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Effect of phytoestrogen-rich fraction of *Millettia aboensis* on lipid profile, oxidative stress, and platelet count in ovariectomized rat model of menopause

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ABSTRACT

Milletia aboensis has long-standing ethnopharmacological indications for the treatment of symptoms and diseases related to menopause. This investigation examined the effects of its phytoestrogen-rich fraction on some predictors of cardiovascular risk in an ovariectomized rat model of menopause. In vitro, antioxidant activity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. Ovariectomy was used to trigger menopause by surgically removing the ovaries under anesthesia. After recovery, the animals were treated orally with the phytoestrogen-rich fraction (PERF) of *M. aboensis* daily for 30 days. Blood samples were obtained for the estimation of serum lipid profile, antioxidant enzyme activities, lipid peroxidation, and platelet count. The PERF showed the strongest inhibition of DPPH radical with an IC_{50} value of 19.65 µg/ml. At 200 and 400 mg/kg, the PERF dose-dependently showed significant increase in high-density lipoprotein (HDL) (42.86 and 43.05 mg/dl) and a significant ($p < 0.05$) decrease in low-density lipoprotein (LDL) levels (3.78 and 1.24 mg/dl) compared with the ovariectomized (OVX) control (31.82 and 20.32 mg/dl, respectively). Being dose-dependently, the PERF increased catalase (CAT) and superoxide dismutase (SOD) enzyme activities as well as significantly reduced malondialdehyde (MDA) levels compared to OVX control. At 200 and 400 mg/kg, the PERF restored platelet count with values (316.54 and 343.3 103/IU, respectively) close to that of sham-operated control (365.17 103/IU). The ability of *M. aboensis* to reverse lipid abnormalities associated with ovariectomy coupled with its reduced platelet count, and antioxidant effects make it a potential therapeutic phytoestrogen remedy for the management of cardiovascular complications associated with estrogen deficiency.

1. Introduction

Cardiovascular disease is a leading cause of death in both men and women (Thomas, 2020). Dyslipidemia is characterized by an increase in low-density lipoprotein cholesterol and serum total cholesterol or triglyceride and reduced serum high-density lipoprotein cholesterol concentrations are routinely determined to estimate cardiovascular risk (Hedayatnia et al., 2020). Primary and secondary cardiovascular events such as atherosclerosis, myocardial infarction, ischemic stroke, and coronary death are prevented by proper management of dyslipidemia (Phan & Toth, 2014). Similarly, abnormalities in the oxidative and antioxidant status causing oxidative stress were found to contribute to atherosclerosis and cardiovascular

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death (Tan et al., 2018). Dyslipidemia in combination with endothelial damage due to oxidative stress has also been established to be a critical stage in the majority of pathogenic processes underlying cardiovascular diseases (Senoner & Dichtl, 2019).

Premenopausal women have a much lower incidence and prevalence of heart disease compared to age-matched men (Rodgers et al., 2019). However, this sex difference in favor of women gradually reverses after menopause with older women being at higher risk of cardiovascular diseases and death than men of the same age (Merz & Cheng, 2016). Multiple lines of evidence from both observational and experimental research have indicated that reduced levels of ovarian hormones constitute a crucial risk element for the emergence of cardiovascular diseases (dos Santos et al., 2014; Maric-Bilkan et al., 2014; Rodgers et al., 2019). These evidences explain the loss of cardiovascular protection in postmenopausal women.

To compensate for the loss of ovarian estrogen at menopause and its associated symptoms and diseases including cardiovascular diseases, postmenopausal women are usually administered hormone replacement therapy (HRT). Unfortunately, the therapeutic potentials of unopposed estrogen-based hormone replacement therapy are impeded by its effects on the reproductive systems, which in turn results in negative consequences such as endometrial hyperplasia and breast cancer (Valdes & Bajaj, 2021). Progesterone can be added to help with these negative effects, but doing so will reduce estrogen's ability to lower cholesterol. The tendency of estrogen to increase coagulability further questions its overall benefit in the management of menopause-associated cardiovascular problems (Bacon, 2021; Jiang & Tian, 2017).

Considering the hazards that have been established for conventional HRT, naturally occurring plant estrogens (phytoestrogens) have been shown in numerous studies to be an effective alternative treatment for postmenopausal women at risk of cardiovascular diseases and other menopausal symptoms and diseases (Poluzzi et al., 2014) (Poluzzi et al., 2014). *Milletia aboensis* is one of such plants with long-standing ethnopharmacological indications for the treatment of symptoms and diseases related to menopause. We have previously reported the phytoestrogen quantification of its root extract as well as the characterization of its abundant phytoestrogens (Anwuchaepe et al., 2019). Its estrogenic effective and toxic doses have also been previously reported in our earlier study (Anwuchaepe et al., 2019). We have equally reported its osteoprotective effect in ovariectomy-induced osteoporosis (Ajaghaku et al., 2021a). To explore further beneficial effects of this plant in other menopause-associated disease conditions, this study examined the effects of a phytoestrogen-rich fraction of *M. aboensis* on some predictors of cardiovascular risk in an ovariectomized rat model of menopause.

2. Materials and methods

2.1. Plant material

Roots of *M. aboensis* were collected from Anambra State (Nigeria). They were identified by a senior taxonomist Mr. Felix Nwafor of the Department of Pharmacognosy and Environmental Medicine, University of Nigeria, Nsukka. A voucher specimen was deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka with herbarium specimen number PCG 474/A/021. *M. aboensis* roots were cut into small pieces, air-dried at room temperature, and then ground.

2.2. Animals

Swiss female Albino rats (150–200 g) used for the study were acquired from the Animal House of the Department of Pharmacology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu State, Nigeria. All animal experiments were conducted in compliance with the 2010/63/EU Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU) and approved by the institution's animal ethical committee (NAU/FPS/PHAT/016-19).

2.3. Extraction and fractionation

Extraction and fractionation were carried out as previously reported (Ajaghaku et al., 2021a, 2021b). Using a soxhlet extractor, methanol was used to extract the powdered *M. aboensis* root, this was filtered and concentrated in vacuo using a rotary evaporator (RE300 Model, United Kingdom) at 50 °C. An aqueous solution of the extract (70 g in 200 ml of water) was subjected to liquid–liquid partitioning successively with 1000 ml of *n*-hexane, ethyl acetate, and butanol in ascending order of polarity. The fractions were filtered and concentrated in a vacuo using a rotary evaporator to obtain fractions soluble in these solvents.

2.4. DPPH activity test

The free radical scavenging activity of the plant extracts and fractions was evaluated using the method of Ajaghaku et al. (2017).

2.5. High-performance liquid chromatography (HPLC) analysis

Dereplication studies on ethyl acetate fraction (phytoestrogen-rich fraction) chromatographic sub-fractions were carried out using HPLC-DAD and HPLC-ESI-MS analyses for the detection and identification of the phytoestrogens following previously reported protocols by Ajaghaku et al. (2021a).

2.6. Experimental design

Thirty female rats, three months old, were used in this study. Ovariectomy was used to induce menopause by surgical removal of the ovaries under anesthesia as described by Shailajan et al. (2016). After 3 weeks of recovery, the animals were separated into 5 groups of 6 animals each and treated orally with the phytoestrogen-rich fraction (PERF) of *M. aboensis* daily for 30 days. Group 1 received 5 ml/kg of distilled water (negative control); Group 2 received 1 mg/kg of estradiol valerate (positive control); Group 3 received 5 ml/kg of distilled water (sham-operated control); Groups 4 and 5 received 200 and 400 mg/kg of the phytoestrogen-rich fraction, respectively. Dose selection was based on our previous study on the estrogenic effective dose (ED_{50}) of the root extract of *M. aboensis* (Ajaghaku et al., 2021a). On the 31st day of treatment, blood samples were collected from all the animals through retro-orbital puncture for the estimation of serum lipid profile, antioxidant activities, and platelet count.

2.6.1. Determination of in vivo anti-lipidemic and antioxidant activities

Serum high-density lipoproteins, triglycerides, and total cholesterol were assayed using standard lipid assay kits (Fortress Diagnostics Ltd, Antrim, UK) as described by Mbagwu et al. (2020). The LDL

concentrations were derived from the formula proposed by Friedewald et al. (1972).

$$LDL\ Conc. = Total\ cholesterol\ conc. - \frac{Triglyceride\ conc.}{5} - HDL\ cholesterol\ conc.$$

Serum catalase activity was estimated by the visible light method as described by Weydert and Cullen (2010) using a catalase assay kit (Elabscience Biotechnology Co. Ltd., China) while serum superoxide dismutase activity (SOD) was estimated by hydroxylamine method as described by Weydert and Cullen (2010) using SOD assay kit (Elabscience Biotechnology Co. Ltd., China).

2.6.2. Quantitative determination of platelet count

Platelet count was determined by the Rees and Ecker method of direct counting of platelets as described by Brown (1976). A 4 ml of the platelet diluting fluid was added to a clean test tube using an automatic pipette. To the 4 ml, platelet diluting fluid, 0.02 ml of blood samples were then added to make a 1:200 dilution. The diluted blood was shaken gently for 3-5 min. The Neubauer chamber was thoroughly cleaned and then filled with diluted blood. The filled Neubauer chamber was placed in a moist chamber created using a moist filter paper and petri dish and allowed to stay there for 15 min to permit the platelets to settle. The moist chamber prevents evaporation of the fluid in the counting chamber. After 15 min the Neubauer chamber was placed on the microscope stage and the platelets were counted at the high dry objective (X45). Five primary squares were counted on the red cell counting area of the Neubauer chamber – 4 from the 4 edges of the Neubauer chamber and one from the center. The platelets appeared as light bluish-coloured, round, oval, or elongated refractile particles which are much smaller than the red blood cells. The total blood platelet counting was calculated by multiplying the total number counted on the 5 squares by 10.000 to give the number of platelets per microlitre (µl) of blood.

2.7. Statistical analyses

Data obtained were expressed as mean ± SEM and analyzed by the Kruskal-Wallis ANOVA test. The differences between the various treatments were analyzed with multiple comparisons of mean ranks for all groups. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance. Statistical analysis was performed using SPSS 20 software, while a graphical plot was performed using Microsoft Excel 2010.

3. Results and discussion

3.1. Phytochemical analysis

Phytochemical analysis of the extract and fractions of *M. aboensis* are shown in Table 1. *M. aboensis* extract revealed the presence of all the tested phytochemicals. Phytochemical analysis of *M. aboensis* root revealed the presence of flavonoids and other phenolic constituents like tannins and anthraquinones in the plant extracts and ethyl acetate fractions. Phenolic compounds generally possess numerous biological activities one of which is cardioprotective activity (Altemimi et al., 2017; Grippo et al., 2007). Several flavonoids (e.g. isoflavones) are phytoestrogens based on their similarity to estrogens suggesting their potential cardiovascular benefits (Nestel, 2004). The presence of these aforementioned phytochemicals bestows high medicinal importance to *M. aboensis*, thus, is responsible for the observed cardioprotective potential of this plant.

Table 1. Phytochemical constituents of methanol extract and fractions of *M. aboensis* root

Phytochemical	Methanol Extract	*n*-Hexane fraction	Ethyl acetate fraction	Butanol fraction	Water fraction
Alkaloids	++	++	-	-	-
Tannins	++++	-	++++	++++	++++
Flavonoids	+++	+	+++	++	++
Steroids	++	+	-	+	+
Terpenoids	+++	+	++	++	++
Cardiac glycosides	++	-	+	+	+
Saponins	+	-	+	+	+
Anthraquinones	+	-	+	+	+

Key: ++++ = Abundantly present, +++ = Present in high concentration, ++ = Present in moderately high concentration, + = Present in small concentration, – = Not present

3.2. Antioxidant activity

3.2.1. In vitro antioxidant activity (DPPH) of extract and fractions and in vivo antioxidant activity of the PERF

In this study, the extract and fractions of *M. aboensis* produced a concentration-dependent inhibition of DPPH radical (Figure 1). Among the fractions, the ethyl acetate fraction showed the strongest inhibition of this radical with IC_{50} (concentration that showed 50% inhibition of the DPPH radical) values of 19.65 µg/ml (Figure 1). The extract and other fractions showed IC_{50} values greater than 100 µg/ml. Ovariectomy induced oxidative stress in the animals which manifested as a significant decrease in the activities of antioxidant enzymes [catalase (CAT) and superoxide dismutase (SOD) enzymes] ($p < 0.05$) and increase in lipid peroxidation shown by significant increase in malondialdehyde (MDA) level in the OVX animals compared to the sham-operated control ($p < 0.05$) (Figure 2). Supplementation with different doses of the phytoestrogen-rich fraction (PERF) significantly increased serum CAT and SOD activities (Figure 2A and 2B, respectively) as well as decreased MDA level (Figure 2C) in a dose-dependent manner. However, there was a non-significant increase in SOD activity at 200 mg/kg ($p > 0.05$). At 400 mg/kg, the PERF showed a non-significant increase in CAT (48.67 IU/ml) and SOD (69.43 IU/ml) activities as well as a reduction in MDA (6.98 nMol/ml) level compared to estradiol control (40.74, 65.66 IU/ml and 7.82 nMol/ml, respectively). The oxidative state of postmenopausal women and the development of metabolic illnesses such as osteoporosis, cardiovascular disease, and liver and kidney diseases are well documented (Doshi & Agarwal, 2013). Similarly, ovariectomy in rats produces long-term negative effects on several organs like the kidney and liver due to estrogen deficiency-induced oxidative stress (Kasımay et al., 2009). Estrogen deficiency has been linked to a rise in cytokine production in peripheral blood mononuclear cells which also contributes to increased oxidative stress associated with ovariectomy (Kim et al., 2012). Apart from estrogenic activity, phytoestrogens like isoflavones exhibit considerable antioxidant activity which in most cases is independent of their estrogenic properties (Krizova et al.,

2019). In this study, *M. aboensis* produced antioxidant activity both in vitro and in vivo. These activities are in tandem with its total phenolic content and phytoestrogen content reported in our previously published study on this plant (Ajaghaku et al., 2021a), in this study, the ethyl acetate fraction of *M. aboensis* showed the highest phenolic and phytoestrogen contents. These phytoestrogens include but are not limited to genistein, daidzein, isoprunetin, and 9-alpha-OH-pinoresinol.

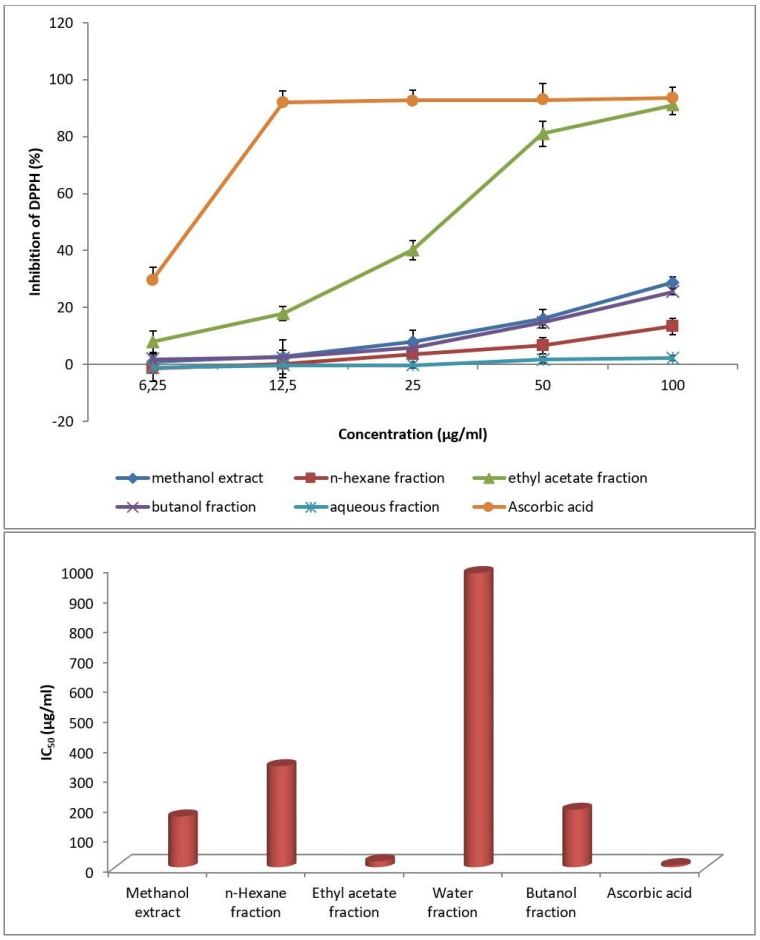

Figure 1. Concentration-response curve of inhibition of DPPH by *M. aboensis* and median inhibition concentration (IC_{50}) of DPPH radical by *M. aboensis* extract and fractions

The antioxidant activity of isoflavone has been proven both in vitro and in vivo (Krizova et al., 2019). For example, increased levels of antioxidant enzymes were described in the mouse epidermis and small intestine following genistein administration (Amigo-Benavent et al., 2008) and in rats following isoflavone administration (Yoon & Park, 2014). The positive influence of isoflavones on the antioxidant system has been also described in several in vivo human studies (Krizova et al., 2019). The ability of the antioxidant molecules to donate electrons is one of the most significant elements impacting antioxidant capability (Huyut et al., 2017). This potency is mostly demonstrated by phenolic compounds (Huyut et al., 2017). The antioxidant activity of *M aboensis* might have been contributed in part by its electron-donating and reducing abilities as demonstrated by the DPPH and FRAP activities, respectively. These properties might also explain the inhibition of lipid peroxidation by *M. aboensis*.

In the defense against oxidative stress, the antioxidant enzyme system of cells plays an important role (Kurutas, 2015). Phytoestrogens like daidzein have been known to increase catalase mRNA through transcriptional activation of the catalase promoter (Sun et al., 2016). It has been demonstrated that genistein reduces the generation of hydrogen peroxide and boosts the activity of antioxidant enzymes like catalase and SOD (Mittal et al., 2014). The ability of *M. aboensis* to produce an increase in these antioxidant enzyme activities may have been mediated through the induction of transcriptional activation of antioxidant enzymes' mRNA production.

3.3. Anti-lipidemic activity

3.3.1. Effect of the PERF on total cholesterol, triglycerides, LDL and HDL

Ovariectomy caused a non-significant increase in the serum total cholesterol (TC) ($p > 0.05$) **(Figure 3A)** and triglyceride (TG) levels **(Figure 3B)**, a substantial decrease in high-density lipoproteins (HDL) **(Figure 3C)** and a significant increase in low-density lipoproteins (LDL) ($p < 0.05$) **(Figure 3D)** compared with the sham-operated control. Administration of the PERF attenuated the increase in TC and TG though not significantly ($p > 0.05$). The PERF at 200 and 400 mg/kg, dose-dependently induced a significant increase in HDL and caused a significant decrease in LDL levels ($p < 0.05$) compared with the OVX control. There were no significant changes in HDL and LDL levels in the PERF-treated groups ($p > 0.05$) compared with estradiol and sham-operated control. Cardiovascular defects are among the major diseases associated with menopause (Ko & Kim, 2020). In postmenopausal women, significant changes in lipoprotein

metabolism are linked to estrogen deficiency. (Ko & Kim, 2020). Even though there are many risk factors associated with cardiovascular diseases, the main factors are lipid abnormalities (Upadhyay, 2015). Low-density lipoproteins (LDL) penetrate the blood vessel walls, where they are oxidized by free radicals. LDL then accumulates and plugs into the blood vessels, thereby causing thrombosis. Platelets express surface receptors that influence platelet-platelet and platelet-vessel wall interactions and provide the membrane surface necessary for the production of thrombin (Periayah et al., 2017).

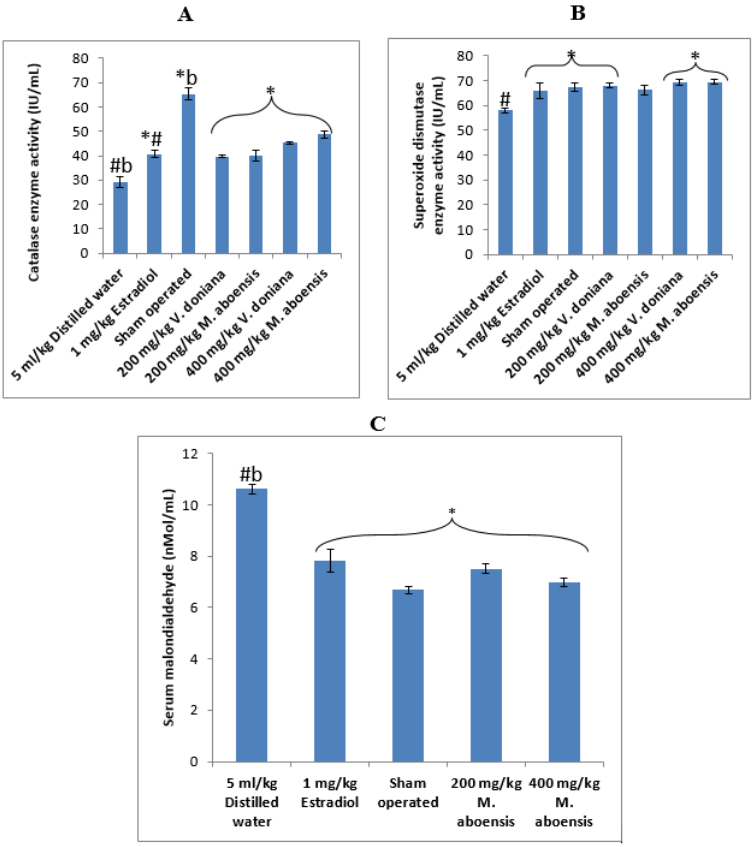

Figure 2. Effect of ethyl acetate fraction of *M. aboensis* on redox predictors

Serum catalase enzyme activity (A), serum superoxide dismutase enzyme activity (B), and serum malondialdehyde level (C)

* $p < 0.05$ compared to vehicle control, # $p < 0.05$ compared to sham-operated, b $p < 0.05$ compared to estradiol

The best-documented effect of isoflavones is on plasma concentrations of lipids and lipoproteins, with a decline in LDL cholesterol and an increase in HDL cholesterol (Ramdath et al., 2017). These effects on plasma lipids have been connected with vascular effects, like enhanced flow-mediated arterial dilation and systemic arterial compliance (Ramdath et al., 2017).

3.4. Effect of the PERF on platelet count

The effect of ovariectomy on platelet count is presented in the Figure 4. Ovariectomy significantly increased platelet count in OVX control ($p < 0.05$) compared with the sham-operated control however, the PERF at 200 and 400 mg/kg restored platelet count with values close to that of sham-operated control. There was no significant reduction in platelet count in the estradiol control group ($p > 0.05$) compared to OVX control. When compared with the sham-operated control group, estradiol control significantly increased platelet count ($p < 0.05$). In addition to effects on lipids, isoflavones like genistein prevents coagulation, a crucial factor in plaque development through inhibition of platelet aggregation and its growth factors, such as platelet-derived growth factor, which then influences thrombin production (Sargeant et al., 1993). The ability of *M. aboensis* to reverse lipid abnormalities associated with ovariectomy coupled with its reduced platelet count, and antioxidant effects make them a potential therapeutic phytoestrogen remedy for the management of cardiovascular complications associated with estrogen deficiency. Their lipid-lowering effect may be mediated through decreased lipogenic gene expression (acetyl-CoA carboxylase, fatty acid synthase, and glycerol-3-phosphate acetyltransferase) whose expression has been documented by other studies to be increased in estrogen-depleted mice (Fu et al., 2016). Findings from this study are in agreement with other studies which have demonstrated the antilipidemic, antioxidant and antithrombotic activities of plant estrogens (Gencel et al., 2012; Terzic et al., 2012; Wu et al., 2009), in fact, Wu et al. (2009) reported that the antioxidant activity of the ethyl acetate fraction of *Cajanus cajan* showed greater activity than the main phytoestrogen compounds of this plant in both DPPH radical-scavenging assay and a β-carotene-linoleic acid test systems, such results might be attributed to the synergistic effects of the components of the plant.

4. Conclusions

Findings from this study revealed that *M. aboensis* has a beneficial effect on lipid metabolism in the ovariactomized model of menopause. Its additional antioxidant and reduced platelet count effects make them a potential therapeutic phytoestrogen remedy for the management of oxidative stress and thrombocytosis associated with menopause.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

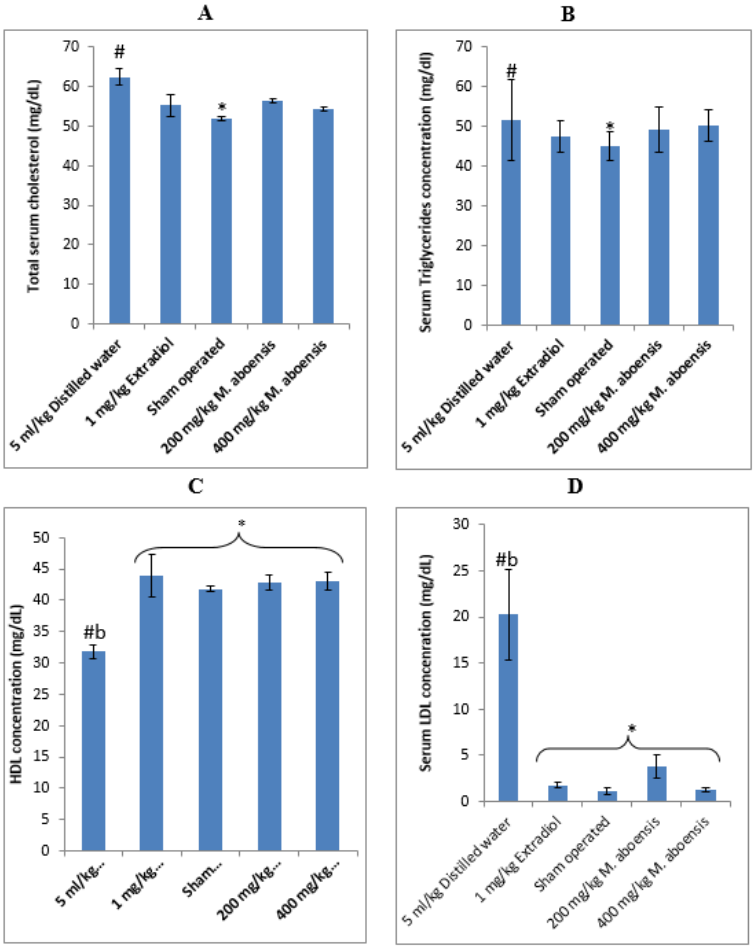

Figure 3. Effect of ethyl acetate fraction of *M. aboensis* on lipid profile
Serum total cholesterol level (A), serum triglycerides level (B), serum HDL level (C) and serum LDL level (D)
* $p < 0.05$ compared to vehicle control, # $p < 0.05$ compared to sham-operated, [b] $p < 0.05$ compared to estradiol

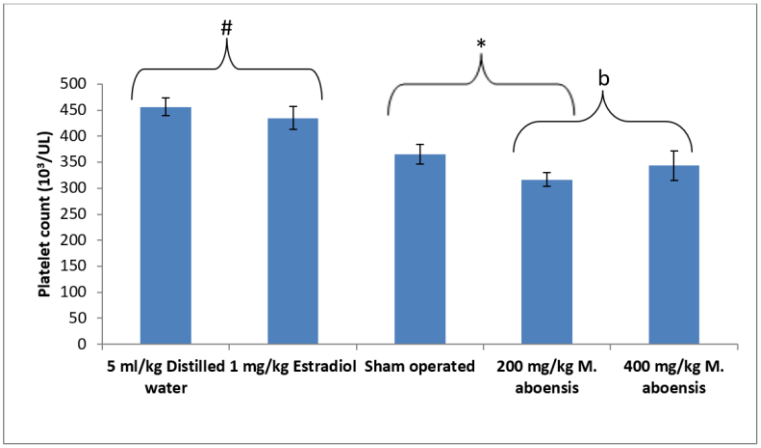

Figure 4. Effect of ethyl acetate fraction of *M. aboensis* on platelet count
* $p < 0.05$ compared to vehicle control; # $p < 0.05$ compared to sham-operated, [b] $p < 0.05$ compared to estradiol

Statement of ethics

All animal experiments were conducted in compliance with the 2010/63/EU Directive on the protection of animals used for scientific purposes and approved by the institution animal ethical committee (NAU/FPS/PHAT/016-19).

Availability of data and materials

All data generated or analyzed during this study are included in this published article. Additional information can be supplied by the corresponding author on request.

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CRediT authorship contribution statement

Amara Anwuchaepe Ajaghaku: Conceptualization, Data curation, Investigation
Daniel Lotanna Ajaghaku: Methodology, Editing original draft, Data analysis
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Supplementary File

None.

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