: *Diethylstilbestrol, progesterone, Tetracarpidium conophorum, uterine fibroid*

INTRODUCTION

Uterine fibroids (UFs), also known as leiomyomas, are benign tumors of smooth muscle cells that develop within the wall of the uterus or on the outer wall.[1] It affects the uterus of up to 80% of women in their reproductive years and causes morbidity in about 30% of them.[2] UF is characterized by increased proliferation of the smooth muscle cells, altered extracellular matrix deposition, and enhanced responsiveness to sex steroid

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hormones.[3] Ethnic origin has been considered one of the known risk factors for uterine leiomyoma (UL). Report indicates that the disorder is prevalent among Black women[4] in their reproductive-age bracket. A study by Baird *et al*.[5] reported that between 80% and 90% of African-American women and 70% of White women will develop fibroids by the age of 50, while another study also suggests that Black women who are obese coupled with high blood pressure are more likely to have fibroids.[6] In Nigeria, there is an increased incidence of fibroid among reproductive-aged women, which ranges from 17.9% to 26.0% as compared to 5%–11% reported in Europe and the United States.[7]

Common symptoms shown by UF patients include abnormal uterine bleeding, pelvic pain, or pressure, bloating, urinary frequency, and constipation.[8] The major factors responsible for the development of fibroids have not been well explained. However, evidence has shown that ovarian steroids, such as estrogen and progesterone, are important factors for the growth.[9] A study by Schwartz *et al*.[10] reported that increased level of estrogen is the most common cause of fibroid and painful menstruation. This was also corroborated by Obochi *et al*.[11]

Most mechanisms that have been proposed for the development of UFs are based on animal studies, which present a powerful tool to help investigators develop models for disease mechanism. Diethylstilbestrol (DES) is a synthetic estrogen used during the early 40s by pregnant women to prevent miscarriages, premature labor, and other pregnancy-related complications.[12] DES is now known to be one of the recognized endocrine-disrupting chemicals (EDCs). EDC is an exogenous compound that may interfere with synthesis, secretion, transport, metabolism, receptor binding, or elimination of endogenous hormones and alter the endocrine and homeostatic systems.[13] These chemicals have been reported to cause cancer, birth defects, and other developmental abnormalities, and the effects have been noted to be more severe when exposure occurs during fetal development.[14] Neonatal exposure to EDCs such as DES and genistein during reproductive tract development has been shown to increase the incidence, multiplicity, and overall size of UL in the Eker rat model, concomitantly reprogramming estrogen-responsive gene expression.[15]

Although surgery (hysterectomy or myomectomy) is the gold standard treatment for symptomatic ULs, these medical treatments still fail to deliver satisfactory results when it comes to the treatment of the disorder.[16] It has been reported that ULs are the most common indication for hysterectomy in women of childbearing age and therefore are a major health issue.[17] A United States of America report indicated that of the 600,000 hysterectomies performed annually, one-third are due to fibroids and UFs are a common reason for surgical removal of the uterus in the United States,[18] with yearly cost estimates of $5.9–34.4 billion.[19] Moreover, even with surgery, recurrence from underlying risk factors is still possible. Hence, it is of utmost importance that natural, nonsurgical therapies be investigated for the cure of this important global health challenge.

Plant-based medicines have long been used in the prevention and treatment of diseases[20,21] and are regarded as potential therapeutic options for UL. Isoliquiritigenin, a natural chalcone flavonoid, richly present in licorice and shallots, has an inhibitory effect on UL growth by inducing the apoptotic pathway activation.[22] Resveratrol, widely present in red wine and grape skin, has an anti-metastatic effect on malignant cancer cells.[23]

The African walnut, known as *Tetracarpidium conophorum*, belongs to the family *Euphorbiaceae*.[24] Common names of this plant include African walnut, black walnut, and Nigerian walnut.[25] Among the Yorubas in Nigeria, African walnut is known as awusa or asala. The Igbos refer to the plant as ukpa or oke okpokirinya, while the Nigerian Hausas refer to it as gawudi bairi. It is known as okhue or okwe among the Bini tribe of Edo State.[26] In Sierra Leone, it is known as musyabassa while the Cameroonians refers to it as kaso or ngak.[27] The African walnut is widely grown in the western and eastern parts of Nigeria. It is also an indigenous plant in Cameroon, Central African Republic, Congo, Gabon, and Niger.[28]

The seed of *T. conophorum* is reportedly used in the treatment of fibroids, while the boiled seeds are also eaten to improve sperm count in men, while the leaf juice is used to improve fertility in women and regulate menstrual flow.[29] The bark is brewed as a tea for use as a laxative and is chewed for toothache. The fruits are edible and used for various purposes, including masticatory, thrush, anti-helminth, and syphilis and also as an antidote against snake bites.[30] The phytochemical analysis of the nuts, leaves, and roots indicated that it has bioactive compounds such as oxalates, phytates, tannins, saponins, alkaloids, flavonoids, and terpenoids,[31,32] and the medicinal effect of the plant has been attributed to these phytoconstituents.

The increased prevalence of UF cases among women of reproductive age in Nigeria and the saddened report of no permanent treatment options apart from the surgical removal of the fibroid or the complete removal

of the uterus are of great concern. Although medicinal plants have potential to serve as cure for UF and large proportion of women with the disease, particularly in developing countries like Nigeria depending on them, there is a paucity of information in the literature on the efficacy of medicinal plant with acclaimed anti-UF effect. Although traditional use of the leaves of *T. conophorum* in the treatment of UF is well known in some Nigeria communities, this also has not been supported by the scientific study.

Materials and Methods

Chemicals and reagents

DES, progesterone, and diagnostic kits were all products of AK Scientific, Inc., USA. All other reagents used were of analytical grades.

Collection and preparation of plant materials

The leaves of *T. conophorum* were harvested from a farmland in Boluwaduro Village, Oriire Local Government Area, Oyo State, Nigeria. The plant sample was identified and authenticated at the Department of Plant Biology, University of Ilorin, Kwara State, with a voucher number UILH/001/1158/2020.

The leaves were processed as described by Ibrahim *et al*.[33] Briefly, samples of *T. conophorum* fresh leaves were air-dried in the laboratory at room temperature for a week after which it was pulverized using an electric blender. The powdered leaves (100 g) were soaked in 2 L of distilled water for 24 h. The mixture was then filtered through Whatman No. 1 filter paper, and the filtrate was freeze-dried at a temperature of − 50°C and a pressure of 52 Pa for 72 h, under vacuum condition. The resultant yield was stored at 4°C. The percentage yield of the extract was calculated using the formula:

$$\%\text{yield} = \frac{\text{Weight of Extract}}{\text{Weight of Sample Extracted}} \times 100$$

Experimental protocol

Experimental animals

Sixty-four nonpregnant female Wistar rats with an average body weight of 200 g were used for the study. The animals were obtained from the Central Research Laboratory, University of Ilorin, Kwara State. They were housed in cages (8 animals per cage) at the Animal House of the Department of Medical Biochemistry and Pharmacology, Kwara State University, Malete. The animals were kept under the standard environmental condition of 12/12-h light/dark cycle and were maintained with rat feed and water *ad libitum.* Before the commencement of the study, the animals were made to acclimatize to the animal house condition for 2 weeks. All of the animal protocols used during the study were performed in strict accordance with the Guidelines for the Care and Use of Laboratory Animals established by the Animal Ethical Committee, Kwara State University Malete, Ilorin, Nigeria.

Induction of uterine fibroid

UF was induced in all rats in Groups 3–8 according to the method of Zhao *et al*.[34] Rats in these groups were orally administered 1.35 mg DES/kg daily and injected with 1.0 mg of progesterone via the lower limb lateral muscle 3 times in a week (Monday, Wednesday, and Friday) for 5 weeks.

Experimental design and extract administration

The nonpregnant female Wistar rats randomized to 8 groups of 8 rats each and labeled as Group 1 (normal control); Group 2 (model group 1); Group 3 (model group 2); Group 4 (pretreatment group 1); Group 5 (pretreatment group 2); Group 6 (treatment group 1); Group 7 (treatment group 2); and Group 8 (self-recovery group) were used for the study. All rats received daily treatment with their test solutions between the hours of 10:00 am and 12:00 pm. Group 1 was administered normal saline orally for 8 weeks. Groups 2 and 3 were treated with progesterone and a combination of DES and progesterone, respectively. Groups 4 and 5 were pretreated with 200 and 400 mg/kg *T. conophorum* extract, respectively, for 3 weeks before the administration of DES and progesterone for 5 weeks. Groups 6 and 7 were administered DES and progesterone for 5 weeks before being treated with 200 and 400 mg/kg *T. conophorum* extract, respectively, for 3 weeks. Group 8 was the self-recovery group-administered DES and progesterone for 5 weeks after which they were given normal saline orally for 3 weeks.

Procedure for sacrificing the rats and serum preparation

At the end of the last administration, food was withdrawn from all the rats for 12 h. The rats were then euthanized in a desiccator using chloroform. Blood samples were withdrawn from the rats via cardiac puncture and stored at 4°C for 1 h. The blood samples were then centrifuged at 3500 rpm for 5 min to obtain the serum, and the serum was stored at 4°C until analyzed.

Tissue and sample preparation

The uteruses were excised from the animals, cleaned of blood and fat, and then weighed. Uteruses were divided into two parts with one part stored in liquid formalin for histology and the other part homogenized in ice-cold sucrose solution to obtain homogenates used for other assays.[33] The homogenates were centrifuged at 3000 rpm for 5 min, and the resulting supernatant was stored frozen for 24 h.

Gross uterus morphology and uterus weight

To obtain the relative uterus weight and gross morphology, the animals were weighed just before sacrifice and after the sacrifice, following tissue excision; the weight of the uterus was also measured using a 1 kg digital weighing scale. Pictures of the uterus were also taken in JPEG format to depict gross morphological images of the fibroid and nonfibroid uteri.

Estimation of the uterine coefficient

The uterine coefficient was calculated by comparing the uterine weight with the total body weight of the animals.[33]

Biochemical analysis

Determination of lipid profile

High-density lipoprotein (HDL)-cholesterol (HDL-c) level was determined following the procedure described by Friedwald *et al*.[35] The serum low-density lipoprotein (LDL)-cholesterol (LDL-c) concentration was determined using the formula described by Friedwald *et al*.[35] Total cholesterol was determined following the procedure described by Richmond.[36] Serum triglycerides (TGs) level was determined following the procedure described by Bablok *et al*.[37] and Bucolo and David.[38]

Enzyme immunoassay technique for progesterone, estrogen, and prolactin

The levels of serum progesterone, estrogen, and prolactin were determined by immunoenzymometric assay using Calbiotech assay kits (El Cajon, CA, USA). Assays were carried out as described by the manufacturer.

Determination of uterus calcium ATPase activity

The Ca^{2+}-ATPase activity in the uterus was measured using the method described by Lin and Way.[39]

Statistical analysis

GraphPad Prism, version 5.0 for windows (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses. One-way analysis of variance followed by Bonferroni's *post hoc* test for multiple comparison was used for the statistical evaluation of the data. Data are presented as mean ± standard error of mean, and the values were considered statistically significant at $P < 0.05$ (confidence level = 95%).

Results

Effect of treatment on uterine weight

The results of the effects of treatment on uterine weight showed that the mean uterine weight of the normal control group was not significantly different from that observed in the progesterone-administered group but significantly lower than that observed in rat co-administered progesterone and DES. Rats pretreated and treated with the extract at 200 mg/kg did not show any significant change in their uterine weight when compared to the normal control and the self-recovery group. However, rats pretreated and treated with the extract at 400 mg/kg showed a significantly increased uterine weight compared to the normal control [Figure 1].

Effect of treatment on uterine coefficient

Figure 1 reveals the results of the effect of treatment on uterine coefficient. The mean percentage uterine coefficient of the normal control group was 0.367 ± 0.036. Sole administration of progesterone did not cause significant change in the uterine coefficient of the rats. However, co-administration of DES and progesterone resulted in a significantly higher uterine coefficient (0.706% ± 0.031%). Rats pretreated and posttreated with the extract at 200 mg/kg showed no significant difference in their uterine coefficient when compared to that of the normal control group. Similarly, rats allowed to self-recover from the effect of the inducing agents also showed no significant difference in uterine coefficient (0.342% ± 0.065%) when compared to the observed value in the normal control group. Pretreatment and posttreatment with the extract at 400 mg/kg resulted in a significantly higher uterine coefficient (1.511% ± 0.058% and 2.179% ± 0.566%, respectively) when compared to the normal control.

Effect of treatment on uterine gross morphology

Figure 2 depicts the representative image of the uterus of all groups after treatment. Uterus of the

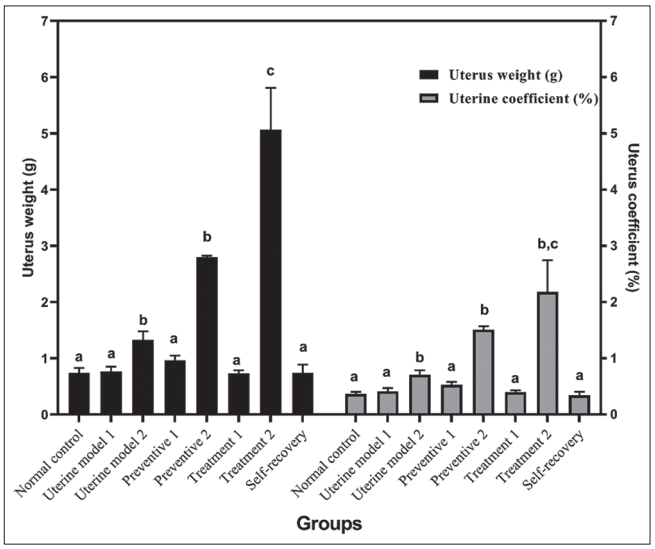


Figure 1: Effect of treatment on uterine weight and uterine coefficient. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

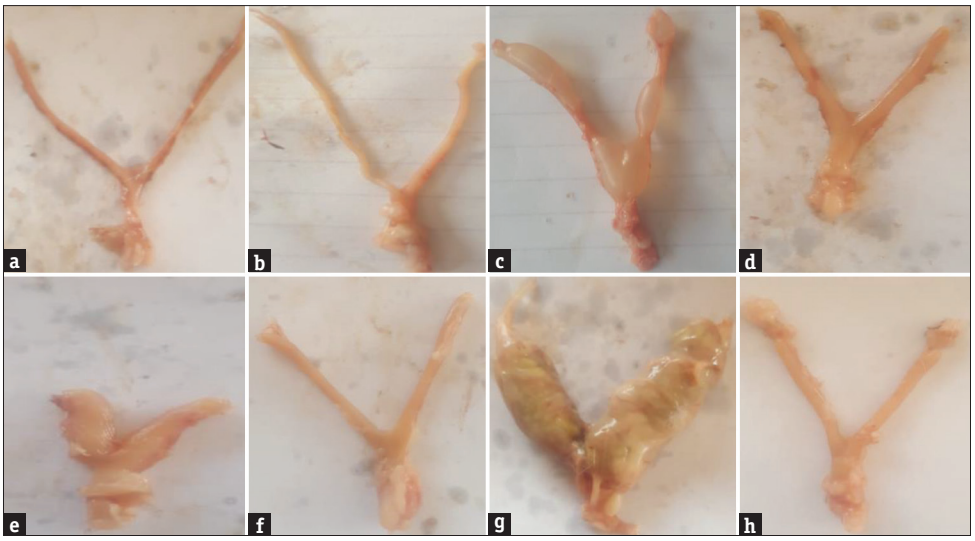


Figure 2: Gross morphology of the uterus after treatment. (a) Normal control (normal saline); (b) uterine model 1 (progesterone only); (c) = uterine model 2 (data ethylstilbestrol + progesterone); (d) pretreatment 1 (200 mg extract + data ethylstilbestrol + progesterone); (e) pretreatment 11 (400 mg extract + data ethylstilbestrol + progesterone); (f) treatment 1 (data ethylstilbestrol + progesterone + 200 mg extract); (g) treatment 11 (data ethylstilbestrol + progesterone + 400 mg extract); (h) self-recovery (data ethylstilbestrol + progesterone + normal saline)

normal control rat showed a bright color and normal texture of the uterus with no cyst, nodules, or swelling [Figure 2a]. Uterus of the rats treated with progesterone only also showed similar results to that of the normal control group [Figure 2b]. However, there was large accumulation of fat deposits observed around the uterine area after dissection. The uteruses of rats administered DES and progesterone only revealed the development of cyst and swelling filled with fluid within the body of the uterus and the uterine horns [Figure 2c]. It also had a faded color and texture different from what was observed in the normal control group. Uterus of the rats pretreated with 200 mg/kg of the extract showed a normal length of the uterus with a thicker texture compared to the normal control group but no obvious swelling [Figure 2d]. Uterus of the rats pretreated with 400 mg/kg of the extract had an obvious cyst and swelling and an asymmetry uterus length compared to the normal control group [Figure 2e]. Treatment with 200 mg/kg of the extract after combined administration of DES and progesterone showed uterus with a normal length, thicker texture with no obvious swelling [Figure 2f] in contrast to what was observed in the group-administered DES and progesterone only but thicker than the normal control group [Figure 2c]. Uterus of the rats treated with 400 mg/kg of the extract had an obvious swelling and cyst, asymmetry uterus length, and a thicker texture compared to the normal control group [Figure 2g]. Uterus of the rats in the self-recovery group showed uterus features similar to what was observed in rats treated with 200 mg/kg of the extract after combined DES and progesterone administration [Figure 2h].

Effect of treatment on lipid profile

The results of the effect of treatment on serum total cholesterol are shown in Figure 3. The value of total cholesterol level observed in the normal control group was 130.20 ± 39.60 mg/dl. This was not significantly different from the values of 76.96 ± 4.48 and 76.55 ± 6.38 mg/dl observed in the rats administered progesterone only (uterine model 1) and those co-administered DES and progesterone (uterine model 2), respectively. Rats pretreated and posttreated with the extract at the tested doses also showed no significant difference in the level of serum total cholesterol when compared with the normal control group. Rats in the self-recovery group had serum total cholesterol level of 76.79 ± 7.38 mg/dl, which was also not significantly different from the observed value in the normal control group.

The results of the effect of treatment on serum TG level revealed that neither the administration of progesterone alone nor co-administration of DES along with progesterone changed the TG level from the control value of 54.92 ± 5.45 mg/dl. Pretreatment with the extract and administration of the extract at all the tested doses after DES and progesterone administration did not also alter the TG level. Comparison with the uterine model 2 (Group 3) also revealed no significant difference with all the experimental groups.

Figure 4 shows the results of the effect of treatment on serum HDL-c. The result showed that the uterine model 1 group had serum HDL level of 37.700 ± 3.560 mg/dl which was not significantly different from what was observed in the normal control

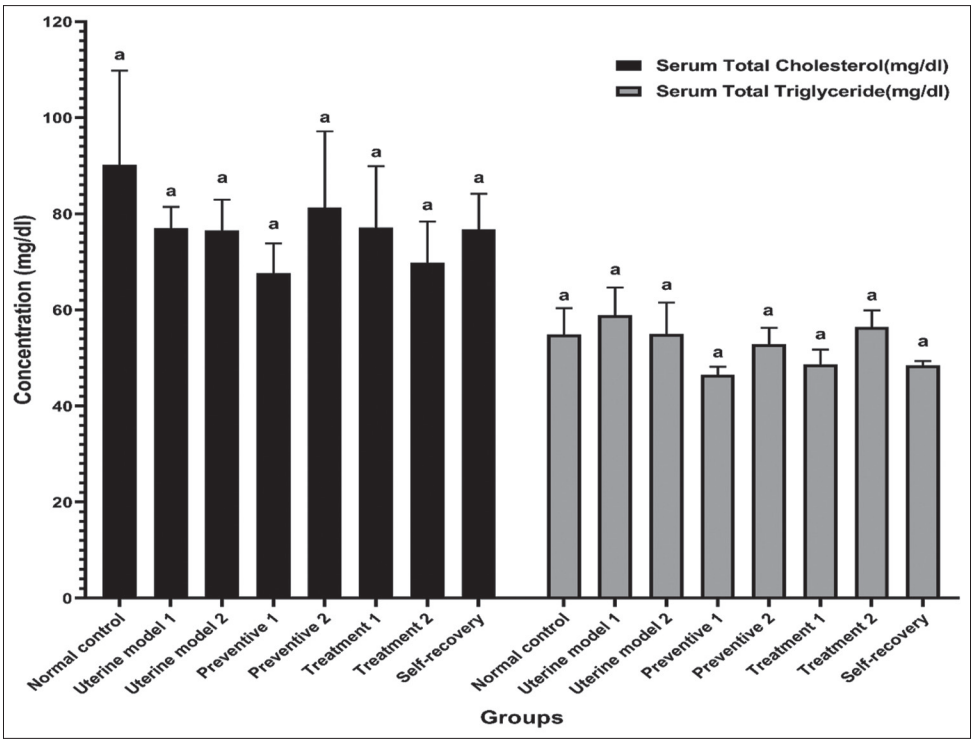


Figure 3: Effect of *Tetracarpidium conophorum* extract on serum total cholesterol and triglyceride level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

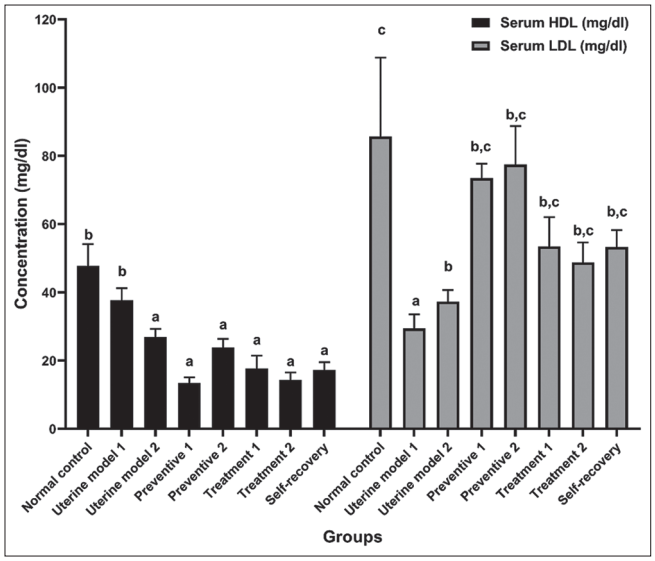


Figure 4: Effect of *Tetracarpidium conophorum* extract on serum high-density lipoprotein and low-density lipoprotein level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

group (47.740 ± 6.400 mg/dl). Co-administration of DES and progesterone (uterine model 2) significantly reduced the level of serum HDL-c (26.870 ± 2.450 mg/dl) when compared to the normal control group. The observed value was however not different from that of rats administered progesterone alone. Rats treated and pretreated with the extracts also showed significant decrease in the level of HDL-c when compared to the value observed in the normal control group. Similarly, rats in the self-recovery group had significantly reduced HDL-c level (17.250 ± 2.310 mg/dl) compared to the normal control group.

The result of the serum LDL-c showed a significant difference ($P < 0.05$) in the level of serum LDL-c of the experimental groups. Further analysis using Bonferroni *post hoc* test for multiple comparison showed that administration of progesterone alone and co-administration of DES and progesterone lowered serum LDL-c significantly to 29.440 ± 4.150 and 37.260 ± 3.450 mg/dl, respectively, compared to the control value of 85.700 ± 23.130 mg/dl. Pretreatment with and postextract treatment showed no significant difference in serum LDL-c level when compared to the normal control value. Similarly, the self-recovery group also shows no significant difference in the serum LDL-c level when compared to the normal control group.

The ANOVA result of the serum coronary risk [Figure 5] showed that there exists a significant difference in the serum coronary risk and atherogenic risk of the experimental groups ($P < 0.05$). Analysis of serum coronary risk and atherogenic risk using Bonferroni *post hoc* test for multiple comparison showed that the administration of progesterone alone significantly lowered the coronary risk index (1.105 ± 0.204) when

compared with the normal control (2.796 ± 0.460). Co-administration of DES and progesterone did not cause any significant change in the coronary index and atherogenic index. Similarly, rats pretreated and posttreated with the extract did not show any significant change in the coronary and atherogenic index compared with the normal control. This was not also different from what was observed in the self-recovery group.

Effect of treatment on hormones

The results of the effect of treatments on serum and uterus hormone level are shown in Figures 6-8. Figure 6 indicates that the serum progesterone of the normal control group (1.252 ± 0.267 ng/ml) was not significantly different from that of the progesterone-administered group and that of rats co-administered DES and progesterone. Pretreatment with the extract at the two tested doses (200 and 400 mg/kg) also did not alter the progesterone level. The observed serum progesterone levels in the two pretreated groups were also not significantly different from that of the self-recovery group. Rats posttreated with 200 and 400 mg/kg of the extract after DES and progesterone co-administration also showed serum progesterone (1.457 ± 0.385 and 1.604 ± 0.988 ng/ml, respectively) comparable to the value observed in the normal control group.

The results of the effect of the treatment on uterus progesterone level showed that the level of progesterone (5.193 ± 0.824 ng/ml) in the progesterone = administered group is significantly higher than that of the control group (2.721 ± 0.522 ng/ml). Co-administration of DES and progesterone also raised the level of uterus progesterone (11.530 ± 3.323 ng/ml) to a value higher than what was observed in the normal control and progesterone-administered group. The progesterone level of the retreated with the extract at the two tested doses compared significantly with each other and with the value observed in the normal control group.

The results of the effect of treatment on serum estradiol are presented in Figure 7. The results showed that the estradiol level of 12.970 ± 1.200 pg/ml observed in the serum of progesterone-administered rat and 14.150 ± 1.760 pg/ml observed in the serum of rats co-administered DES and progesterone were significantly higher than the value observed in the normal control group (10.210 ± 0.230 pg/ml). Rats administered the extract, and the recovery group showed no significant difference in the level of serum estradiol when compared to the control group.

Analysis using Bonferroni test for multiple comparison shows that the uterus estradiol was also significantly raised when the rats administered DES and progesterone were compared with the normal control group. Rats treated with the extract at the two tested doses also showed no significant difference in the value of uterus estradiol when compared between each other and when compared with the normal control value.

The results of the effect of treatment on serum prolactin [Figure 8] showed that there was no significant difference in the level of serum prolactin of the rats administered progesterone only and those co-administered DES and progesterone when compared to the normal control value (2.414 ± 0.285 ng/ml). However, rats pretreated with the extract showed a significantly lower level of serum prolactin when compared to the observed value in the control

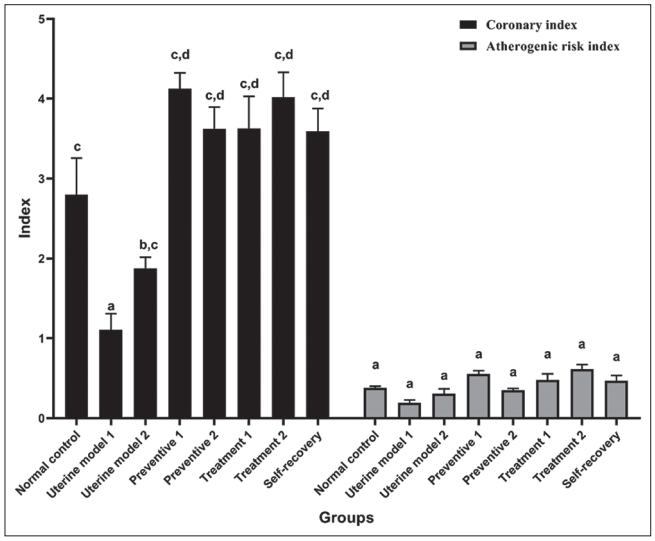


Figure 5: Effect of *Tetracarpidium conophorum* extract on coronary and atherogenic risk indices. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

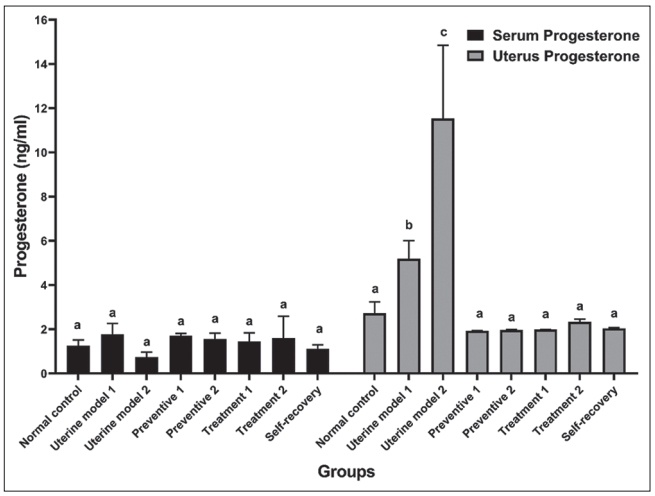


Figure 6: Effect of *Tetracarpidium conophorum* extract on progesterone level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

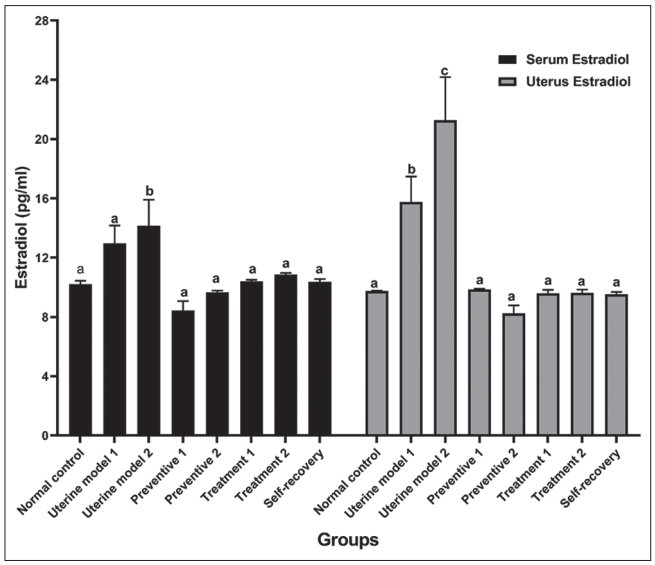


Figure 7: Effect of *Tetracarpidium conophorum* extract on estradiol level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

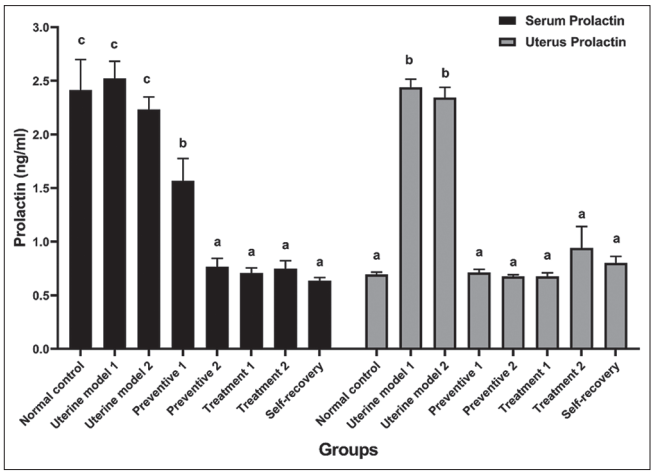


Figure 8: Effect of *Tetracarpidium conophorum* extract on prolactin level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

group (2.414 ± 0.285 ng/ml). Similarly, rats treated with the extract after co-administration of DES and progesterone also showed significantly lower levels of serum prolactin when compared with the normal control group. This was also what was what observed with the recovery group.

The results of the level of prolactin in the uterus revealed that the levels of prolactin in the uterus of rats administered progesterone only (2.439 ± 0.077) and those treated with DES and progesterone (2.344 ± 0.096 ng/ml) were significantly higher than the value observed in the normal control group (0.693 ± 0.024 ng/ml). However, uterus prolactin levels of rats that were pretreated with the extract and those treated with the extract after DES and progesterone administration were not significantly different from the normal control value (0.693 ± 0.024 ng/ml). This was similar to the result observed from comparison of the self-recovery group to the normal control group.

Effect of treatment on Ca^{2+} ATPase activity

Figure 9 is the results of the activities of Ca^{2+} ATPase in the experimental groups. The activity of Ca2+ ATPase in the progesterone-administered group (0.001 ± 0.177 × 10^{-3} µmol Pi/mg protein/h) and the group co-administered DES and progesterone (0.005 ± 0.964 × 10^{-3} µmol Pi/mg protein/h) was significantly lower than the value observed in the normal control group (0.013 ± 0.486 × 10^{-3} µmol Pi/mg protein/h). Rats pretreated with 400 mg/kg of the extract had Ca^{2+} ATPase activity of 0.023 ± 2.323 × 10^{-3} µmol Pi/mg protein/h, which was significantly higher than the value observed in the normal control group. Treatment with the extracts at all the tested doses showed no significant difference in the activity of Ca^{2+} ATPase when compared with the normal control group (0.013 ± 0.486 × 10^{-3} µmol Pi/mg protein/h). This result is similar to what was observed in the rats pretreated with the extract at 200 mg/kg and the self-recovery group.

Discussion

Medicinal plants have played an important role in disease prevention and treatment over the years due to their special attributes as large source of phytochemicals. The extraction and isolation of these phytochemicals from plants, though tedious and time-consuming, provide numerous bioactive compounds which has proven to have positive impact on health and important in the development of novel therapeutic drugs.[40,41]

T. conophorum has been reported to possess numerous bioactive compounds which may account for its reported use in disease treatment and prevention. The leaves of the plant have been reported to be used traditionally for the improvement of fertility and menstrual flow and the seeds for the treatment of fibroid.[29] The yield obtained from the aqueous extract of *T. conophorum* leaves in this study was 10.01% (w/w). Previous study by Akomolafe *et al*.[42] on the yield of *T. conophorum* leaves reported a yield of 25% (w/w) which is higher than the value reported in this study. The observed decreased yield in this study could be as a result of the difference in the extraction technique used.[43]

The weight and uterine coefficient were monitored to evaluate the potential for uterine toxicity. Results of the uterine weight and uterine coefficient obtained in this study revealed that administration of progesterone alone did not produce any significant change in the weight and

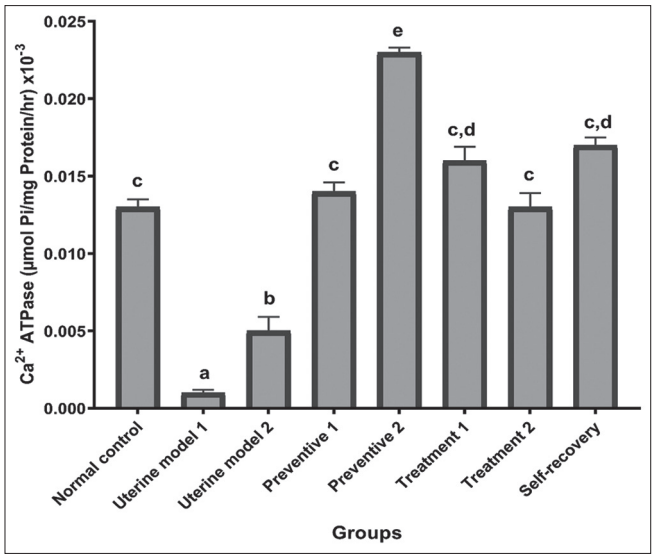


Figure 9: Effect of *Tetracarpidium conophorum* extract on uterus Ca^{2+} ATPase level. Values were expressed as mean ± standard error of mean. Bars with the same letter are not significantly different from each other

coefficient of the uterus. However, co-administration of DES and progesterone resulted in a significant increase in uterine weight and uterine coefficient. This result correlated with the report of Lin *et al*.,[44] who reported that the weight of the uterus and uterus coefficient increased with combined administration of DES and progesterone. This suggests a condition of organ toxicity. This finding is also supported by the observed alterations in the morphology of the uterus. Rats pretreated and posttreated with *T. conorphorum* at 200 mg/kg did not show any significant difference in uterine weight and coefficient when compared to the normal control group.

Gross morphology of the uterus showed increased uterus density, edema, necrosis, hyperplasia, and malformation in rats co-administered DES and progesterone. These changes could be attributed to cellular proliferation induced by prolonged intake of DES. DES changes the transcriptional profile of the uterus so that it responds abnormally to ovarian hormones (estrogen and progesterone) which may ultimately lead to uterine disorders. UF growth and development have been reported to be dependent on these sex steroid hormones.[44] Studies have also reported that DES exposed uterine epithelium resulted in morphological changes of the epithelium from simple columnar to stratified epithelium.[46] Uterus of rats pretreated with the extract showed evidence of hyperplasia but no obvious edema. This is more prominent at dose of 200 mg/kg. This result suggests that *T. conophorum* could have mitigated the edema and reduced the hyperplasic texture of the uterus induced by DES administration. A suggested mechanism for the amelioration of edema in the extract preadministered rats could be similar to that of a class of antiuterine medications, gonadotropin releasing hormone (GnRH) agonists which are known to shrink fibroid and stop heavy bleeding in fibroid patients by blocking the production of the female hormones, estrogen, and progesterone.[47]

The results of the lipid profile revealed that treatments did not cause any significant change in total cholesterol and TG level. This result agrees with the result of Hussam and Zwain,[48] who reported in a study conducted on the relationship between lipid profile and UF among premenopausal women in Iraq; there was no significant difference between fibroid women and controls regarding serum cholesterol, serum TG, and serum LDL-c levels. We also did not observe any alterations in the total cholesterol and TG level in rats administered progesterone and DES. This study however observed that rats co-administered DES and progesterone showed a significantly reduced level of LDL-c. This observed decrease could be attributed to the effect of estrogen in promoting LDL clearance and consequently decreasing LDL-c concentration.[49] Estrogen increases the expression of hepatic LDL receptors and reduces proprotein convertase subtilisin/keexin type 9 levels which leads to the degradation of LDL receptors, thereby increasing the number of hepatic LDL receptors and leading to accelerated clearance of LDL and reduction in plasma LDL levels.[51] The observed serum level of LDL-c in the rats treated with the extract at the two tested doses was not significantly different from the normal control value. This suggests that *T. conophorum* extract could mitigate and prevent alterations in LDL-c in UF disorder.

Co-administration of progesterone and DES resulted in significantly reduced level of serum HDL-c and treatment with *T. conophorum* extract at the two tested doses was not seen to mitigate this decrease. Administration of progesterone only, however, did not result in any significant change in the serum HDL-c level. This result agrees with the previous report of Yamagushi *et al*.,[51] who showed that DES administration caused a reduction in HDL-c due to the inhibition of apolipoprotein E (APOE) secretion from the liver. APOE plays an important role in cholesterol and TG homeostasis by serving as a receptor-binding ligand that mediates the clearance of dietary chylomicrons and hepatically derived very low-density lipoprotein (VLDL) and their remnant from the circulation.[52] It activates enzymes involved in lipoprotein metabolism such as hepatic lipase, cholesteryl ester transfer protein, and lecithin-cholesterol acyltransferase. Hence, it enhances phospholipid hydrolysis in HDL, lipid exchange

between VLDL and HDL, and conversion of discoidal to spherical mature HDL particles.[53] The present study affirms that the administration of DES to adult female rats altered this role performed by APOE.

The atherogenic index of the blood is an important index composed of TGs and HDL-c.[54] This index has been used to quantify blood lipids, as an indicator of dyslipidemia and its associated diseases such as cardiovascular diseases[15,55] and as a stand-alone index for cardiac risk estimation.[56] The result of the coronary and atherogenic risk index revealed that co-administration of DES and progesterone did not affect the coronary and atherogenic index. Treatment with the extract was also not observed to alter the coronary and atherogenic index. Our study suggests that neither fibroid disorder may predispose to cardiovascular diseases associated with dyslipidemia nor treatment of the disorder with the extract may also lead to cardiovascular diseases.

The results obtained from the analysis of estradiol in this study revealed that UF disorder may alter the level of serum and uterus estradiol. This result is in consonance with what was observed by Zhao *et al*.[34] Several studies have reported estradiol to be an important hormone necessary for fibroid growth and development. Estrogen receptors (ERs) have also been reported to be increased in fibroid tissue compared to the normal myometrium.[45,57] DES has a binding affinity for the ER that is much higher than that of the naturally occurring estrogen 17-estradiol,[58] suggesting that DES functions as a strong estrogen. Estradiol is produced when aromatase enzyme converts androgens to estrogens. Pretreatment with the extract significantly lowered the level of serum estradiol compared to the level observed in the model group. Although the mechanism of this reduction is not yet known, it could be suggested that the extract acted as an aromatase inhibitor which exert its effect by inhibiting the conversion of androgens into estrogens.[59] The inhibition of aromatase enzyme has been reported to be a potent mechanism in hormone-dependent fibroid growth,[8,60] suggesting that the extract can be effective in preventing the rise in estradiol level associated with fibroid, thus serving as a preventive treatment measure to fibroid growth and development.

Progesterone is an endogenous steroid hormone known to play a role in the coordination of the female reproductive physiology.[61] It is secreted by the corpus luteum which develops in the ovary after ovulation. Progesterone affects multiple tissues and organs including the breast, uterus, and ovary.[62] Clinical trials on animal models have reported that progesterone and progesterone receptor (PR) promote the growth and development of UF and breast cancer.[9,63] The physiological actions of progesterone are mediated by its interaction with PR.[64] The ligand-occupied PR binds to DNA[65] and recruits coregulatory proteins that activates or represses transcription via interaction with the general transcription apparatus.[66] The present study revealed that although induction of fibroid may not cause any significant change in the level of serum progesterone, it may significantly raise the level of uterus progesterone. This finding is in consonance with the result of Zhao *et al*.,[34] who also reported an increase in the level of uterus progesterone in rats administered DES and progesterone. Similar finding was also reported in the uterus of human fibroid.[8] The increase in uterus progesterone level could be attributed to the role of estradiol in inducing the production of PRs, which in turn lead to the response of the uterine tissues to progesterone secreted by the ovaries.[67] Progesterone can promote the growth of UF by upregulating endothelia growth factor, transforming growth factor (TGF) beta 1, and TGF-beta 3 and promote their survival through the upregulation of Bcl-2 expression and downregulation of tumor necrosis factor-alpha.[68] It is therefore possible to say here that the efficacy of co-administration of DES and progesterone in inducing UF may be partly due to the progesterone effect on the growth factor.

DES is a synthetic estrogen which acts in similar manner as the endogenous estrogen. Ishikawa *et al*.[9] and Kim *et al*.[69] reported that estrogen plays a permissive role by enhancing PR expression to augment progesterone action in the process of tumorigenesis. Although the detailed mechanism of action of DES is not well understood, DES has been reported to mediate its action by binding to the ERs and ER alpha plays a role in mediating DES-induced toxicity and phenotypic changes in the reproductive tract of female rats.[70]

It is important to note that rats pretreated with the *T. conophorum* extract at the two tested doses had serum and uterus progesterone level not significantly different from that of the normal control group. This shows the efficacy of *T. conophorum* leaves in stalling progesterone increase during UF development. The observed decrease in the level of uterus progesterone in the treated groups indicates the efficacy of *T. conophorum* leaves extract to reduce progesterone level in patients with UF condition. The exact mechanism as to how the extract prevented progesterone increase is not known, but it could be suggested that the extract has acted as a GnRH agonist which triggers a temporary menopause with amenorrhea by means of reduction in progesterone and estrogen levels, which eventually leads to the reduction in the size of the fibroids.[71]

Prolactin is a hormone secreted not only by the anterior pituitary gland but also from the hypothalamus, placenta, and uterine muscle. It is known to mediate its function

by interacting with Type-1 cytokine receptors and signals through Janus kinase signal transducers and activators of transcription pathways.[72] Although isolated as a pituitary hormone, it is expressed in other tissues including UF.[73] Although UF has been rarely reported to play a role in hyperprolactinemia, cultured UF cells and cell lines derived from UF muscle cells have been reported to produce prolactin mRNA and to secrete immunoreactive prolactin into culture medium with the secreted prolactin similar to that of the pituitary gland.[74,75] A report by Sachdev *et al*.[76] showed that the increased level of ectopic prolactin in UF patients was corrected by the excision of UFs and immunostaining done on the fibroid showed that the fibroid was responsible for the hyperprolactinemia. This study showed that the levels of serum prolactin in the group administered progesterone only and the group co-administered DES and progesterone were not significantly different from what was observed in the control group, whereas increases were observed in the uterus prolactin level. The increase observed could be attributed to the ability of fibroid cells to secrete prolactin. The mechanisms suggested to be associated with this secretion are the activation of the genome expression for prolactin synthesis in the myometrial smooth muscle cell during the transformation of normal myometrial cells to fibroid cells.[73] Treatment with *T. conophorum* leaves extract significantly reduced the level of prolactin both in the serum and uterus when compared to the observed value in the UF condition. Reduction in the level of serum and uterus prolactin by the extract suggests that this extract could play a role in suppressing prolactin production and consequently hindering tumorigenesis influenced by increased prolactin production. The result of this study shows the ability of *T. conophorum* leaves extract to reduce serum prolactin level that may be elevated during UF condition.

Ca^{2+} ATPases are proteins involved in regulating local intracellular Ca^{2+} dynamics and maintaining Ca^{2+} homeostasis by mediating Ca^{2+} extrusion from the cell.[76] One molecule of ATP is hydrolyzed for every Ca^{2+} ion transported with concomitant release of energy.[77] Specific calcium channel proteins have been reported to be significantly expressed in fibroids and the surrounding smooth muscles and their modification affect proliferation rate of UF.[78] Ca^{2+} ATPase dysfunction in the sarcoplasmic reticulum leads to the elevation of cytoplasmic calcium which triggers ER stress,[79] a condition reported to play a role in the UFs.[80] The results of the present study revealed that administration of progesterone alone and the combined administration of DES and progesterone significantly reduced the activity of Ca^{2+} ATPase in the uterus of the rats. DES has been reported to be a potent inhibitory agent of Ca^{2+} ATPase activity in the sarcoplasmic reticulum, the hydroxyl groups at the para positions of the two benzene rings of DES has the capacity to develop an inhibitory effect on the Ca^{2+} ATPase protein such that an increase in the concentrations of DES caused a decrease in the affinity and the total calcium binding to Ca^{2+} ATPase.[81] It has been reported that progesterone did not affect either C^{2+} uptake or ATPase activity in the human myometrial mitochondria but DES inhibited Ca^{2+} uptake by causing a release of Ca^{2+} stored by the mitochondrial.[68,82] This suggests that the reduced activity of Ca^{2+} ATPase observed in this study could be attributed to the role of the DES in decreasing the affinity of calcium binding to Ca^{2+} ATPase. Rats in which fibroid was induced and thereafter treated with the extract at the two tested doses did not show any significant change in the activity of Ca^{2+} ATPase when compared with the normal control. This suggests that the extract was able to reverse the decreased Ca^{2+} ATPase activity induced by co-administration of DES and progesterone.

Conclusion

Findings from this study have shown that *T. conophorum* leaf extract contains important phytochemical constituents with antiproliferative proprieties. The study also confirmed that co-administration of DES and progesterone was able to induce UF condition. Treatment with *T. conophorum* extract was shown to be able to mitigate the alterations induced by DES and progesterone co-administration. The most remarkable morphological and biochemical changes in the uterus were observed at 200 mg/kg. Hence, administration of *T. conophorum* extract at this dose could serve as a feasible, inexpensive, and effective option for UF treatment and may offer possible alternative to surgery.

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Conflicts of interest

There are no conflicts of interest.

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