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Evaluation of Antiosteoporotic potential of Sesbania grandiflora Linn ethanolic fraction in Ovariectomized Rats

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ABSTRACT

Sesbania grandiflora (SG), an Indian herbal plant, has a long history of traditional use for managing female-related hormonal disorders and alleviating menopausal symptoms. In this study, researchers investigated the potential antiosteoporotic effects of the ethanolic extract of Sesbania grandiflora using an ovariectomized (OVX) rat model. Additionally, the study assessed the safety of the extract in relation to the uterus. The research involved thirty 6-month-old female Sprague-Dawley rats, which were randomly divided into five groups. These included a Sham-operated group and four OVX subgroups (n=6 each), all of which underwent bilateral ovariectomy. The OVX rats were further subdivided into different treatment groups, which received either the vehicle, raloxifene at a dosage of 5.4mg/kg/day, Sesbania grandiflora ethanolic leaf extracts at dosages of 250mg/kg/day, or 500mg/kg/day, respectively. The researchers evaluated the extract's effects on osteoporosis through various parameters, including body weight, uterus wet weight, serum and urine biochemical markers, bone mineral density, biomechanical strength, trabecular microarchitecture, histomorphology, and uterus immunohistochemistry. The results indicated that the daily oral administration of the ethanolic leaf extract notably ameliorated the symptoms of ovariectomy. This was evident from the reduction in serum ALP, TRAP, hydroxyproline, and urinary calcium levels in the treatment groups. Moreover, the extract exhibited positive effects on femur parameters, such as increased bone strength, bone mineral density (BMD), trabecular bone mass, and microarchitecture, which were comparable to the effects of raloxifene. Additionally, the histopathological data revealed significant restorative progress, as there was an increase in the ossification and mineralization of trabecular bone without any signs of uterine hypertrophy. In conclusion, this study demonstrated the remarkable antiosteoporotic activity of Sesbania grandiflora ethanolic extract. It suggests that the plant extract holds promise as a potential treatment for postmenopausal osteoporosis induced by estrogen deficiency, offering a natural approach through herbal resources. However, further research is warranted to fully understand the mechanisms and confirm the safety and efficacy of this herbal remedy for managing osteoporosis in menopausal women.

Keywords: *Sesbania grandiflora Linn., Ethanolic fraction, Ovariectomised rats, Osteoporosis, Bone health, Herbal medicine, Phytochemicals, Bone mineral density, Bone metabolism, Estrogen deficiency, Fracture risk, Bone remodeling.*

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INTRODUCTION

Osteoporosis is a systemic skeletal disorder characterized by the reduction of bone mass and deterioration of bone tissue architecture, resulting in increased bone fragility and the risk of fractures. It predominantly affects postmenopausal women and elderly individuals within 10-15 years after menopause. The development of osteoporosis is influenced by various factors, including biological, endocrinological, genetic, nutritional, and environmental elements, which can predispose both men and women to the condition.

While there are several available treatments for osteoporosis, such as hormone replacement therapy, bisphosphonates, selective estrogen receptor modulators (e.g., raloxifene and droloxifene), strontium ranelate, denosumab, calcitonin, synthetic parathyroid hormone, and other anabolic therapies, each of them carries potential adverse effects that may lead to non-traumatic fractures.

Therefore, there is an urgent need for alternative approaches in the form of natural extracts and constituents derived from medicinal plants for the prevention and treatment of osteoporosis. Phytoestrogens, including isoflavones (such as genistein, daidzein, glycitein, equol, and biochanin A), lignans (such as enterolactone and enterodiol), flavonoids (such as quercetin and kaempferol), and coumestans, possess structural and functional similarities to naturally occurring or synthetic estrogens. These compounds exhibit estrogenic activity through binding to estrogen receptors, thereby potentially preventing postmenopausal osteoporosis and reducing cardiovascular risks by enhancing tical conditions such as leucorrhoea, amenorrhea, anemia, emaciation, postpartum lactation stimulation, and gonorrhea in males. Additionally, the plant has been utilized for treating night blindness, ulcers, headaches, swellings, anemia, bronchitis, pain, liver disorders, as a laxative and analgesic, for fever reduction, as an astringent, and for tumor-related conditions. Scientific studies have demonstrated its pharmacological properties, including anticancer, antiurolithiatic, hepatoprotective, anxiolytic, anticonvulsive, cardioprotective, anti-inflammatory, hypotensive, depressant, diuretic, hypoglycemic, and hemolytic effects. The plant and its products are utilized in both traditional medicine and modern formulations for the alleviation of leucorrhoea and fever relief. However, the potential of S. grandiflora as an alternative preventive medicine for osteoporosis has not been explored. In this study, we conducted a systematic investigation on the effects of the ethanolic leaf extract of S. grandiflora (EQSG) using an ovariectomy-induced osteoporosis model. Our hypothesis was that S. grandiflora could potentially prevent bone loss associated with estrogen deficiency. The findings from our research indicate that treatment with EQSG is safe and effectively inhibits bone deterioration in the ovariectomy-induced osteoporosis rat model. This effect is likely attributed to the promotion of new bone formation, suggesting that S. grandiflora may serve as a protective agent for managing bone diseases.he body's defense mechanisms.

Sesbania grandiflora (L.) Pers. (Leguminosae) is a medicinal plant native to India, commonly known as "sesbania," "agathi," or "hummingbird tree." It has been traditionally used in Ayurvedic medicine. Studies have indicated that S. grandiflora contains phytoestrogens such as quercetin and kaempferol, along with other compounds like catechin, epicatechin, luteolin, myricetin, naringenin, betacarotene, grandiflorol, leucocyanidin, neoxanthin, and oleanolic acid. Traditionally, different parts of the plant have been used forvarious gynecology.

MATERIALS AND METHODS

Chemicals Reagents and Diagnostic kits

All chemicals and reagents used in this study were of analytical grade and were obtained from Sigma–Aldrich (St. Louis, MO, USA). Phosphorus, calcium, tartrate-resistant acid phosphatase, and alkaline phosphatase kits were purchased from Coral Biosystems in India. Raloxifene was obtained from CIPLA Ltd. in Goa, India, while ketamine and xylazine were obtained from Themis Medicare Ltd. in Haridwar, India, and Indian Immunologicals Ltd. in Hyderabad, India, respectively.

Extract preparation

Sesbania grandiflora leaves were procured from Bhugaon, Pune, Maharashtra. The leaves were properly identified, authenticated, and submitted as a voucher specimen (BSI/WRC/Cert./2014, ATP15) to the Botanical Survey of India located in Pune, Maharashtra. To obtain the extract, fresh leaves were shade-dried, powdered, and

subjected to extraction using an appropriate alcohol solvent, such as ethanol or methanol, instead of distilled water. The extraction process was carried out in a Soxhlet apparatus for 24 hours at a temperature range of 50-60°C. Subsequently, the obtained extract was concentrated using a rotary evaporator and stored in an airtight container at -20°C until further use. A fresh extract solution was prepared daily for conducting antiosteoporotic studies on ovariectomized rats.

Constituent identification

was carried out using high-performance liquid chromatography (HPLC) analysis. The analysis was performed using a Dionex Ultimate 3000 liquid chromatograph, which consisted of an LPG 3400 SD pump, a diode array detector (DAD 3000) with a 5 cm flow cell, a manual sample injection valve equippedwith a 20µl loop, and the Chromeleon (c) Dionex Version 7.2.5.9377 system manager used as a data processor. For separation, a reversed-phase AcclaimTM 120 C18 column with a particle size of 5µm and dimensions of 250 x 4.6mm was employed. The detection wavelength the analysis was 272nm.

In vivo studies

The present study was conducted in accordance with the guidelines provided by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. The study protocol (Research Project No. 43/1415) was approved by the Institutional Animal Ethical Committee (IAEC) at the National Toxicology Centre in Pune (Registration No. 40/CPCSEA/1999).

Thirty female Sprague-Dawley rats, aged 6 months and weighing 220±20g, were bilaterally ovariectomized to induce osteopenic condition. Following the surgery, the rats were allowed a recovery period of 2 months. The ovariectomized rats were randomly divided into four groups (n=6) for the 90-day oral treatment phase as follows: Sham-operated + vehicle (distilled water), Ovariectomized (OVX) + vehicle (distilled water), OVX + Raloxifene (5.4mg/kg/day), OVX + EQSG (250mg/kg/day), and OVX + EQSG (500mg/kg/day).

All rats were housed under standard conditions with a 12-hour light/dark cycle, maintained at an ambient temperature of 22±2°C, and relative humidity of 50-60%. They had ad libitum access to a standard diet and water throughout the experimental period. At the end of the 12-week treatment period, the rats were anesthetized using intraperitoneal injections of ketamine HCl (50mg/kg) and xylazine (25mg/kg). Bone mineral density (BMD) was measured using dual-energy X-ray absorptiometry (DEXA).

Following the BMD measurement, the rats were euthanized, and blood samples were collected through cardiac puncture. The collected blood was centrifuged at 1900xg for 10 minutes to obtain serum samples, which were then stored at -70°C for further biochemical analysis. Additionally, urine samples were also collected and kept at -70°C. Autopsies were performed to collect bones for subsequent biomechanical testing and structural analysis.

Serum and urine biochemistry

Its measurements were conducted using various diagnostic kits and methods. Serum calcium (S-Ca), alkaline phosphatase (S-ALP), urinary calcium (U-Ca), and urinary phosphorus (U-P) levels were determined using a semi-automatic analyzer (Pathozyme Smart-7, India) with diagnostic kits from Coral Biosystems, India.

Tartrate-resistant acid phosphatase (S-TRAP) levels were measured using a diagnostic kit obtained from Labcare Diagnostic Pvt. Ltd., Gujarat, India. Urinary hydroxyproline (U-HOP) and serum hydroxyproline (S-HOP) levels were measured using a modified Neuman and Logan method (Neuman and Logan, 1950).

Serum estradiol (E2) levels were determined using an enzyme-linked fluorescent assay (ELFA) method. The assay was performed with a kit from Mini Vidas BioMerieux, France, following the manufacturer's instructions.

Bone mineral density

Bone mineral density (BMD) of the total femora was measured using a dual-energy X-ray absorptiometry (DEXA) machine, specifically the Lunar iDXA from GE Healthcare, USA. BMD was calculated using the bone mineral content (BMC) of the measured area with enCORE Version 16 software (GE Healthcare, Madison, WI, USA), utilizing the small animal scan mode.

Micro-CT analysis

Micro-computed tomography (micro-CT) analysis was performed to assess the trabecular

bone microarchitecture of the right distal femoral metaphysis. High-resolution micro-CT imaging was conducted using a Tri-Foil imaging system (CA, USA), and bone histomorphometric analysis was carried out using MicroView version ABA 2.4 Software. The scanning parameters were set as follows: 1x1 binning FLY mode, Voltage 60Kev, Current 175µA, Exposure 1700ms, focal spot 32 µm magnification 4x4 with FOV 29.59mm, Frames 3, Number of projections 1024, and a total acquisition time of 37 minutes.

Biomechanical evaluation

The dried right femora were weighed using a digital balance, and the length was measured with a vernier caliper, starting from the proximal tip of the femur head to the distal tip of the medial condyle. Biomechanical strength assessment of the bones was conducted using a Universal Tensile Testing Machine (Veekay Testlabs, Mumbai, India), specifically the Model No. UTMG410B. The testing was performed ata speed of 2 mm/min. Various tests were conducted to evaluate the biomechanical properties of the femur, including three-point bending, femoral neck loading, and lumbar compression of the 4th lumbar vertebra. The tests were conducted following the method described by Shirwaikar et al.

Histomorphometric and Immunohistochemical Analysis

Histomorphometric and immunohistochemical analyses were conducted to evaluate the samples. The left femur was fixed in 10% formalin and subjected to decalcification using a 10% ethylenediaminetetra-acetic acid (EDTA) solution for a period of 10 days. The distal femur was longitudinally sectioned at a thickness of 5 µm using a rotary microtome (Leica RM22, Germany). The sections were then processed for hematoxylin and eosin staining to observe microarchitectural changes. Microscopic examination of the stained sections was performed to analyze the histopathological features.

Immunohistochemical (IHC) analysis was conducted to assess estrogen receptor (ER) expression in the uteri. ER monoclonal antibody (Lab Vision Corporation, Fremont, USA) was used for the immunohistochemical evaluation, following the instructions provided by the manufacturer. Qualitative examination of the stained cells was performed using light microscopy (Nikon H550S Eclipse Ci-L, Japan) equipped with NIS Elements Imaging Software version 4.

Statistical analysis

The data obtained from the study were presented as mean±SEM (standard error of the mean) and subjected to statistical analysis. One-way analysis of variance (ANOVA) was performed, followed by a post hoc Dunnett's test, to compare all treatment groups with both the normal control and OVX control groups. Group means were considered significantly different at a level of significance of 5% ($P<0.05$). The statistical analysis was conducted using GraphPad Prism software, version 5.03.

RESULTS

Identification of Phytoconstituents

Analysis using high-performance liquid chromatography (HPLC) demonstrated the presence of gallic acid,catechin, and quercetin in the ethanolic extract of Sesbania grandiflora, as shown in Fig. 1a and 1b. The identity of the compounds in the test sample was confirmed by comparing their retention times with those of the corresponding standards: gallic acid (retention time: 5.059), catechin (retention time: 9.179), and quercetin (retention time: 24.833).

Effect of S. grandiflora on body weight and uterine weight

The initial and final body weights of all animals in each group are provided in Table 1. While no significant difference was observed in the initial mean body weight, at the end of the study, the mean weight of the OVX (estrogen-deficient) rats increased significantly compared to the sham group ($p<0.001$), showing an approximately 37% increase relative to the initial body weight. Administration of the ethanolic extract of S. grandiflora (EQSG) at both doses significantly inhibited the increased body weight by approximately 30% and 26% ($p<0.05$) respectively, compared to the OVX group. The raloxifene group also exhibited significant inhibition ($p<0.05$) of approximately 20% after 90 days.

As expected, ovariectomy resulted in a significant reduction in uterus weight in the OVX group ($p<0.001$),with an approximately 86.5% decrease compared to the sham group, indicating uterine atrophy due to estrogen deficiency. Daily

oral administration of 250 mg/kg and 500 mg/kg of the ethanolic extract of S. grandiflora did not cause a significant increase or decrease in uterine weight compared to the OVX group, suggesting that EQSG did not have any uterotrophic effects. Raloxifene also demonstrated a significant prevention of uterine weight loss compared to the OVX group ($p<0.05$).

HPLC analysis revealed the presence of gallic acid, catechin and quercetin in the ethanolic extract of Sesbania grandiflora as presented in Fig. 1a and 1b. Visible peaks in the test sample was confirmed with retention time against those of the standards: gallic acid (5.059), catechin (9.179) and quercetin (24.833) respectively.

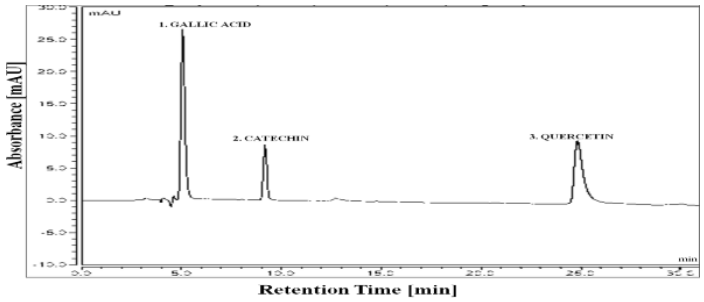

FIG. 1A. HPLC Chromatogram of standard Gallic acid, Catechin and Quercetin. 1. Gallic acid, 2. Catechin, 3. Quercetin.

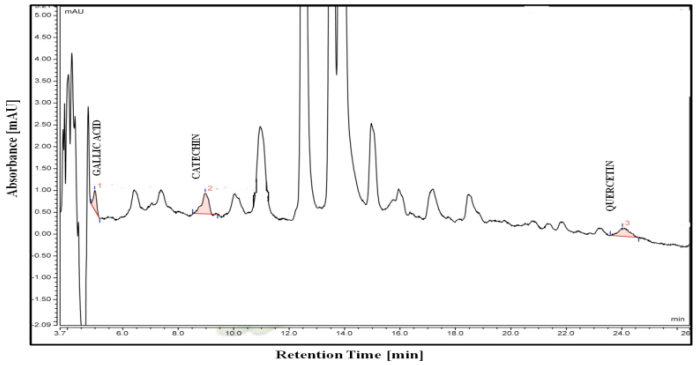

FIG. 1B. HPLC Chromatogram of the ethanolic extract of S. grandiflora.

TABLE 1. Effect of ethanolic extract of S. grandiflora on body and uterus weight

Parameters	Body weight (g)			Uterus weight (g)
Groups	Initial	Final	Difference	
Sham	224.6±2.9	251.3±7.4	26.71	0.68±0.109
OVX	225.3±6.1	324.2±4.8***	98.7***	0.091±0.007***
RLX	233.8±5.1	273.5±8.5#	40.7	0.130±0.016#
EQSG 250	221.9±3.9	291.6±9.1	69.8	0.126±0.009
EQSG 500	220.3±7.3	295.3±2.5	75.3	0.127±0.008

Body weight difference and uteri wet weight changes were assessed in the ovariectomy (OVX) model of osteoporosis. OVX rats were assigned to different treatment groups: OVX (no treatment), RLX (raloxifene, 5.4mg/kg/day), and EQSG (ethanolic extract of Sesbania grandiflora, 250 and 500 mg/kg/day) for a duration of 12 weeks. The results are presented as mean ± S.E.M with n=6 in each group. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's t-test. Statistical significance was indicated as *p< 0.05, **p< 0.01, ***p< 0.001 compared to the Sham group. Additionally, the symbol # indicated statistical significance of p< 0.05 compared to the OVX group.

Effect of S. grandiflora on biochemical parameters

The effects of the ethanolic extract of Sesbania

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grandiflora (EQSG) on serum and urine biochemical parameters were evaluated and are presented in Table 2. Ovariectomy led to a significant increase in the levels of serum alkaline phosphatase (S-ALP), urinary calcium (U-Ca), serum tartrate-resistant acid phosphatase (S-TRAP), urinary hydroxyproline (U-HOP), and serum hydroxyproline (S-HOP) excretion ($p<0.001$, $p<0.05$) compared to the sham group. Treatment with raloxifene (RLX) and EQSG (250 mg/kg and 500 mg/kg) significantly decreased urine calcium, hydroxyproline, and serum TRAP levels ($p<0.05$), indicating a reduction in bone resorption markers. However, there was no significant effect observed on urinary phosphorus (U-P) and serum calcium (S-Ca) levels. Serum alkaline phosphatase (S-ALP), a bone formation marker, was not affected bythe treatments.

TABLE 2. Effect of ethanolic extract of S. grandiflora on biochemical parameters

Parameters	Sham	OVX	RLX	EQSG 250	EQSG 500
S-ALP(U/L)	111.5±17.07	370.43±44.73***	318.33±17.34***	251.60±23.57*	317.00±45.86***
S-TRAP(IU/L)	2.11±0.31	5.05±0.18***	2.69±0.32###	2.53±0.38###	3.12±0.36##
S-Ca (mg/dl)	6.69±0.18	6.91±0.25	6.07±0.20#	6.42±0.34	6.16±0.41
S-HOP(ug/ul)	0.985±0.09	1.347±0.11**	0.689±0.05###	1.01±0.08#	0.86±0.09##
Estradiol (pg/ml)	3.58±0.16	2.66±0.26**	4.72±0.46##	3.68±0.12	2.56±0.26
U-Ca(mg/dl)	2.55±0.17	3.23±0.12*	2.19±0.08###	2.16±0.19###	2.26±0.15###
U-P(mg/L)	3.37±0.10	3.46±0.02	3.31±0.06	3.39±0.02	3.32±0.01
U-HOP(ug/ul)	0.52±0.03	0.83±0.11**	0.319±0.03###	0.19±0.02###	0.16±0.02###

The data presented are expressed as mean ± S.E.M with n=6 in each group. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's t-test for multiple comparisons. Significance levels were denoted as *p< 0.05, **p< 0.01, and ***p< 0.001 when comparing each group to the Sham group. Significance levels of #p< 0.05, ##p< 0.01, and ###p<
0.001 were used when comparing each group to the OVX group.

The impact of the ethanolic extract of Sesbania grandiflora on femur parameters

According to the findings presented in Table 3, the femur ash, ash percentage, and ash calcium content exhibited significant reductions ($p<0.05$) of approximately 12%, 9%, and 18% respectively in the ovariectomized (OVX) rats compared to the sham group. However, these reductions were significantly improved ($p<0.05$) after treatment with raloxifene (RLX) and the ethanolic extract of Sesbania grandiflora (EQSG) at a dose of 250 mg/kg. The reductions in ash content were partially reversed by approximately 15% and 10% respectively with EQSG treatment at doses of 250 mg/kg and 500 mg/kg compared to the OVX group.

Furthermore, the serum estradiol levels (pg/ml) were notably increased in the RLX and EQSG (250 mg/kg and 500 mg/kg) treatment groups compared to the OVX group ($p<0.05$). This suggests a potential attenuation of high bone turnover and resorption. Based on these results, it can be concluded that EQSG effectively reduced the bone loss associated with estrogen deficiency. No significant differences were observed in femur length among the groups. Additionally, ovariectomy led to a significant reduction in femur weight by approximately 30%, which was improved by approximately 20%, 22%, and 34% with RLX, EQSG (250 mg/kg), and EQSG (500 mg/kg) treatments, respectively, compared to the OVX group.

TABLE 3. Effect of ethanolic extract of S. grandiflora on ash content of femoral bone

Parameters	Ash weight (g)	Ash (%)	Calcium (mg/cm^3)	Femur Length (mm)	Femur weight(g)
Sham	489.0±14.72	58.49±0.56	128.76±4.66	35.41±0.36	1.03±0.12

OVX	451.48±6.61***	51.50±0.76***	103.92±2.87***	34.23±0.81	0.72±0.05***
RLX	469.40±2.83$^{\#}$	56.78±0.19$^{\#}$	115.38±0.81$^{\#}$	34.45±0.38	0.86±0.02$^{\#\#}$
EQSG 250	518.77±29.33$^{\#\#}$	59.30±1.51$^{\#\#}$	115.60±2.67$^{\#}$	34.65±0.30	0.92±0.04$^{\#\#}$
EQSG 500	503.24±11.46$^{\#}$	56.57±0.87$^{\#}$	116.57±3.00$^{\#\#}$	34.09±0.27	0.97±0.03$^{\#\#}$

The data presented are expressed as mean ± S.E.M with n=6 in each group. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's t-test for multiple comparisons. Significance levels were denoted as *p< 0.05, **p< 0.01, and ***p< 0.001 when comparing each group to the Sham group. Significance levels of #p< 0.05, ##p< 0.01, and ###p<0.001 were used when comparing each group to the OVX group.

The impact of the ethanolic extract of Sesbania grandiflora on biomechanical parameters

The effect of the ethanolic extract of Sesbania grandiflora on biomechanical parameters was evaluated, as shown in Table 4. In the ovariectomized (OVX) group, a significant decrease (p<0.05) was observed in the mechanical strength of the femur shaft, while the femur neck loading and lumbar vertebra parameters showed non-significant decreases compared to the sham group.

After 90 days of treatment, an increasing trend of improvement was observed in the mechanical strength of the femur and vertebra with the administration of EQSG at a dose of 250mg/kg (p<0.05). However, the femoral neck loading test showed a non-significant increase. Similarly, significant improvements (p<0.05, p<0.001) were observed in the femoral neck loading and lumbar vertebra compression tests with raloxifene treatment compared to the OVX control group.

TABLE 4. Effect of ethanolic extract of S. grandiflora on biomechanical parameters.

Parameters	Three point bending of Femur (N)	Femoral neck load (N)	Compression of 4th lumber vertebra (N)
Sham	106.71±6.74	158.15±9.62	77.80±3.97
OVX	70.25±1.03**	136.83±5.99	57.08±5.27
RLX	88.20±5.07	172.75±11.07$^{\#}$	97.17±5.61$^{\#\#\#}$
EQSG 250	94.25±4.96$^{\#}$	186.00±12.12	87.75±5.59$^{\#\#}$
EQSG 500	95.25±5.90	150.00±10.64	67.40±6.38

The data presented are expressed as mean ± S.E.M with n=6 in each group. Statistical analysis was conducted using one-way analysis of variance followed by Dunnett's t-test for multiple comparisons. Statistical significance was denoted as *p< 0.05 when comparing each group to the Sham group. Significance levels of #p< 0.05 and ###p< 0.001 were used when comparing eachgroup to the OVX group.

Bone mineral density

Bone mineral density (BMD) is widely recognized as a crucial parameter for assessing osteoporosis. In this study, the total femoral BMD in OVX rats decreased significantly from 0.114 ± 0.005 g/cm2 to 0.104 ± 0.001 g/cm2 compared to Sham rats ($p < 0.05$), representing an 9.04% reduction (Fig 2). However, treatment with ethanolic extract of Sesbania grandiflora (EQSG) at doses of 250 and 500mg/kg/day resulted in a notable increase in BMD by 5.53% and 5.57% ($p < 0.05$), respectively, compared to the OVX group. Furthermore, rats treated with raloxifene also exhibited a significant elevation in BMD by 8.97% ($p < 0.05$) compared to OVX rats.

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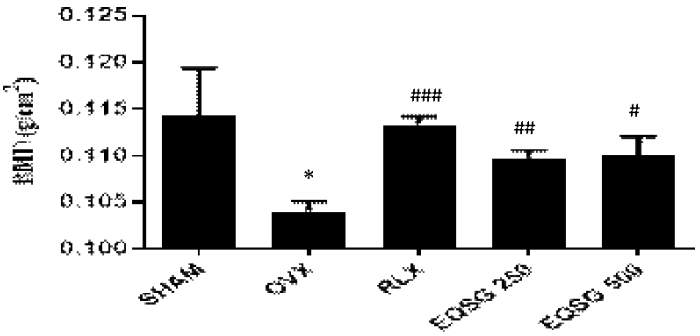

FIGURE 2 Effects of EQSG or RLX on bone mineral density (BMD) of total femora in OVX rats as evaluated by DEXA.

The data presented are expressed as mean ± S.E.M with n=6 in each group. Statistical analysis was conducted using one-way analysis of variance followed by Dunnett's t-test for multiple comparisons. Statistical significance was denoted as p< 0.05 when comparing each group to the Sham group. Significance levels of #p< 0.05 and ###p< 0.001 were used when comparing eachgroup to the OVX group.

Micro-CT analysis

The quantitative evaluation of distal femur Micro CT scan results is presented in Table 5. The morphometric parameters of the femur demonstrated the deterioration of bone microarchitecture caused by estrogen deficiency in the OVX group. These findings were characterized by a significant decrease in trabecular bone volume fraction (BV/TV), bone mineral content (BMC), tissue mineral content (TMC), tissue mineral density (TMD), and total bone volume (BV) (p<0.05). Additionally, there was a reductionin trabecular thickness (Tb.Th) and connectivity density (Conn.D), along with an increase in trabecular separation (Tb.Sp).

Treatment with raloxifene, EQSG at a dose of 250mg/kg, and EQSG at a dose of 500mg/kg resulted in improvements in these parameters. Based on the data, it can be suggested that EQSG has a bone preventive action in maintaining bone mass and trabecular microarchitecture at skeletal sites (Fig 3).

TABLE 5. Effects of S. grandiflora on microarchitecture of right distal femoral metaphysis

Parameters	Sham	OVX	RLX	EQSG 250	EQSG 500
BV/TV	0.49±0.04	0.38±0.004*	0.41±0.002	0.43±0.006$^{\#}$	0.45±0.003$^{\#\#}$
BMC (mg)	1149.9±67.1	360.2±110.7***	659.6±85.9*	981.03±167.2$^{\#}$	1565.3±47.09$^{\#\#\#}$
TMC (mg)	1081.9±100.9	331.58±54.2***	618.2±89.1$^{\#}$	938.6±15.07$^{\#\#\#}$	1264.37±77.74$^{\#\#}$
TMD (mg/cc)	799.3±6.2	358.7±21.24***	513.2±30.1$^{\#}$	708.29±14.07$^{\#\#\#}$	394.22±21.11
Tb.Sp (mm)	2.35±0.19	2.83±0.35	2.57±0.59	2.91±0.03	1.97±0.16
Tb. Th (mm)	1.21±0.12	1.02±0.17	1.2±0.16	1.31±0.32	1.13±0.03
CD (1/mm^3)	0.076±0.003	0.018±0.006***	0.020±0.003$^{\#}$	0.017±0.004	0.016±0.001
BV (mm^3)	1342.9±118.1	977.98±198.92*	1283.4±292.3	1328.01±47.65	3267.07±372.16$^{\#\#\#}$

The results are presented as mean ± S.E.M with n=4 in each group. Statistical analysis was conducted using one-way analysis of variance followed by Dunnett's t-test for multiple comparisons. The symbols *p< 0.05, **p< 0.01, and ***p< 0.001 indicate significant differences when comparing each group to the Sham group. The symbols #p< 0.05, ##p< 0.01, and ###p< 0.01 represent significant differences when comparing each group to the OVX group. The parameters assessed include bone mineral content (BMC), tissue mineral content (TMC), tissue mineral density (TMD), bone volume

fraction (BV/TV), trabecular thickness (Tb. Th), trabecular separation (Tb. Sp), bone volume (BV), and connectivity density (CD).

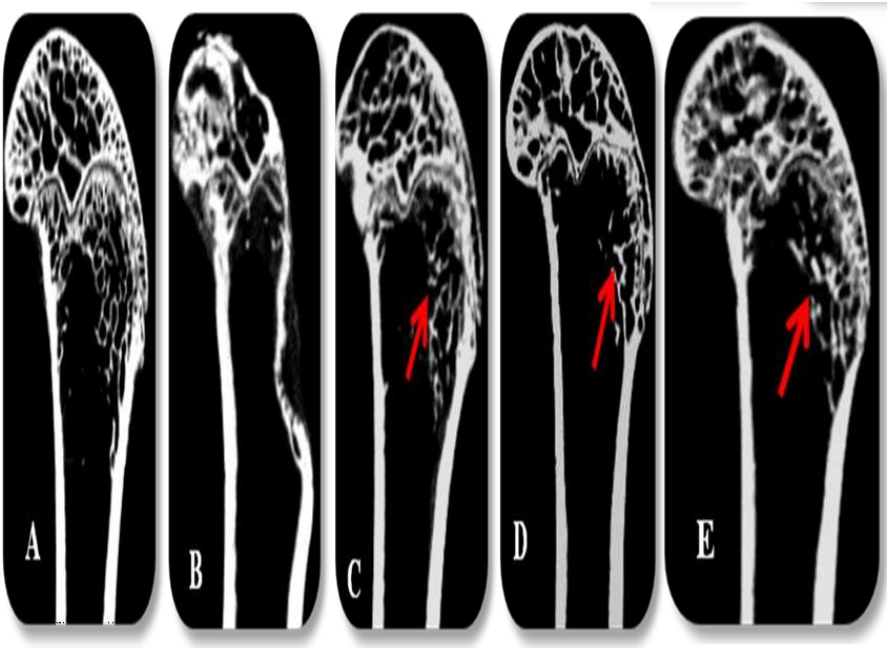

FIG 03. D Micro CT scan of distal metaphyseal femur region in each group (n = 4)

Sham; (B) OVX; (C) RLX; (D) EQSG250; (E) EQSG500. OVX group showed a notable decrease in the trabecular area. RLX and EQSG group prevented OVX-induced bone loss and significantly improved the microarchitecture after 90 days treatment (red arrow).

Histological and Immunohistochemical examination

The evaluation of bone microarchitecture and new bone formation over the 12-week treatment period was assessed through histological analysis of the left femur diaphysis (Fig 4).Osteodystrophy, characterized by trabecular disruption, lytic changes, trabecule thinning, and widening of intertrabecular spaces, was observed in the OVX group, indicating bone deterioration compared to the sham group, which exhibited normal trabecular microarchitecture and bone compactness. In the photomicrographs, significant restorative progress was observed in the raloxifene and EQSG (250, 500mg/kg) treated groups. These groups showed increased ossification, mineralization, osteoblastic activity, compactness, reduced bone resorption, and increased proliferation of trabecular fibrocartilaginous tissue.

Histological examination of uterine sections revealed degenerative changes in uterine mucosal epithelium, loss of uterine glands, and increased atrophy in the OVX group. However, RLX and EQSG-treated groups showed reversed uterotrophic changes with restoration of uterine mucosal epithelium and glands. This suggests that EQSG treatment did not exhibit uterine estrogenicity or antiestrogenicity.

Immunohistochemical analysis (Fig 5) showed yellow-brown cytoplasmic staining indicating ER expression in the endometrium, interstitial cells, and smooth muscle cells across all groups. The OVX group exhibited decreased ER labeling compared to the Sham group, while the RLX and EQSG (250, 500mg/kg) treated groups showed a relative increase in ER expression after 12 weeks of treatment.

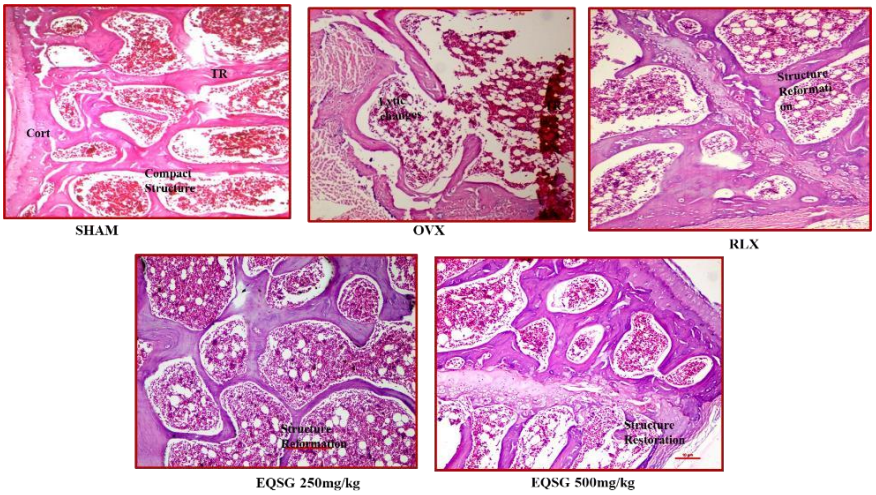

FIG 04. Effects of S. grandiflora on histology of left femur diaphysis microarchitecture (100×).

(A) The Sham group exhibited normal, compact, and uniform trabecular microarchitecture.
(B) The OVX group showed trabecular disruption, lytic changes, trabecule thinning, widening of intertrabecular spaces, and loss of interconnectivity, indicating bone deterioration.
(C) The Raloxifene group displayed less trabecular disruption, thinning of trabeculae, loss of interconnectivity, and narrowed intertrabecular spaces, indicating partial restoration of normal architecture in the epiphyseal femoral region.
(D) (D-E)The EQSG 250mg/kg and 500mg/kg treatment groups exhibited trabecular reformation and restoration of normal architecture, along with an increase in bone cells. TR: Trabecular bone, Cort: Cortical bone.

DISCUSSION

According to the World Health Organization (WHO), osteoporosis is recognized as a significant global public health concern, ranking second only to coronary heart disease in terms of its impact on individuals worldwide, regardless of race or ethnicity, and affecting individuals of all ages. The International Osteoporosis Foundation estimates that the annual cost burden of osteoporosis worldwide will rise to USD 131.5 billion by 2050.

The ovariectomized (OVX) rat model is widely accepted as an experimental model for postmenopausal osteoporosis. Ovariectomy leads to increased body weight, elevated bone turnover, expansion of the marrow cavity in the femoral diaphysis, decreased bone mineral density (BMD), and increased bone resorption on the endocortical surface. These effects are similar to the characteristics observed in human osteoporosis.

In this study, the effects of the ethanolic extract of Sesbania grandiflora (EQSG) were compared to raloxifene, which acts as a hormone modulator with fewer side effects. Previous research has shown that estrogen plays a protective role in maintaining bone structure and density. Estrogen binds to estrogen receptors (ERα and ERβ) located on bone cells, leading to the activation of the estrogen-receptor complex. This activation promotes osteoblast differentiation by suppressing the receptor activator of nuclear factor kappa-B ligand (RANKL) and upregulating osteoprotegerin (OPG), thereby inhibiting osteoclastogenesis. Estrogen deficiency, on the other hand, enhances osteoclastogenesis through RANKL activation and OPG downregulation, ultimately resulting in bone loss.

Previous research has indicated that in an estrogen-deficient state, there may be an increase in fat accumulation and altered metabolism due to reduced levels of leptin, a hormone involved in regulating energy intake and suppressing appetite. Leptin has also been found to have direct effects on bone metabolism, including enhancing osteoblastic differentiation, inhibiting osteoclastogenesis, and reducing trabecular bone loss in ovariectomized rats.

In our study, treatment with the ethanolic extract

of Sesbania grandiflora (EQSG) and raloxifene for 12 weeks significantly prevented an increase in body weight compared to the OVX rats, despite similar food intake. This suggests that EQSG may have an impact on body weight regulation. The effects of various drugs on bone remodeling are often assessed by evaluating bone turnover markers. Studies in postmenopausal women and laboratory animals have shown associations between levels of alkaline phosphatase (ALP), a marker of osteoblast-specific bone formation, and tartrate-resistant acid phosphatase (TRAP), a marker of osteoclast-specific bone resorption, with changes in bone microarchitecture.

Consistent with these findings, our data showed that levels of ALP, TRAP, and hydroxyproline (HOP) were significantly increased in the OVX rats. Urinary HOP levels, a marker of collagen degradation, were particularly elevated, reflecting increased activity of osteoclasts. These findings indicate an imbalance in bone turnover favoring bone resorption in the OVX group.

The bone-sparing effects of the ethanolic extract of Sesbania grandiflora (EQSG) and raloxifene treatment were observed in estrogen-deficient rats, resulting in a significant reduction in levels of alkaline phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), and urinary hydroxyproline (HOP). This indicates a decrease in bone resorption. Furthermore, both RLX and EQSG treatments reversed the increase in urinary calcium excretion, while serum calcium and urine phosphorus levels remained unchanged, suggesting that EQSG does not disrupt mineral homeostasis. The serum estradiol (E2) level, which was markedly decreased in ovariectomized (OVX) rats, was restored following EQSG administration. These findings suggest that EQSG has both antiresorptive and osteogenic properties, contributing to the amelioration of OVX-induced bone loss and promoting new bone formation.

Loss of bone mass is a significant factor that compromises bone integrity, reduces bone strength, and increases the risk of fractures. In our study, we observed a significant decrease in femur bone mineral density (BMD) in ovariectomized (OVX) rats, indicating increased bone turnover compared to the sham group. However, oral administration of the ethanolic extract of Sesbania grandiflora (EQSG) showed a significant improvement in BMD and had the potential to prevent the progression of bone loss caused byovariectomy. This was supported by the enhanced femur compressive strength and vertebral compression values observed in the treatment groups compared to the OVX rats. Additionally, the increased calcium content in the bones of the treatment groups further reinforced these findings.

Previous studies have demonstrated that ovariectomy (OVX) leads to uterine atrophy. Phytoestrogens have been widely considered as an alternative therapy for postmenopausal osteoporosis. However, concerns have been raised regarding endometrial hyperplasia, which refers to excessive cell growth in the uterus and may potentially progress to a precancerous stage. In our study, treatment with raloxifene and the ethanolic extract of Sesbania grandiflora (EQSG) at doses of 250 and 500 mg/kg resulted in an increase in uterus weight without inducing hypertrophy, as confirmed by microscopic examination of uterine cells. No cell proliferation or hyperplasia was observed. Furthermore, both EQSG and raloxifene treatment led to an increased expression of estrogen receptors (ER) in the rat endometrium.

Based on these findings, it can be suggested that EQSG is a safe option (non-uterotrophic) for postmenopausal osteoporosis treatment, exhibiting similar potential to raloxifene but with a lower risk of endometrial carcinoma.

The present findings on Sesbania grandiflora shed light on the potential mechanisms underlying its antiosteoporotic effects. Firstly, the presence of phytoestrogens such as quercetin and kaempferol, along with other phytocompounds like saponins, catechin, myricetin, and triterpenoids, may contribute to boneprotection. These compounds are believed to inhibit tyrosine kinase, DNA topoisomerase, and possess antioxidant activity. They can also inhibit angiogenesis, stimulate sex hormone binding globulin, and modulate enzymes involved in steroid metabolism. Furthermore, they may enhance the secretion of insulin-like growth factor-I (IGF-I) and osteoprotegerin (OPG) from osteoblasts through the activationof the estrogen receptor beta (ERβ) 42.

Secondly, the polyphenolic compounds present in Sesbania grandiflora, including quercetin, myricetin, catechin, and kaempferol, exhibit antioxidative potential by scavenging reactive oxygen species (ROS).

Moreover, HPLC analysis revealed the presence of (+)-catechin and quercetin in the ethanolic

extract of Sesbania grandiflora. Quercetin, in particular, has been shown to inhibit osteoclastic differentiation induced by receptor activator of nuclear factor kappa-B ligand (RANKL) through the modulation of transcription factors NFκB and AP-1. It also exerts bone-protective effects without affecting the uterus

Additionally, (+)-catechin has been reported to stimulate the growth of osteoblasts in vitro and inhibit TNF-α-induced apoptosis and production of inflammatory cytokines.

Collectively, these mechanisms may contribute to the observed antiosteoporotic effects of ethanolic extract of Sesbania grandiflora.

CONCLUSION

This study demonstrates, for the first time, that daily administration of ethanolic extract of Sesbania grandiflora (EQSG) in estrogen-deficient female rats for 12 weeks resulted in improved serum E2 levels, preservation of trabecular microarchitecture, maintenance of bone mass, and enhanced biomechanical quality. EQSG offers the advantage of being easily prepared, readily available, and cost-effective, making it a promising alternative medicine for preventing postmenopausal osteoporosis. However, further research is needed to identify the specific bioactive compounds responsible for the bone-protective effects of EQSG in vivo and elucidate the molecular mechanisms underlying its therapeutic approach as a potential preventive agent for postmenopausal osteoporosis.

CONFLICT OF INTEREST

The authors assert no conflict of interest associated with this project.

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