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Osteoprotective Effect of Phytoestrogen Rich Fraction of Ochna schweinfurthiana Stembark Extract on Ovariectomized Animal Model of Menopause

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Abstract
Osteoporosis is one of the major disease conditions associated with estrogen deficiency in postmenopausal women. This study evaluated the osteoprotective effect of phytoestrogen rich fraction (PERF) of *O. schweinfurthiana* back extract on ovariectomized animal model of menopause. The methanol plant extract was partitioned into n-hexane, ethyl acetate, butanol and water fraction using liquid-liquid fractionation. The phytoestrogens in the fractions were quantified using genistein as standard. Ovariectomy induced osteoporosis was achieved by surgical removal of the ovaries. Body weight changes, relative uterine weight, serum estradiol, bone turnover markers (Alkaline phosphatase - ALP and Acid Phosphatase – ACP), bone mineral content and density as well as lipid peroxidation assays were used to establish ability of ovariectomy to induce osteoporosis and dose response effect of the PERF. Water fraction showed the highest content of phytoestrogen which was more than 10 fold higher than the content of other fractions. Ovariectomy induced weight gain was significantly ($P<0.05$) lowered by PERF compared to vehicle control. Significant ($P<0.05$) increase in serum estradiol following treatment with PERF produced corresponding uterotrophic effect with median effective dose (ED50) of 197 mg/kg. osteoprotective effect of PERF was evident from the significant ($P<0.05$) increases in both bone mineral content and density which corresponded with reduction in bone turnover markers. Ovariectomy induced oxidative stress was reduced by PERF with ED50 of 716 mg/kg. *O. schweinfurthiana* exhibited osteoprotective effect that may have been mediated by downstream signaling effects through estrogen receptor.
Keywords: Osteoporosis; Menopause; Phytoestrogen; bone turnover
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1. Introduction

Osteoporosis is a systemic skeletal disorder characterized by low bone mass and microarchitectural deterioration of bone tissue predisposing to an increased risk of fracture (Sozen et al., 2017). Due to its prevalence worldwide, osteoporosis and associated fractures are significant public health issues that are expected to become more important as the population ages (Cauley, 2013). It afflicts an estimated 200 million people globally, placing a heavy burden on financial and health-care resources (Hilliard, 2016). Gender and age are important risk factors for osteoporotic fractures. Of the millions of people with osteoporosis, two-third are women mostly at their postmenopausal stage (Alswat, 2017). An osteoporotic hip fracture can result in up to 10-20% excess mortality within 1 year (Glinkowski et al., 2019). At least one in two of all women with hip fractures spends some time in a nursing home; one in five requires long-term nursing care (Rockville, 2004). Osteoporotic fractures can also result in chronic pain, disability, deformity, and/or depression with huge economic consequences (Rockville, 2004).

Hormonal alterations (particularly decline in ovarian estrogen production) that occur during menopause have been traced to trigger several alterations in the body with bothersome symptoms such as hot flashes, night sweats, vaginal atrophy and dryness, sleep disturbances and mood swings (Dalal and Agarwal, 2015). Besides these symptoms, osteoporosis is the most prevalent disease in menopausal women (Ji and Yu, 2015). Estrogen affects bone through many mechanisms that include lowering of the sensitivity of bone mass to parathyroid hormone thus reducing bone resorption; increasing the production of calcitonin thereby inhibiting bone resorption; accelerating calcium absorption by the intestine while reducing its excretion from the kidney which contributes to improved bone mineralization and through other mechanisms mediated through estrogen-receptor-responsive elements on promoters for genes involved in bone matrix biosynthesis including collagen type-1. Estrogen acts directly on osteoclasts (bone resorption cell) to promote its apoptosis (Khosla et al., 2012). Similarly, the bone forming activity of osteoblast is enhanced by estrogen (Khosla et al., 2012). The drop in ovarian production of estrogen at

menopause is therefore accompanied by increased lifespan and activity of osteoclast and decrease in bone forming cellular activity leading to overall fall in bone mass associated with osteoporosis.

To compensate for the estrogen deficiency associated with osteoporosis in postmenopausal women, estrogen based hormone replacement therapy (HRT) has been the major therapeutic approach (Mehta et al., 2021). This therapy has been shown to prevent postmenopausal bone loss and reduce fracture risk (Mehta et al., 2021). However, the multiple tissue targets of estrogen with both positive and negative outcomes has created a complex risk-benefit situation. The beneficial effect on fracture risk in WHI study was counterbalanced by adverse cardiovascular, cerebrovascular, venous thromboembolic events as well as increased risk of breast cancer (Manson et al., 2013). The benefits derived from the synthetic estrogen hormone have also been shown to be time bound. The discontinuation of therapy probably due to side effects often relapse the deterioration thus requiring the switch to other therapies to prevent osteoporosis (Ascott-Evans et al., 2003). This complexity has caused many patients and physicians to loose enthusiasm for estrogen based hormone replacement as a bone-protective therapy in postmenopausal women even though its efficacy is beyond question.

Phytoestrogens (PE) are plant-derived compounds that structurally and/or functionally mimic mammalian estrogens and can bind to estrogen receptors (ER) (Lecomte et al., 2017). The binding of phytoestrogen to ER may result in partial activation of the same (agonist effect) or displacement of an estrogen molecule, which reduces receptor activation (antagonistic effect) (Lecomte et al., 2017). They have an affinity for ER that is lower than that of estradiol. The affinity and the time of occupation of phytoestrogens like isoflavones by the β receptor is about 30 times higher than by α receptor. In tissues, there is a different distribution of these receptors, suggesting that they exert selective tissue effects depending on the tissue in which they act. In the reproductive tissue, especially the uterus and breast, the α type predominates which contributes to most of the side effects associated with hormone replacement therapy in postmenopausal women, while the bone tissue has a greater amount of β receptors (Paterni et al., 2014). Also, the incidence of breast and prostate cancers is lower among consumers of phytoestrogen rich foods in comparison to others (Marugame and Katanoda, 2006).

Ochna schweinfurthiana F. Hoffm is a tropical small tree that measures up to 4 metres and belongs to the Ochnaceae family. It is highly patronized in ethnomedicine for the treatment of different metabolic diseases involving mechanisms of oxidation or inflammation such as rubella, burns, stomach ache, and multiple sclerosis (Abdullahi et al., 2014). Several studies revealed that the Ochnaceae family is a rich source of complex metabolites such as flavonoids, chalcones, steroids, terpenoids, and alkaloids (Anuradha et al., 2006; Pegnyemb et al., 2003). The evaluation of the antioxidant activity of the leaves, stem-barks extracts, and fractions of *O. schweinfurthiana* has been already done (Nyegue et al., 2016). A study has reported on the phytochemistry of the stem bark of *O. schweinfurthiana* and demonstrated cytotoxicity against HeLa cells of the methanolic and ethyl acetate extracts, as well as the isolation of amentoflavone and agathisflavone (Ndongo et al., 2015). Messi et al. (2016) isolated three new antiplasmodial and antioxidant agents from the roots of *O. schweinfurthiana*. Our preliminary pilot screening study reveals presence of phytoestrogens in the stem bark extract of *O. schweinfurthiana* which is in line with other studies that have isolated phytoestrogens from the same stem bark (Djova et al., 2019). These observations coupled to reported activities of the plant extract against metabolic diseases involving mechanisms of oxidation and inflammation informed our decision to evaluate its effect on ovariectomy induced osteoporosis.

2. Materials and Method

2.1. Plant Materials

The bark of *Ochna schweinfurthiana* was collected from Agulu in Anambra State Nigeria and authenticated by a trained taxonomist, Mr Felix Nwafor of Department of Pharmacognosy and Environmental Medicine, University of Nigeria Nsukka, Enugu State, Nigeria. A voucher specimen (No. PCG 521/A/043) was deposited at the herbarium of the Department of Pharmacognosy and Traditional Medicine, Nnamdi Azikiwe University Awka for future reference. The plant materials were subsequently air-dried under room temperature and pulverized with a mechanical grinding machine (GX160 Delmar 5.5HP).

2.2. Animal Source

Swiss female Albino rats were employed for the study. All the animals were obtained from the Animal House of the Department of Pharmacology and Toxicology, Nnamdi Azikiwe University, Agulu Campus. The animals were housed in standard laboratory conditions of 12 h light, room temperature, 40-60% relative humidity and fed with rodent feed (Guinea Feeds Nigeria Ltd). They were allowed free access to food and water. Maintenance and care of all animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Guide for the care and use of Laboratory Animals, DHHS Publ. # (NIH 86-123 were strictly adhered to. Ethical approval was obtained from the Animal Ethical Committee of the Enugu State University of Science and Technology (Approval number: ESUT/AEC/0169/AP204)

2.3. Extraction and Fractionation

Ochna schweinfurthiana bark powder was macerated in methanol for 72 h with intermittent shaking. The resulting solution was filtrated using Whatman filter paper and the filtrate concentrated *in vacuo* using rotary evaporator (RE300 Model, United Kingdom) at 40°C. Two-third of the extract dissolved in water was subjected to liquid-liquid partition successively with n-hexane, ethyl acetate and butanol. The fractions were concentrated *in vacuo* using rotary evaporator at 40°C to obtain the n-hexane, ethyl acetate, butanol and water soluble fractions. The extracts and all the fractions were stored in refrigerator between 0-4°C for further use.

2.4. Quantification of phytoestrogen content using genistein as standard

The phytoestrogen contents of the extract and fractions were quantified using the method described by Cesar *et al*. (2008). One milliliter each of the extracts and fractions (100 ug/ml) was mixed with 1 ml of 2% $AlCl_3$ (dissolved in methanol) in duplicate. Ten minutes after the addition of $AlCl_3$ absorbance was recorded at 382 nm. Blank solutions of the standard and samples without addition of $AlCl_3$ (only methanol) was also prepared. Phytoestrogen content was estimated from the genistein calibration curve and expressed as milligrams of genistein equivalent (GE) per gram of the extract/fractions.

2.5. In vivo Pharmacological assay

2.5.1. Acute Toxicity Studies

Acute toxicity analysis of the phytoestrogen rich fraction was performed using Lorke's method as described by Mbagwu et al. (2019). The sample was tested at doses ranging from 100 to 5000 mg/kg. Observation for 24 hours for obvious signs of toxicity and death was taken. The LD_{50} was calculated using the formula:

$$LD_{50} = \sqrt{(D_0 \times D_{100})}$$

D_0 = Highest dose that gave no mortality,
D_{100} = Lowest dose that produced mortality.

2.5.2. Ovariectomy induced Osteoporosis model

2.5.2.1. Induction of menopause

Vaginal cytology was performed on all the animals for the confirmation of regular estrus cycles using the method described by Ajaghaku et al., (2021). Vaginal smears from the animals were examined under the microscope every morning (8 am – 10 am) to observe at least two consecutive 4-day estrus cycles. The animals were ovariectomized (OVX) in order to induce menopause and to investigate reproductive changes following the supplementation with the phytoestrogen rich fraction. After ovariectomization, vaginal smears of all the animal were examined under the microscope to ensure complete removal of the ovaries evident in the absence of epithelial cell cornification (Diestrus phase).

Animals from all the groups were OVX except the animals that served as sham-operated control. In the sham-operated control (without OVX), the ovaries were exposed and gently manipulated but not excised. The rest of the animals were subjected to bilateral OVX via a dorso-lateral approach with a small lateral vertical skin incision using surgical blade followed by ligation and excision of the ovaries along the upper horn under general anesthesia with ketamine and xyaline (80 and 10 mg/kg body weight respectively, i.m). The incisions were joined together using suture materials (chromic catgut and silk) followed by applying oxytet spray for quick healing of wound. Care was taken to avoid any infection throughout the OVX procedure. Animals were allowed to heal for 4 weeks before dosing. During the healing period, the animals were observed for any abnormal behavior or side effects.

2.5.3. Experimental Design

Forty-eight female rats, 3 months old, were used for this study (42 ovariectomised and 6 sham operated) were randomly allocated to the following groups: sham group, vehicle 10 ml/kg 5% Tween 80 (as the osteoporosis model control), 50, 100, 200, 400 and 800 mg/kg of the phytoestrogen rich fraction of *O. schweinfurthiana* and estradiol (1 mg/kg). Each group comprised of 6 animals and treatment was done through oral route daily for 60 days. During the two-months dosing period, all the animals were monitored daily for clinical signs and any symptoms of toxicity, once before dosing, immediately after dosing and up to 1 h after dosing.

After 2 months treatment period, all animals were anesthetized using a mixture of ketamine at dose of 75 mg/kg with xylazine at dose of 10 mg/kg and their blood, uterus and femurs collected for analyses.

2.5.4. Uterotrophic assay

The uterus was carefully dissected at the level of the vaginal fornix, trimmed of fascia and fat under a stereomicroscope and weighed. The relative uterine weight of each animal was calculated according to El-Alfy *et al*. (2012) using the following formula:

$$Relative\ uterus\ weight\ (g) = \frac{Organ\ weight\ (g)}{Body\ weight\ (g)} \times 1000$$

2.5.5. Body Weight determination

During the experimental period, the body weight of each rat was monitored using a sensitive scale. The animals

were weighed just before the start of the experiment (Pre-induction), after the ovariectomy/healing (post-induction) and after the treatment (post-treatment). Body weights were expressed in grams.

2.5.6. Blood and Serum Preparation

The blood samples were collected from the retro-orbital plexus in separator tubes and were allowed to stand for 1 h to ensure complete clotting. Blood was then centrifuged at 3000 rpm for 10 min and serum samples separated and stored in aliquots

2.5.7. Determination of serum estrogen concentration

Serum estradiol concentration was determined used estradiol ELISA kit (Calbiotech) following the manufacturer's instructions. The test kit is based on the principle of competitive binding between estradiol in the test specimen and estradiol enzyme conjugate for a constant amount of anti-Estradiol polyclonal antibody. Absorbance values for each specimen is used to determine the corresponding concentration of Estradiol in pg/ml from the estradiol standard curve.

2.5.8. Determination anti-osteoporotic activity

2.5.8.1. Bone turnover biomarker determination

Bone restorption and formation were monitored using serum acid phosphatase (ACP) and alkaline phosphatase (ALP) activities. ACP was estimated by alpha naphthyl phosphate kinetic method as described by Panteghini and Bais (2008) using Teco diagnostics test kit (Anaheim, California). While alkaline phosphatase was estimated by the method described by Colville (2002) using ALP test kit (Span Diagnostics Ltd., India).

2.5.8.2. Quantitative determination of serum calcium and Phosphorous

Serum calcium was estimated by ortho-cresolphthalein method as described by Endres and Rude (2008) using test kits [Quimica Clinica Aplicada (QCA), Spain]. While serum phosphorous was estimated by Fiske-SubbaRow method as described by Endres and Rude (2008) using test kits [Quimica Clinica Aplicada (QCA), Spain].

2.5.8.3. Bone Analysis

The femurs was dissected out and was cleaned of adhering flesh and connective tissue. The last fragment of connective tissues were trimmed off with small scissors. The left femur was used for analysis of bone mineral content (BMC) and density (BMD). BMD was calculated from the bone mineral content (BMC) of the measured area and was expressed in g/cm3.

2.5.8.4. Bone mineral content and mineral density

The bone mineral content was determined as weight fraction of the water-free bone using ashing method (Van et al., 2012). Mid segment of the femur (1cm) was defatted in acetone, dried for 24h at 100°C and weighed. The weighed samples were ashed at 800°C for 24 h and reweighed. The weight difference was taken as the mineral ash mass. Archimedes principle was used to determine bone density as ash mass per bone volume (g/cm^3).

2.5.9. Oxidative/Antioxidative Stress Markers Analysis

2.5.9.1. Malondialdehyde (MDA) assay

MDA level in serum was estimated by modified thiobarbituric acid method as described by Draper and Hadley (1990) using malondialdehyde assay kit (Elabscience Biotechnology Co. Ltd., China).

Five milliliter test tubes were labeled appropriately for the samples, standard and blank all in duplicates. 50 ul of the serums, standard and absolute ethanol were placed in their appropriate labeled sample, standard and blank test tubes respectively. Then 50 ul of clarificant was added to all the test tubes followed by the addition of 1.5 ml of acid reagent and then 500 ul of dilute chromogenic agent. The contents of the test tubes were mixed well, covered with a plastic cover and incubated in the water bath (New Life Medical Instruments, England) at 95 ^{O}C for 40 minutes. After incubation the test tubes were cooled with running water and centrifuged at 3000 revolutions per minute for 10 minutes. The supernatants were collected from the centrifuged test tubes. The absorbances of the supernatants were read at 532 nm against a distilled water blank. MDA level of each sample was calculated using the formula below;

$$MDA\ (nmol/ml) = \frac{Absorbance\ of\ Sample - Absorbance\ of\ Blank}{Absorbance\ of\ Standard - Absorbance\ of\ Blank} \times 10$$

2.6. Statistical Analysis

The data obtained were expressed as mean $\pm$ SEM. All data were analysed by Kruskal-Wallis ANOVA test. The differences between the different treatments were subjected to 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 analyses was performed using STATISTICA 12 software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Median lethal dose (LD50)

Acute oral administration of the PERF of *O. schweinfurthiana* did not produce mortality even at the highest tested dose. No obvious sign of toxicity was observed throughout the observational period. The median lethal dose was estimated to be above 5000 mg/kg.

3.2. Phytoestrogen content

The extract showed phytoestrogen content of 1680 mgGenisteinEq/g (Fig. 1). Liquid-liquid partitioning of the phytocompounds in the extract concentrated phytoestrogen compounds in the water fraction. Compared to the phytoestrogens in other fractions more than 10 fold difference exist when compared with water fraction. Phytoestrogen content of the fractions increased with increase in solvent polarity.

3.3. Body weight changes

Ovariectomy induced increase in body weight was observed across groups (Fig. 2). However, treatment with the PERF produced dose dependent reduction that achieved non-significant ($P>0.05$) difference between sham operated control and 800 mg/kg PERF treated groups. Just like the reference standard (1 mg/kg estradiol), treatment with the PERF from 100 mg/kg to 800 mg/kg produced significant ($P<0.05$) reduction in body weight compared to the vehicle control ovariectomized group. No significant ($P>0.05$) difference was observed with effects produced by 400 and 800 mg/kg of PERF compared to the reference standard.

3.4. Effect of PERF on serum estradiol

Ovariectomy produced significant ($P<0.05$) reduction in serum estradiol from 10.2 pg/ml recorded in sham operated control to 2.28 pg/ml in ovariectomised vehicle control (Fig. 3). Compared to the ovariectomised vehicle control group, the PERF produced significant ($P<0.05$) increase in serum estradiol at all the tested doses. However, these concentration increases produced by various doses of PERF were still significantly ($P<0.05$) lower that obtained in the sham operated control. Treatment with 1 mg/kg estradiol (reference standard) produced significantly ($P<0.05$) high serum concentration of the hormone compared to both sham operated control and the PERF treated groups.

3.5. Effect of PERF on uterosomatic index

Significant ($P<0.05$) reduction in uterosomatic index was recorded between ovariectomised control and sham operated control (Fig. 4). Treatment with the PERF led to dose depended increase with median effective dose of 197 mg/kg. The increase in relative uterine weight produced as various doses of the PERF was lower than the sham operated control as well as the reference standard. Compared to sham operated control, the estradiol treated animals showed significantly ($P<0.05$) higher uterine weight (Fig. 4).

3.6. Effect of PERF on bone mineral content and bone mineral density

Reductions in bone mineral content and bone mineral density due to ovariectomy were shown by significant ($P<0.05$) difference between the mean values of the vehicle and sham operated control groups (Fig. 5 and 6). Compared to vehicle administered ovariectomised control group, the PERF produced significant ($P<0.05$) increase in both parameters although lower that the effect produced by the reference standard. The median effective dose of the PERF on BMC was established to be 226 mg/kg while 263 mg/kg was required to produce 50% increase in BMD.

3.7. Effect of PERF on serum calcium and phosphorus

Significant ($P<0.05$) drop in serum calcium was recorded in the ovariectomised control compared to sham operated control group (Fig. 7). Increase in serum calcium was produced by treatment with the PERF as significant ($P<0.05$) difference existed between the treated groups and vehicle control group. Compared to the effect produced by the reference standard (1 mg/kg estradiol), only 800 mg/kg PERF produced similar effect with non-significant ($P>0.05$) difference between both mean values. From the dose response curve, it was estimated that 947,576 mg/kg of the PERF will be required to produce 50% increase in serum calcium. Similar reduction in serum concentration was also recorded for phosphorus (Fig. 8). However, significant ($P<0.05$) increase was only achieved at higher concentrations (200 – 800 mg/kg) of the PERF when compared to vehicle control group. The median effective dose for increase in serum phosphorus was established at 656 mg/kg

3.8. Effect of PERF on bone turnover markers

There were increased serum bone turnover markers (ALP and ACP) in response to ovariectomy (Fig. 9 and 10). Double fold increase in serum ALP was recorded while about 60% increase was recorded for serum ACP compared to sham operated control. The median effective doses required to reduce the bone turnover markers were 262 mg/kg for ALP and 319 mg/kg for ACP. The PERF produced significant ($P<0.05$) dose dependent reduction in serum ALP and ACP compared to vehicle ovariectomised control group. Better effect was achieved with estradiol than the PERF for reducing serum ALP while similar effect was produced by 400 and 800 mg/kg of PERF for serum ACP compared to estradiol.

3.9. Effect of PERF on serum oxidative biomarker

Just as with bone turnover markers, ovariectomy produced elevation of lipid peroxidation as evidenced by double fold increase in serum lipid peroxidation by product – MDA in ovariectomised control compared to sham operated control (Table 1). Significant ($P<0.05$) reductions were recorded following treatment with the PERF compared to vehicle control group. Effective dose required for 50% reduction was established as 716 mg/kg (Fig. 11). Similar activity as with estradiol was produced by 800 mg/kg of the PERF while lower doses of PERF produced significantly ($P<0.05$) less activity.

4. Discussion

Estrogen deficiency in postmenopausal women is accompanied by numerous changes that impact adversely to their quality of life. To evaluate the effect of phytoestrogen rich fraction of *O. schweinfurthiana* on osteoporosis associated with menopause, ovariectomized rat model was used. The choice for this model was informed by the fact that it mimics natural deficiency in ovarian production of estrogen associated with menopause (Brinton, 2012). This model also allowed for evaluation of estrogenicity of test samples as possible mechanism of osteoprotection (Brinton, 2012).

Numerous concerns about menopause exist among women and the fear of an increase in body weight is one of the most important of them (Kozakowski et al., 2017). The menopause transition is marked by phenotypic changes including body weight gain associated with increased fat mass and decreased lean body mass which are connected to reduced skeletal muscle mass and net bone resorption (Zhao et al., 2008). The increase in body weight can contribute to overweight and obesity and can double the menopausal burden for those women that are already overweight or obese (Kozakowski et al., 2017).

Increase in body weight leading to obesity is a major risk factor for musculoskeletal disorders like osteoarthritis and osteoporosis (Zhao et al., 2008). Physiological estrogens increase fat oxidation in the skeletal muscles and inhibit hepatic and muscle lipogenesis (Liu et al., 2019). So it should be expected that the decline/lack of estrogens associated with menopause would result in a tendency for an increase in the visceral fat mass. Studies in mice have also demonstrated that the loss of ovarian function promotes a diet-independent increase in adipose tissue mass and decreased energy expenditure (Rogers et al., 2009). Our result is in tandem with these established effect of ovariectomy on body weight.

Estrogen administration to ovariectomised animals has been documented to normalize body weight earlier increased as a result of the removal of the gonalds and to protect against these changes if estrogen supplementation was initiated on time (Nishio et al., 2019). Due to their structural similarity to 17-β-estradiol (E2), the natural and primary target for phytoestrogens are estrogen receptors (Lecomte et al., 2017). Activation of ERs by phytoestrogen genistein have been reported to repress adipogenic differentiation and maturation; lipid accumulation and the expression of adipocyte-specific genes in adipocytes (Park et al., 2009). Similarly, isoflavone phytoestrogen supplementation is also associated with downregulation of the expression of genes that are involved in lipid synthesis (Kurylowicz, 2021). Apart from inhibition of lipogenesis, several phytoestrogens have been found to induce lipolysis, triggering different molecular mechanisms that include suppression of cAMP-specific phosphodiesterase in adepocytes that lead to decreased triglyceride accumulation; upregulation of genes that are related to fatty acids oxidation such as hormone sensitive lipase, uncoupling protein 2 and increase in energy expenditure (Kurylowicz et al., 2020). Amentoflavone – a biflavonoid phytocompound isolated from stem of *O. schweinfurthiana* have been reported to be highly consumed by young people to build muscle and lose weight (Aliyev et al., 2021). It has also been reported to reduce high fat diet induced obesity by inhibiting lipogenesis through the regulation of signaling pathways like PI3K/AKT pathways as well as decrease in lipogenesis related gene expression (Chen et al., 2016). The weight reductive effect of phytoestrogens from *O. schweinfurthiana* could have been mediated by the combination of both genomic and non-genomic mechanisms that control energy expenditure, lipid metabolism and feeding. Increased estrogen levels in ovarectomized rats treated with various doses of *O. schweinfurthiana* showed the estrogen-like activity of the phytoestrogen rich fraction which may have contributed to weight reduction through estrogen receptor mediated signaling.

The endometrium of the uterus is the primary target for estrogen (Yu et al., 2022). Estrogen causes it to increase in size, weight, cell number, cell types, blood flow, protein content and enzyme activity (Yu et al., 2022). In addition to increased cell division, the morphology of uterine cells is transformed in response to 17 β-estradiol (Yu et al., 2022).These changes account for the uterotrophic effect of estrogen. Thus, lack of estrogen causes the endometrium to thin out and the tissue of the uterus to degenerate, shrink and lose weight. The low uterine weight of our ovariectomized control relative to naïve is an indication of successful ovariectomy induced reduction in estrogen production.

The uterotrophic effect of phytoestrogen rich extract of *O. schweinfurthiana* further established its *in vivo* estrogenic activity. The increase in uterine weight at various doses of the administered phytoestrogen rich fraction of *O. schweinfurthiana* is a strong indication that it is capable of promoting dose dependent estrogenic activity – reversing the effect of ovariectomy. The increased uterine weight exhibited by these doses of phytoestrogen rich

fraction were lower than that of 17 β-estradiol treated group indicating that these doses might have weak estrogenic effect. The weak estrogen activity demonstrated by these doses may be explained by documented higher affinity of phytoestrogens for beta estrogen receptors (ERβ) compared to alpha receptor (ERα) in contrast to estradiol whose affinities for both receptor types are roughly the same (Mahmoud et al., 2015). As shown previously, the known uterotrophic of estrogen is mediated mainly through the ERα mechanisms as have also been demonstrated by ERα selective agonists in contrast to inverse effect exhibited by selective ERβ agonist (Frasor et al., 2003). It could be that the weak estrogenic activity exhibited by the phytoestrogen rich fraction may have resulted from its relative estrogen receptor selectivity with higher affinity for ERβ and partial/low affinity for ERα especially at higher doses. Most adverse effects of estrogen based hormone replacement therapy are mediated mainly through ERα mechanisms (Siersbaek et al., 2018). High uterotrophic effect of estrogen may be linked to endometrial hyperplasia and possibly neoplasia associated with estrogen replacement therapy (Furness et al., 2012). Conversely, the weak estrogenic activity of the phytoestrogen rich fractions may thus be advantageous in producing estrogenic beneficial effects with minimal potential for its neoplastic side effects. The weaker estrogenic effect may also function paradoxically as antiestrogens *in vivo* by protecting estrogen receptors from activation by the more potent steroidal agonists.

The established role of ovariectomy in ovarian estrogen deficiency has made it a widely used model of studying how the decline in endogenous estrogen production by the ovaries at menopause leads to postmenopausal osteoporosis and how potential interventions can preserve bone metabolism in this state (Miao et al., 2012). Estrogen decline contributes to loss of bone mass, architectural integrity and strength (Ji and Yu, 2015). In many animal species, including humans and rats, depletion of estrogen either spontaneously after menopause or surgically induced by ovariectomy results in osteoporosis which can be prevented by estrogen replacement (Seidlova-wuttke et al., 2012). In this study, ovariectomy successfully induced osteoporotic-like changes including reduction in bone mineral content and bone mineral density and increase in bone turnover markers. These changes are consistent with known effect of estrogen deficiency on bone homeostasis.

In bone remodeling, resorption is mediated by osteoclast while bone formation in mediated by osteoblast. The stimulatory effect of estrogen on bone formation is mediated by estrogen receptor responsive elements on promoters for genes involved in bone matrix biosynthesis including type 1 collagen or cytokines believed to be important for coupling of bone resorption and bone formation (Khosla et al., 2012). Similarly, estrogen suppresses bone resorption and its deficiency promotes osteoclastogenesis due to weakened inhibitory effect on osteoclasts (Khosla et al., 2012).

The osteoblatic and osteoclastic roles of alkaline phosphatase (ALP) and acid phosphatase (ACP) makes them important serum markers of bone formation and bone resorption respectively (Hassan et al., 2013). Osteoclasts have high acid phosphatase activity and elevation in serum acid phosphatase is associated with resorption sites on bone. Contrary to the role of ACP, ALP is involved in osteoid formation and mineralization by enzymatic degradation of the inhibitor of mineralization, pyrophosphate at an alkaline pH (Orimo, 2010). The activities of these enzymes have been reported to be increased significantly in rats with estrogen deficiency which is in agreement with the result of this study (Liu et al., 2018). Concomitant elevation in serum ALP enzyme activity which is a marker of bone formation may have resulted from compensatory response to the increased osteoclastic bone resorption activity. Elevated serum ALP as a surrogate marker of high bone turnover associated with post-menopausal osteoporosis has been a significant predictor of decreased bone mineral density (BMD) (Tariq et al., 2019). The increase in these bone turnover makers in ovariectomised (OVX) animals further confirmed the adequacy of this model in inducing osteoporosis and their reduction following treatment with phytoestrogen rich fraction of *O. schweinfurthiana* supports their therapeutic potentials in postmenopausal induced osteoporosis.

Mineralization is an important process required for effective bone formation. Among micronutrients, calcium and inorganic phosphate are two main constituents of hydroxyapatite that are essential for bone formation and bone strength. Both minerals are widely accepted as markers for bone formation and estrogen deficiency adversely affect their serum concentration (Miao et al., 2012). Phytoestrogen rich fraction of *O. schweinfurthiana* through increased Ca and P may contribute to enhance bone mineral density which is expected to counter menopause associated bone loss and osteoporosis.

Several flavonoids are known as phytoestrogens. Among these flavonoids, flavones and isoflavones are known for their estrogenic activity and for preserving bone health (Kiyama, 2023). Although the isolation and characterization phytoestrogens were not done in this study, previous studies identified and isolated flavones and isoflavones such as 6,7-diethyoxy-3-4-dimethoxyisoflavone, agathisflavone and amentoflavone from the stem of *O. schweinfurthiana* (Djova et al., 2019). Methoxyisoflavones such as Genistein (7,4′-dihydroxy-6-methoxyisoflavone), glycitein (7,4′-dihydroxy-6-methoxyisoflavone), biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), and formononetin (7-hydroxy-4′-methoxyisoflavone) belonging to isoflavone phytoestrogens have been reported to have osteoprotective effect on skeletal system, inhibition of bone resorption and increased bone density (Singh et al., 2017). These effect have been demonstrated to be mediated through their estrogen receptor mediated signaling. Among plant derived estrogens, Isoflavones are ranked among the most

estrogenic compounds having more affinity for β-ER isoforms than for α-ER isoforms (Vitale et al., 2013), in contrast to estradiol (E2), whose affinities to both receptor types are roughly the same (Krizova et al., 2019).

The predominant situation of β-ER in the bones makes isoflavones important therapeutic phytoestrogen for maintenance of bone health in postmenopausal women (Krizova et al., 2019). Studies on post-menopausal women showed that increasing soy isoflavone intake reduces bone turnover as well as reduction in osteoclastic factors and increases in osteoblastic factors which are beneficial in prevention/reducing the risk of osteoporosis (Zheng et al., 2016). Similarly agathisflavone a biflavonoid have been reported to act on estrogen receptors and to possess other activities that are beneficial in preservation of hone health such as antioxidant and anti-inflammatory activities (Dos Santos et al., 2018). Of very important is it's pronounce inhibitory effect on cytokines (IL-1, IL-6, TNF-α) and transcriptional factor (NFKβ) that mediate bone resorption (Dos Santos et al., 2018). Its antioxidant and anti-inflammatory effects gives it a positive indication in the treatment of many other diseases related to oxidative stress and inflammatory reactions including osteoporosis and other Musculoskeletal diseases (MSD) (Dos Santos et al., 2018).

Amentoflavone is also a biflavonoid bearing two epigenin molecules (Xiong et al., 2021). Estrogen receptor agonist effect of epigenin is well known as well as its osteoprotective effect (Jung, 2014; Yao et al., 2021). Amentoflavone have been reported to increase osteoblast differentiation, collagen synthesis and increased bone mineralization (Lee et al., 2006). It has also been shown to suppress bone resorption through inhibition of osteoclastogenesis (Zhang et al., 2018). Improved osteoblast function and concomitant suppression of osteoclast activity may have contributed to the mechanism of osteoprotective effect of phytoestrogen rich fraction of *O. schweinfurthiana.*

Ethical Approval
The institutional animal ethics committee gave the experiment the go-ahead with approval, issuing it with the number: ESUT/AEC/0169/AP204.

Data Availability
The corresponding author will provide more details upon request.

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Nil.

Declaration of Competing Interest
The authors affirm that they have no known financial or interpersonal conflicts that would have appeared to have an impact on the research presented in this study.

CRediT authorship contribution statement
Uchechukwu H. Orji: Conceptualization, Methodology, Software. **Emmanuel E. Ilodigwe:** Supervision, Writing –review & editing. **Ikechukwu S. Mbagwu:** Data analysis, Validation. **Amara A. Ajaghaku:** Data curation, Visualization. **Daniel L. Ajahaku:** Writing –original draft, Investigation.

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Supplementary Materials
Nil.

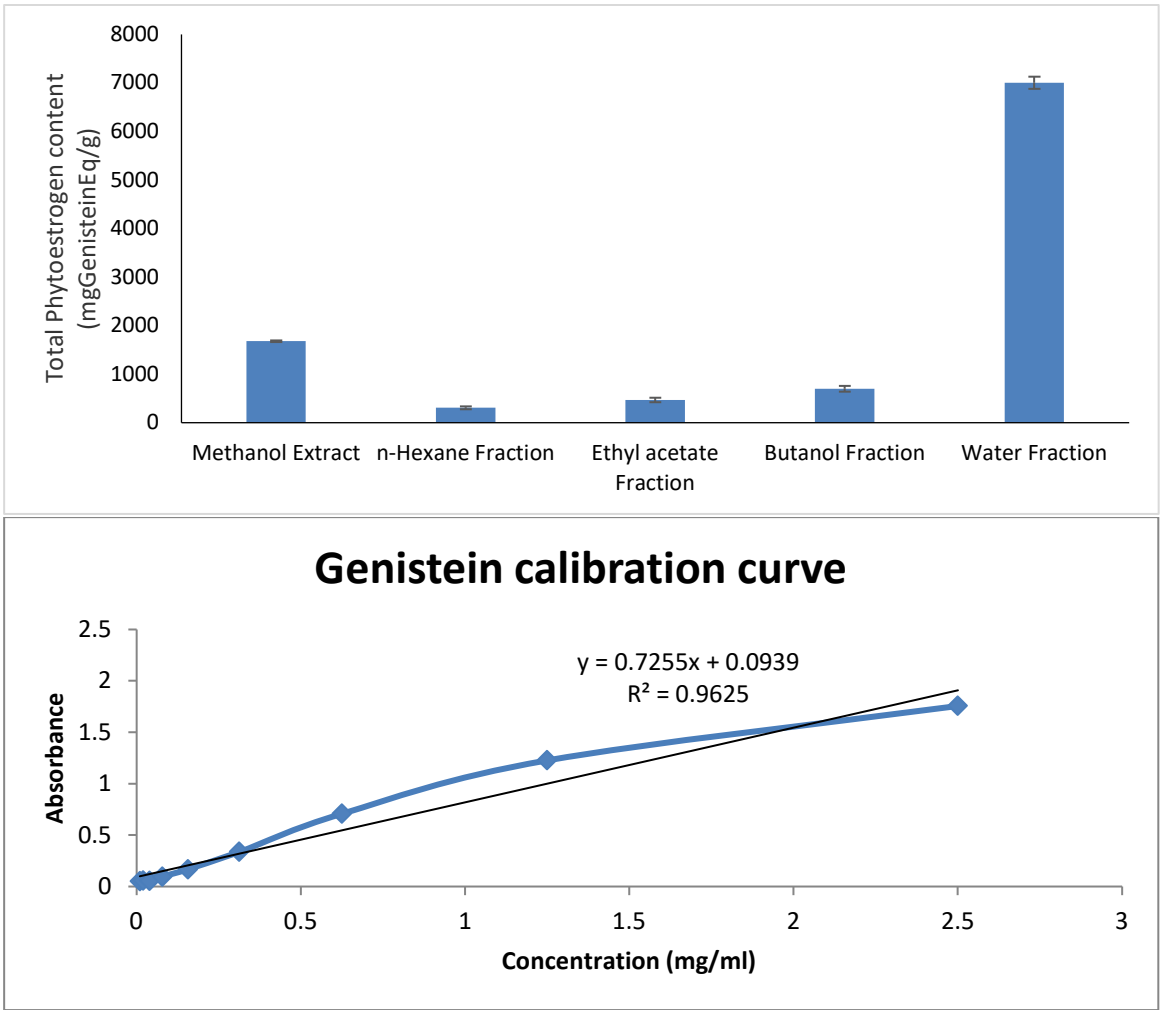


Figure 1: Total phytoestrogen content of the extract and fractions of *O. schweinfurthiana*

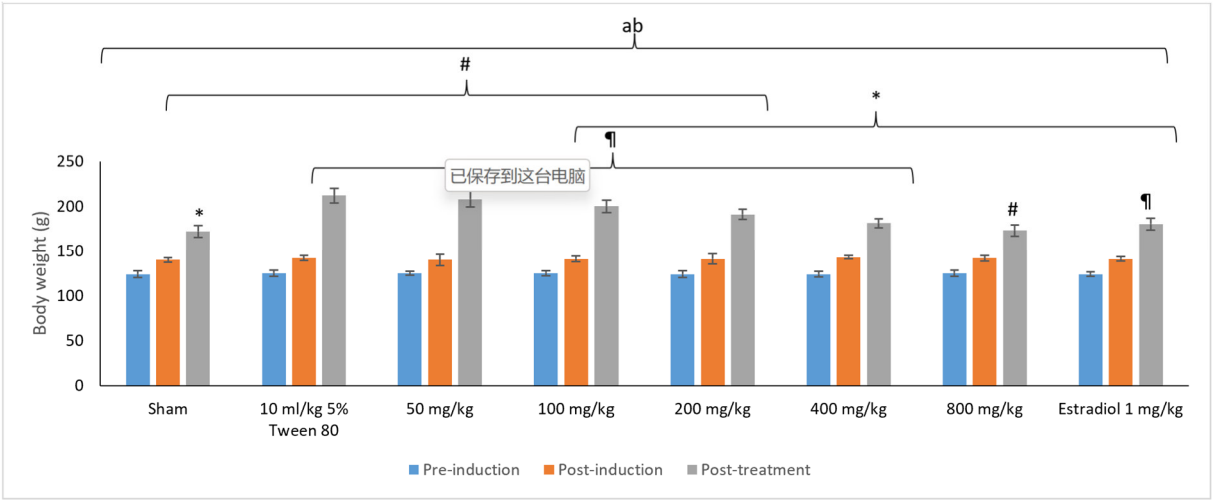


Figure 2: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on body weight

Where: a = P<0.05 compared to pre-induction within group; b = P<0.05 comparing post-induction to post treatment within group; ¶ = P<0.05 comparing post-treatment values to sham; * = P<0.05 comparing post-treatment values to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 comparing post-treatment values to Estradiol (standard reference drug).

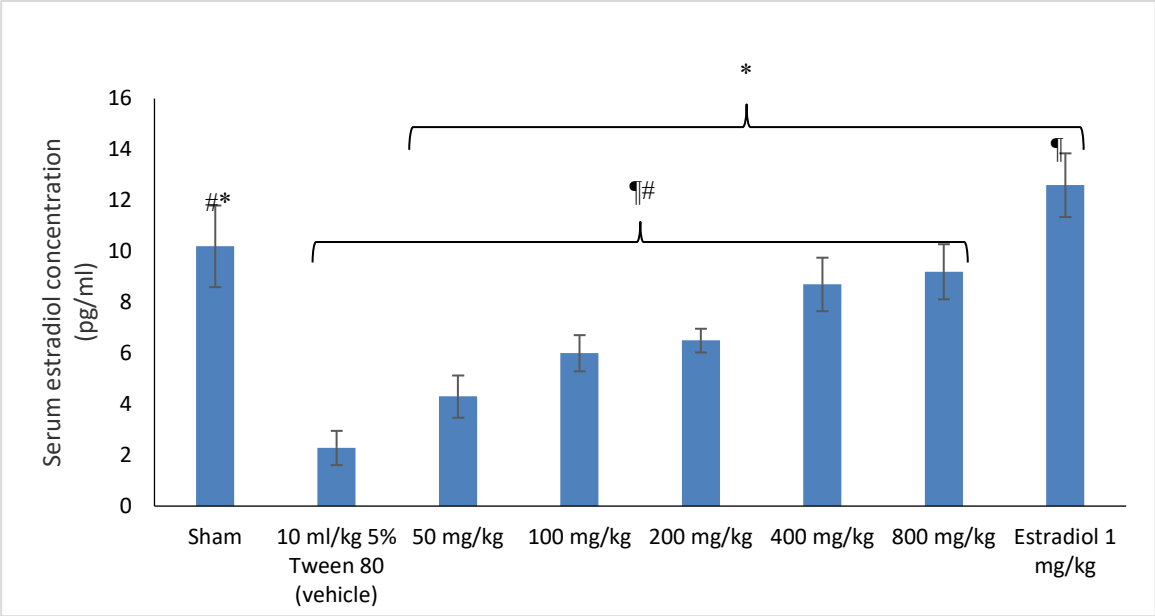


Figure 3: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on serum estradiol
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

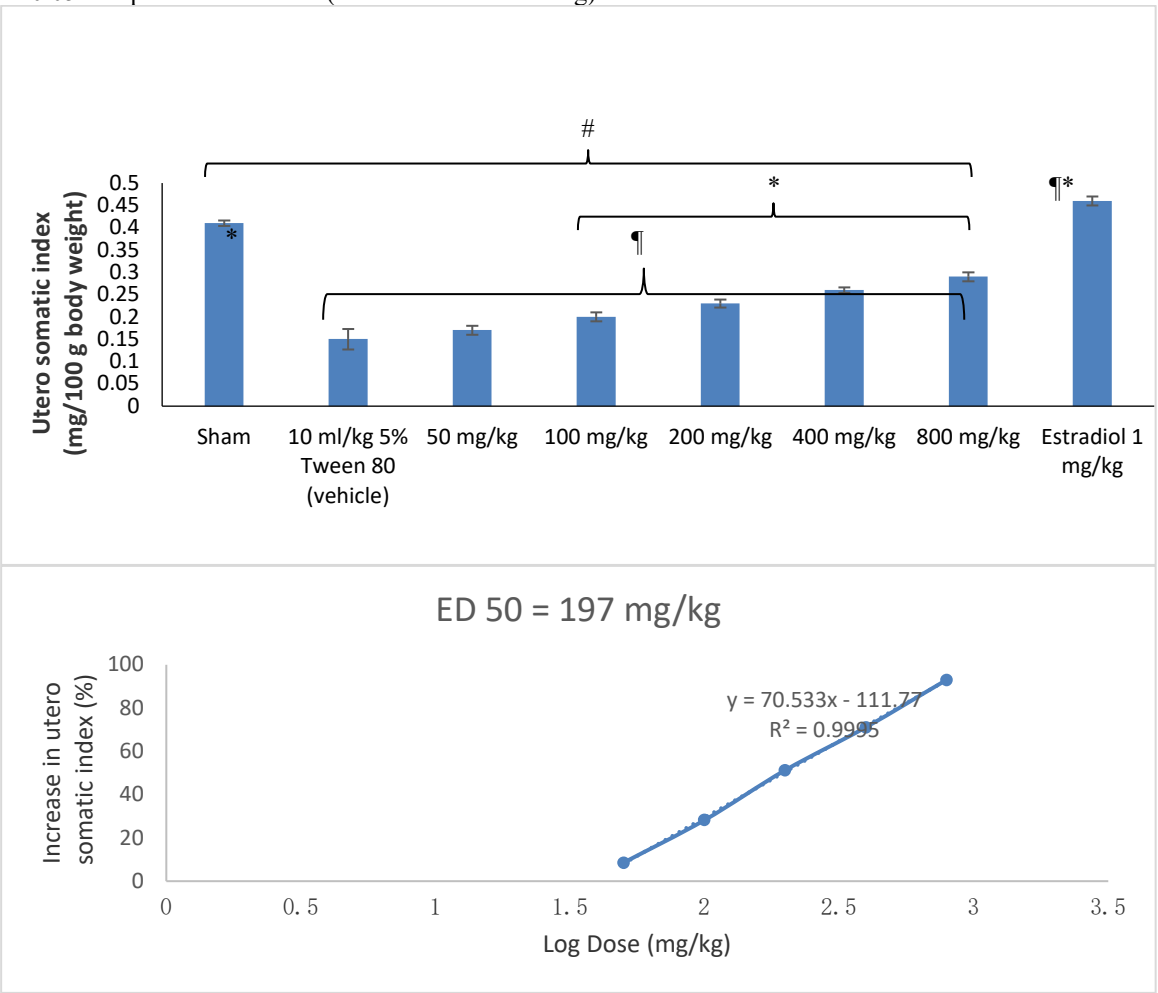


Figure 4: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on utero somatic index
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

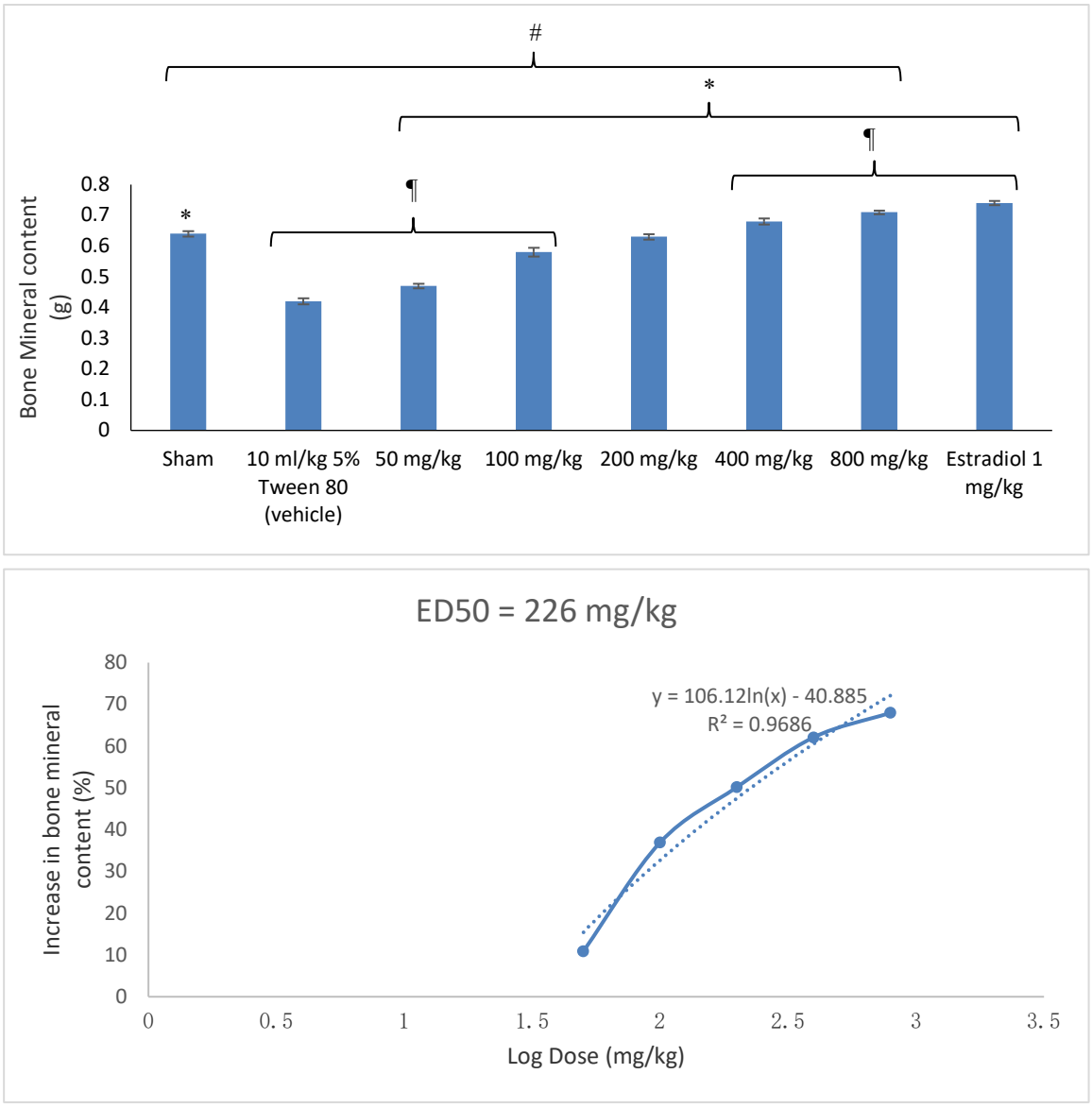


Figure 5: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on bone mineral content
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

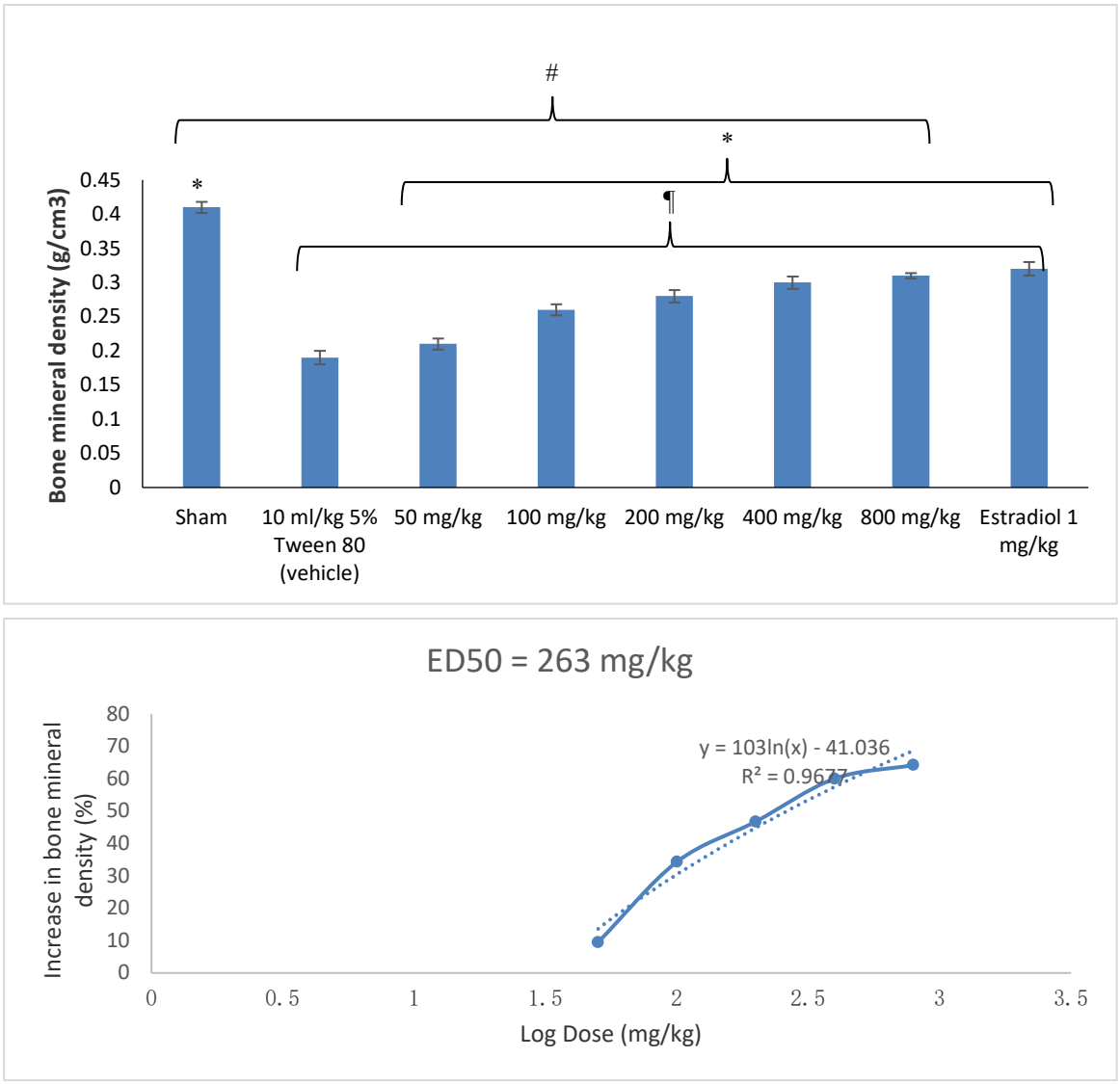


Figure 6: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on bone mineral density
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

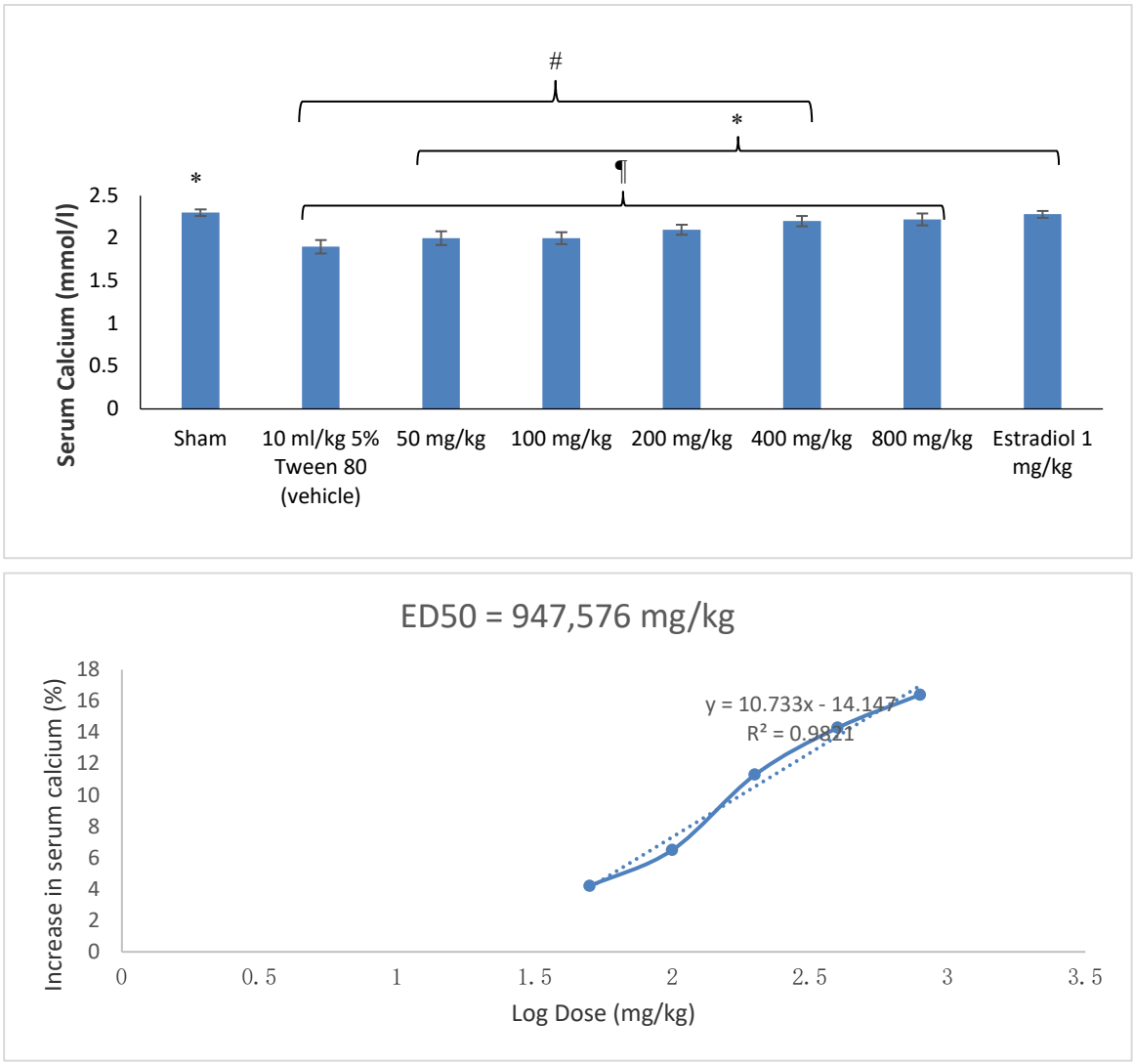


Figure 7: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on serum calcium
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

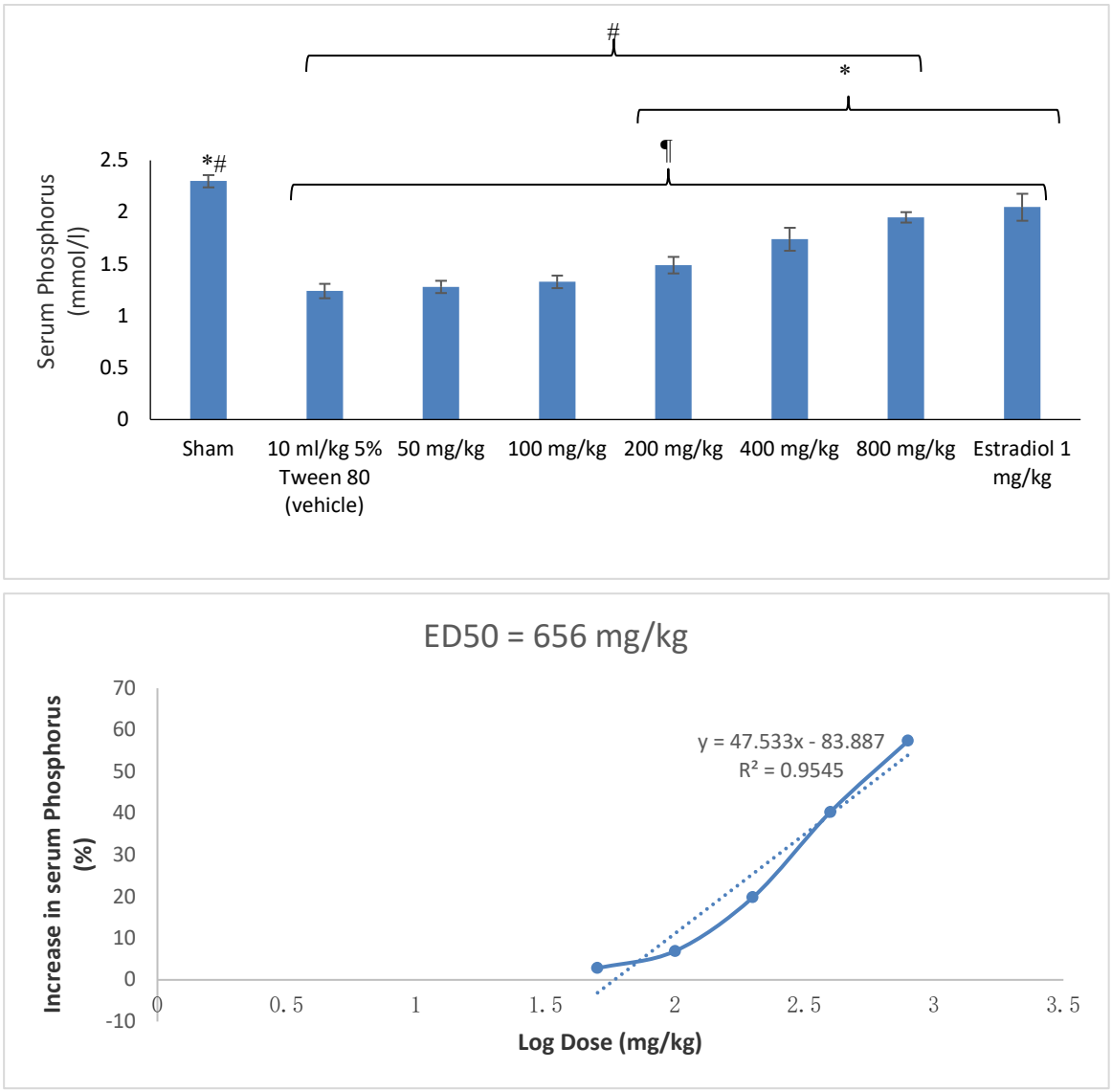
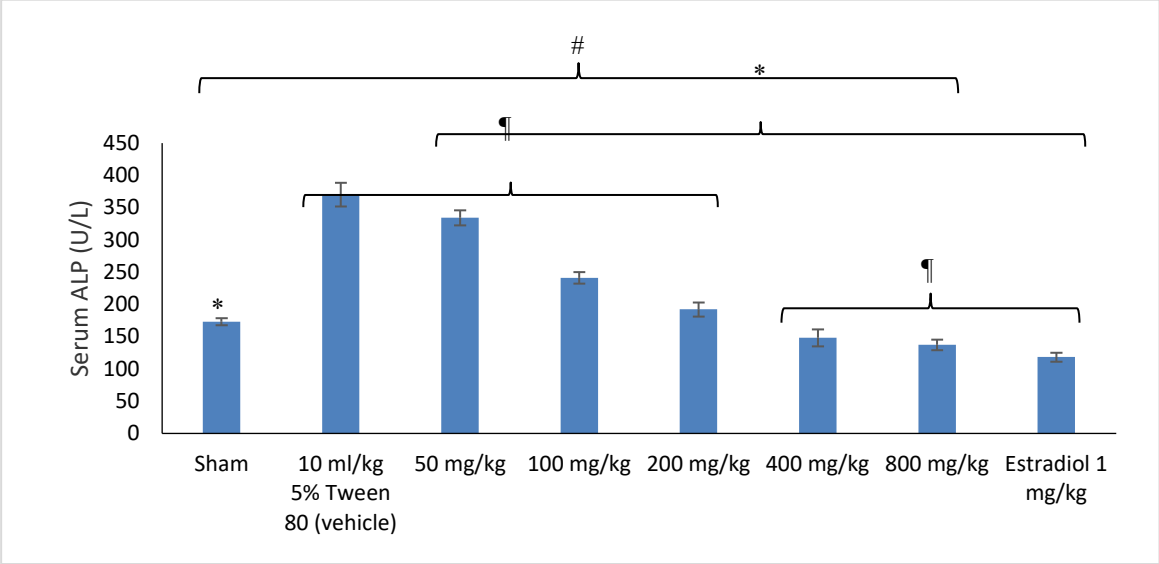


Figure 8: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on serum phosphorus
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).



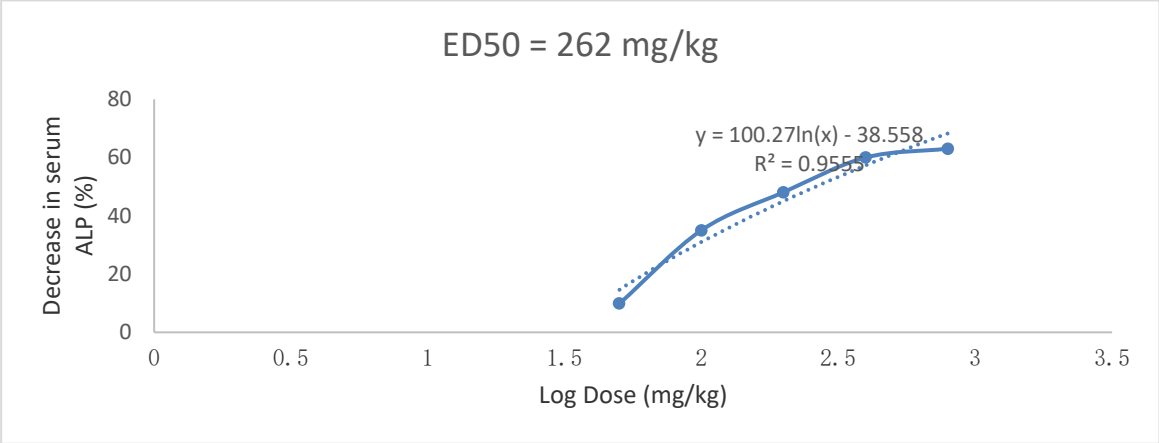


Figure 9: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on serum Alkaline Phosphatase
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

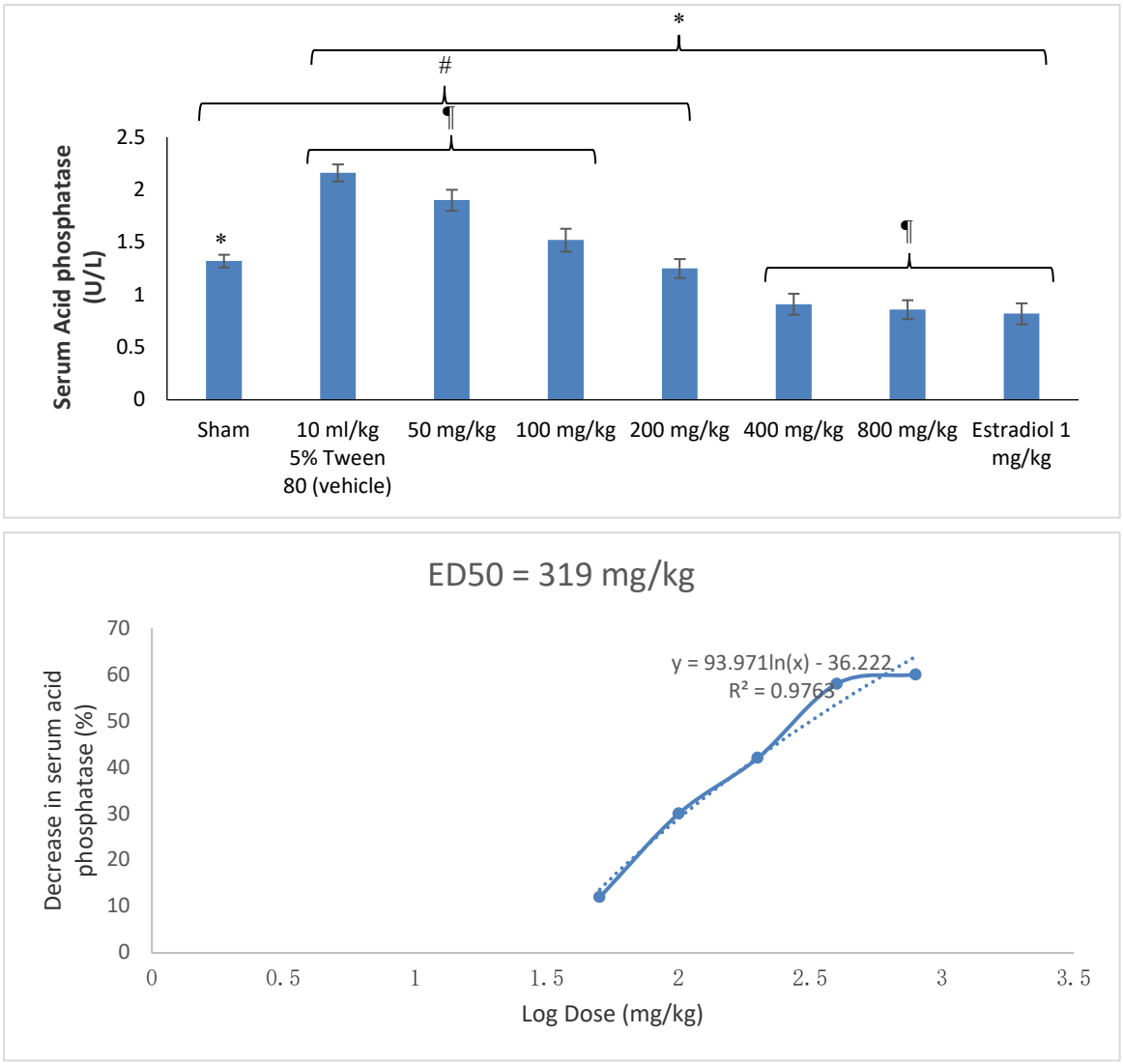


Figure 10: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on serum Acid Phosphatase
Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug).

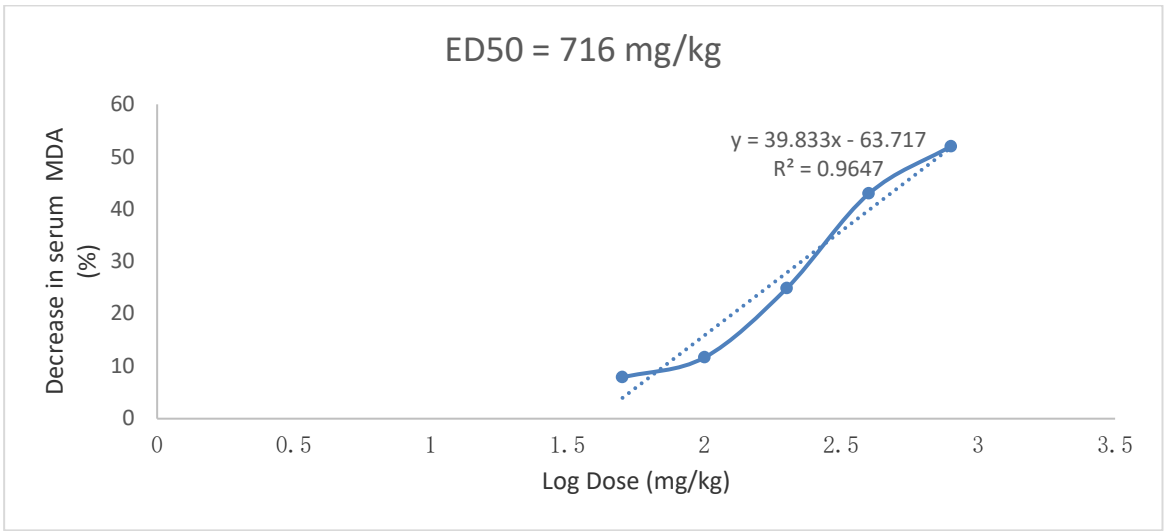


Figure 11: lipid peroxidation effective dose of phytoestrogen rich fraction of *O. schweinfurthiana*

Table 1: Effect of phytoestrogen rich fraction of *O. schweinfurthiana* on lipid peroxidation

Treatment	Dose (mg/kg)	Serum MDA (nmol/ml)
PERF	50	$5.79 \pm 0.21^{\#*¶}$
PERF	100	$5.54 \pm 0.18^{\#*¶}$
PERF	200	$4.72 \pm 0.18^{\#*¶}$
PERF	400	$3.58 \pm 0.19^{\#*¶}$
PERF	800	$3.01 \pm 0.1^{*¶}$
Estradiol	1	$3.02 \pm 0.09^{*¶}$
Sham (5% Tween 80)	10 ml/kg	$2.47 \pm 0.27^{*\#}$
Vehicle control (5% Tween 80)	10 ml/kg	$6.28 \pm 0.08^{\#¶}$

Where: ¶ = P<0.05 compared to sham; * = P<0.05 compared to 10 ml/kg 5% Tween 80 (vehicle control); # = P<0.05 compared to Estradiol (standard reference drug); PERF = Phytoestrogen rich fraction.

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