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Research Article
Salvia officinalis Extract and 17β-Estradiol Suppresses Ovariectomy Induced Osteoporosis in Female Rats

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Abstract

Background and Objective: Osteoporosis is a progressive metabolic disorder characterized by an impaired bone formation that leads to increased morbidity and mortality. *Salvia officinalis* is a source of phytoestrogens that could help mitigate the risk of osteoporotic rat fracture by exerting sex hormones. Therefore, the present study was designed to investigate the curative effect of *Salvia officinalis* Extract (SOE) and 17β-estradiol (E_2) and their combination on bone loss in female rats with ovariectomy-induced estrogen deficiency **Materials and Methods:** Forty adult female albino rats were divided into five groups, which included Sham control (Sham), ovariectomy (OVX), OVX+SOE, OVX+E_2 and OVX +SOE+E_2. SOE (10 mL kg^{-1}) and E_2 (30 μg kg^{-1}) had been daily gavaged in the OVX+SOE, OVX+E_2 and OVX+SOE+E_2, respectively for 6-weeks. **Results:** The model of ovariectomy resulted in osteoporosis as demonstrated by the decreased serum Ca, P, vitamin D, E_2 level associated with a significant increase in PTH levels in comparison to the sham control group. Besides, OVX to rats caused up-regulation in the levels of CTX-1, P1NP, BALP, OC and RANKL comparable to the sham control group. Moreover, SOE and E_2 significantly modulated the calciotropic parameters and improved all bone turnover markers as well as RANKL as compared to the OVX group. However, Histopathological and immunohistochemical results showed defective mineralization with the destruction of the bone matrix and increased TNF-α expression from the OVX group relative to the treated groups. **Conclusion:** These results suggest that both SOE and E_2 or their combined administration are efficient inhibitors against ovariectomy-induced bone loss in female rats.

Key words: Osteoporosis, ovariectomy, *Salvia officinalis*, 17β-estradiol, bone turnover, markers, RANKL

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Osteoporosis is a metabolic bone disease characterized by declined bone mass, bone density and micro-architectural weakening of bone tissue that can be produced by depleting estrogen levels, leading to enhanced bone fragility and susceptibility to fractures[1]. Bone loss is caused by an imbalance between osteoclast and osteoblast activities and by the uncoupling of bone resorption and formation[2]. Loss of the coupling and consequent disruption of bone homeostasis can result in several metabolic bone diseases, including osteoporosis and tumor-associated bone diseases[3].

Currently, osteoporosis has become a severe threat to human health and ultimately leads to reduced daily activity, lowered quality of life and increased mortality[4]. Roman-Blas *et al*.[5] described that in women, remodeling mechanisms are significantly influenced by ovarian function and are remarkably altered by ovarian aging and generative damage. These signs are related to estrogen deficiency and not a genetic origin. There are two types of osteoporosis: postmenopausal women: postmenopausal osteoporosis is the most prominent and is associated with estrogen deficiency. Osteoporosis associated with age (senile osteoporosis) is caused by aging and is usually due to reduced dietary calcium and vitamin D or increased activity of the parathyroid gland[6]. Interestingly, the most accurate approach used for the diagnosis of metabolic bone diseases and growth disorders is various collagenous and non-collagenous bone markers[7]. A marker of bone formation is Osteocalcin (OC), a non-collagenous bone matrix protein secreted by osteoblasts. The Procollagen type 1 N-Propeptides (PINP) released into the circulation during the synthesis of collagen are correlated with bone formation, whereas the carboxy-terminal Cross-link Telopeptide type 1 collagen (CTX-I), a type 1 collagen degradation product, represents bone resorption[8].

It has been shown that various experimental models cause osteoporosis[9]. The most well-known and popular model for studying bone loss is the rat ovariectomized (OVX) model[10]. Osteoporosis therapies aim to minimize bone loss, improve bone strength and reduce fracture incidence. Estrogen is a steroid hormone, is well-known to affect the circulatory, reproductive and bone homeostasis through inhibition of bone resorption and enhancement of bone formation[11]. By raising many cytokines, free radicals and growth factors, estrogen deficiency causes chronic inflammatory conditions, which create an altered bone microenvironment by raising bone loss and osteoclast formation[12]. Besides, estrogen deficiency reduces intestinal calcium (Ca) and Ca absorption[13]. Consequently, Hormone Replacement Therapy (HRT) is also one of the most common and successful methods to reduce the risk of osteoporosis progression[14].

There has been the latest interest in plant-derived compounds that have a comparable structure to estrogen and a similar ability to bind estrogen receptors. Breitman *et al*.[15] defined that the estrogen-like compounds reflect an unusual approach to replacing HRT for osteoporosis treatment. Besides, phenolic compounds show a significant free radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen or electron-donating agents, the steadiness of the resulting antioxidant derived radical, their reactivity with other antioxidants and lastly, their metal-chelating properties[16].

Salvia officinalis (SO) (communal sage) is a common herb generally used for food flavoring and has been used for centuries in traditional medicine for the treatment of a variety of ailments. Several of its pharmacological properties have been related to polyphenolic antioxidants[17]. Some authors have stated that *Salvia officinalis* has been used to treat menopausal symptoms and nervous disorders as an anti-inflammatory, anti-mutagenic, hypoglycemic, antiseptic, estrogenic and even as a drug[18]. Phytoestrogens are plant compounds with biological activity like estrogen, isoflavones, flavonoids, coumestans and lignans are the major groups of phytoestrogens[19]. Phytoestrogens are described to inhibit bone resorption and maintain or increase bone density[20].

The current study was therefore conducted to investigate the possible mechanism and curative impact of SOE and E_2 on bone loss and remodeling in female rats with estrogen deficiency induced by ovariectomy. Additionally, their combined potential therapeutic activity against ovariectomy in female rats was also, investigated.

MATERIALS AND METHODS

Study area: This research was conducted at the biology laboratory of the Department of Zoology, Faculty of Women for the Arts, Science and Education, Ain Shams University, Egypt from February-June, 2019.

Chemicals and drugs: 17β-estradiol (E_2) was obtained from Sigma Chemical Co., St. Louis, MO, USA. All the other reagents, solvents and chemicals used for analysis met the quality criteria in agreement with the International Standards.

Plant preparation and extraction procedures: The dried SO leaves were purchased from an herbal store of the faculty of Agriculture, Ain Shams University, Egypt. Total 10 g of the

dried plant was slowly boiled in 100 mL of distilled water and heated for 30 min. The extract was then filtered and preserved in a refrigerator (-40°C) until used in the biological assay[21].

Animals care: All animal surgical and experimental procedures were performed in conformity with the declaration of Helsinki and the guidelines for the care and use of experimental animals established by the Committee for Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the National Institutes of Health (NIH) protocol (registration number: 13/165).

This work was achieved in the Biology laboratory of the Department of Zoology, Faculty of Women for Arts, Science and Education, Ain Shams University, Egypt. This study was carried out on 40 adults (6-7-month-old) female albino rats, weighing (150-160 g). Rats were caged in a fully ventilated room and exposed to natural daily (12:12 hrs light-dark cycle). The animals were grouped in metabolic cages and maintained under standard laboratory conditions (temperature 25±2°C). They were allowed free access to a standard dry pellet diet and water *ad* libitum. They were acclimated for 1-week pre-experimentation period.

Animal model of osteoporosis and dietary treatments: Rats were surgically ovariectomized[22] under anesthesia by intramuscular (i.m.) application of the mixture ketamine/xylazine (75 and 10 mg kg^{-1} b.wt.), respectively[23]. Eight rats were abdominally resected without removal of the ovaries and served as control (sham-operated group). The surgical procedure was performed under aseptic conditions following ethical regulations for animal care and use. After 3 months of ovariectomy, rats were divided into 5 groups (n = 8 per group) according to the following experimental protocol:

- Sham-operated control group, in which adult normal (regularly) cycling female rats receiving the same vehicle in which the drugs are dissolved in a manner like the treated groups (Sham)
- Ovariectomized control group: OVX rats receiving the same vehicle in which the drugs are dissolved in a manner like the treated groups (OVX)
- OVX+*Salvia officinalis* extract: OVX rats receiving SOE orally by gavage at a volume of 10 mL kg^{-1} b.wt.[21] (OVX+SOE)
- OVX+estradiol group: OVX rats receiving 17β-estradiol (E_2) dissolved in small amounts of ethanol with the volume adjusted with olive oil to give at a concentration of 30 μg kg^{-1} b.wt. subcutaneously[24] (OVX+E_2)
- OVX+*Saliva officinalis* extract+estradiol group: OVX rats receiving SOE orally at a dose of 10 mL kg^{-1} b.wt. then treated with subcutaneously 17β-estradiol at a dose of 30 μg kg^{-1} b.wt. (OVX+SOE+E_2). All treatments were administered daily for 6 weeks

Sample collection: At the end of the experimental period (6 weeks), rats were sacrificed under anesthesia. Blood samples were collected from each rat, in dry, clean centrifuge tubes, permissible to clot at room temperature to get sera then centrifuged at 3000 rpm for 20 min. The clear, non-hemolyzed supernatant sera were quickly removed and frozen at -20°C pending further analysis.

Biochemical analysis

Calcium homeostasis measurements: Concentrations of calcium and phosphorus in serum samples were calorimetrically determined using specific diagnostic reagent kits (ab102505 and ab65622) according to the method described by Zhou and Cai and Cliffe *et al.*[25,26], respectively. Serum vitamin D was assayed by commercial ELISA kits (Mouse 25-hydroxy vitamin D (25(OH) D) ELISA Kit) according to the manufacturer's instruction.

Hormones measurements: Serum parathyroid hormone (PTH) was determined by the ELISA technique using a kit purchased from Ray Biotech, Inc., USA, according to the method of Zheng *et al.*[27]. Serum estradiol (E_2) was determined by the ELISA method using a kit purchased from MyBioSource Inc. (San Diego, CA, USA) according to the manufacturer's instruction.

Bone biomarkers measurements: Serum C-terminal telopeptide (CTX) was assessed by ELISA technique using kits purchased from MyBioSource Inc. (San Diego, CA, USA) according to the manufacturer's instruction. The Procollagen I intact N-terminal (PINP) in serum was performed by ELISA technique following the method of Vasikaran *et al.*[28]. Serum bone alkaline phosphate was estimated by the colorimetric method using kits purchased from Sigma-Aldrich Chemical Co., USA according to the method of Chen *et al.*[29]. Serum osteocalcin was measured by the ELISA technique purchased from Abcam (ab1951214) according to the method of Lee *et al.*[30].

Bone resorbing cytokines measurements: The contents of RANKL in the samples were quantified by sandwich ELISA technique using kits (ab213841 Human TNFSF11 ELISA Kit (RANKL) according to the method of Schramek *et al.*[31].

Bone histopathological studies: The collected right lower limbs femurs of rats were fixed in 10% neutral buffered formalin for 24 hrs, decalcified in 10% EDTA solution (pH = 7.4) at 4°C for 2 weeks and then embedding in paraffin. Thin paraffin sections (4 μm) were prepared and stained with H and E[32].

Immunohistochemical investigations: Formalin-fixed, paraffin-embedded section (4-μm-thick) were immunohistochemically stained for polyclonal antibody tumor necrosis factor-alpha (TNF-alpha) at 1:200 working dilution for 15 min, according to Bloomer *et al.*[33]

Statistical analysis: In the existing study, results are indicated as the mean±S.E of the mean for the eight rats in each group. Statistical Package for the Social Sciences program (SPSS), version 20.0 (SPSS Inc., USA) was used to compare significance between every two groups. The difference was considered statistically significant at $p \leq 0.05$.

RESULTS

Calcium homeostasis measurements: The data presented in Table 1 revealed a significant decline ($p<0.05$) in Ca and P levels of the OVX group when compared to the Sham group (2.05 and 1.45 verses 9.74 and 6.16 mg dL^{-1}) with the percent of difference -78.54 and -76.46%, respectively. In contrast, treatment of the OVX groups with SOE, E_2, or both SOE and E_2 together led to significant elevation ($p<0.05$) in Ca and P levels as compared to the OVX group.

Hormones measurements: The results of PTH, vit. D and E_2 in the Sham and other studied groups are illustrated in Table 2. The recorded values of vit. D and E_2 in the OVX group revealed a significant decrease ($p<0.05$) as compared to the Sham group. Whereas, the serum level of PTH was significantly increased ($p<0.05$) in OVX groups compared to the sham group. On the other hand, treatment with SOE, E_2, or both of SOE and E_2 caused a significant amelioration ($p<0.05$) of the examined parameters PTH and vit. D associated with significant increment ($p<0.05$) in the levels of E_2 When compared to the OVX group (Table 2).

Bone Biomarkers and bone-resorbing cytokines measurements: The data presented in Table 3 revealed significant increment ($p<0.05$) in CTX-1, P1NP, BALP and OC levels of the OVX group as well as RANKL levels when compared with the Sham group. On the other hand, the concurrent administration of SOE or E_2 to the OVX rats significantly ($p<0.05$) reduced the levels of CTX-1, P1NP, BALP, OC and RANKL as compared to the OVX group. The data, however, display that after treatment with a combination of SOE and E_2 significantly ($p<0.05$) restored the levels of CTX-1, P1NP, BALP, OC and RANKL near to normal levels compared to the OVX group (Table 3).

Bone histopathological studies: Rat femur bone tissues of different experimental groups stained with hematoxylin and eosin showed that the trabecular bone of the Sham group was relatively more abundant, with healthier structural arrangement and continuity (Fig. 1a). Whereas, the trabeculae

Table 1: Effect of SOE, E_2 and their combination on serum calcium and phosphorus levels in ovariectomized rats

	Groups				
Parameters Ca (mg dL^{-1})	Sham	OVX	OVX+SOE	OVX+E_2	OVX+SOE+E_2
Ca	9.74±0.13	2.05±0.07^{a}	4.94±0.06^{ab}	7.32±0.12^{abc}	9.56±0.16^{bcd}
P	6.16±0.22	1.45±0.13^{a}	4.03±0.18^{ab}	5.18±0.23^{abc}	5.80±0.24^{abcd}

Data represented as Mean±SE of 8 rats/group, a: Significant change at $p<0.05$ when compared to the Sham group, b: Significant change at $p<0.05$ contra OVX group. c: Significant change at $p<0.05$ against the SOE group, d: Significant change at $p<0.05$ against the E2 group. Sham: Sham control, OVX: Ovariectomy; SOE: *Salvia officinalis* extract; E2: 17β-estradiol

Table 2: Effect of SOE, E_2 and their combination on serum PTH, Vit D and E_2 in ovariectomized rats

	Groups				
Parameters	Sham	OVX	OVX+SOE	OVX+E_2	OVX+SOE+E_2
PTH (pg mL^{-1})	8.84±0.35	28.54±1.53^{a}	20.11±1.34^{ab}	14.55±0.73^{abc}	10.36±0.80^{abcd}
Vit D (ng mL^{-1})	38.47±3.10	9.99±0.99^{a}	24.58±2.39^{ab}	28.32±2.31^{abc}	35.15±2.91^{abcd}
E2 (pg mL^{-1})	78.67±3.57	9.81±1.46^{a}	40.30±1.81^{ab}	63.73±3.23^{abc}	74.36±4.09^{abcd}

Data represented as Mean±SE of 8 rats/group. a: Significant change at $p<0.05$ when compared to the Sham group; b: Significant change at $p<0.05$ contra OVX group. c: Significant change at $p<0.05$ against the SOE group; d: Significant change at $p<0.05$ against the E2 group. Sham: Sham control; OVX: ovariectomy; SOE: *Salvia officinalis* extract; E2: 17β-estradiol

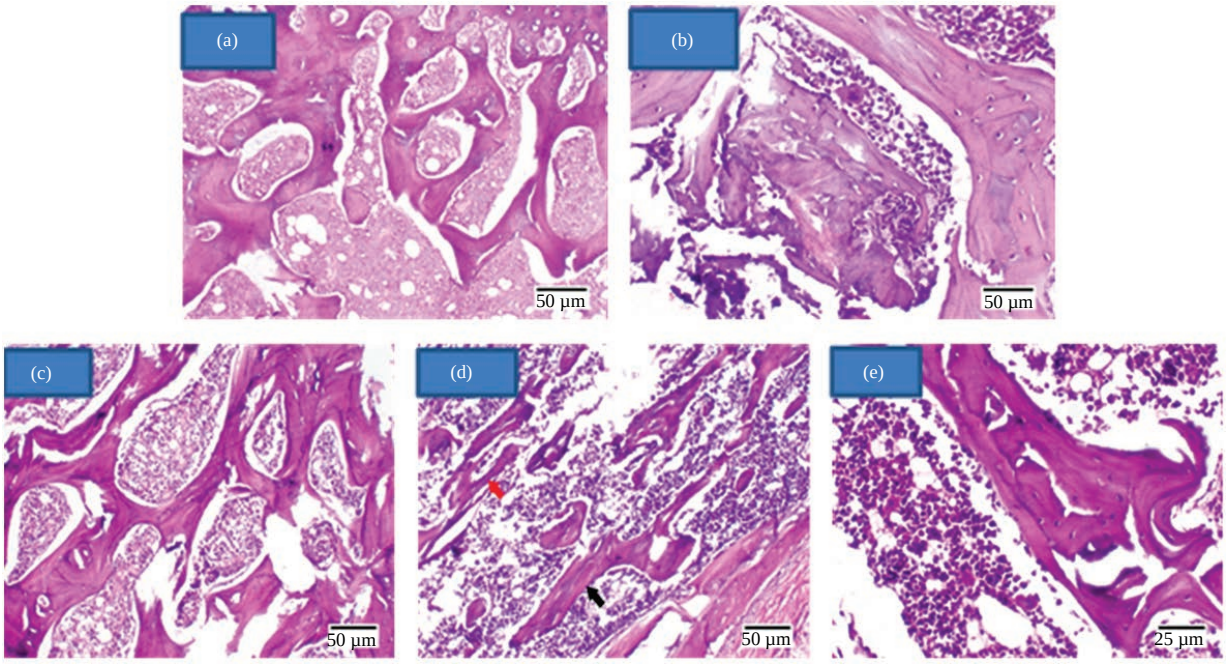

Fig. 1(a-e): Histological examination of bone tissue (H and E)

(A) Sham group showed normal bone structure with a normal density of trabecular medullary bone of endochondral origin with parallel regular cement line. (B) The OVX group showed defective mineralization with disorganization and destruction of the bone matrix. (C) The SOE treated group exhibited thicker trabeculae with increased interconnectivity, despite the presence of the osteoid tissue in some trabeculae. (D) The E_2 treated group showed moderate improvement of the osteoporosis, the examined sections showed thick and thin trabeculae. (E) The SOE+E2 treated group provided the best protection among the different treated groups, while the trabecular bone appeared thick

Table 3: Effect of SOE, E_2 and their combination on CTX-1, P1NP, BALP, OC and RANKL levels in ovariectomized rats

	Groups				
Parameters	Sham	OVX	OVX+SOE	OVX+E_2	OVX+SOE+E_2
CTX-1(ng mL^{-1})	1.22±0.22	3.04±0.49^{a}	1.77±0.30^{ab}	1.54±0.25^{abc}	1.25±0.19^{bcd}
PINP(µg L^{-1})	26.47±2.78	115.13±5.62^{a}	62.91±3.97^{ab}	44.42±3.86^{abc}	29.14±3.27^{abcd}
BALP (ng mL^{-1})	22.23±2.74	70.62±5.77^{a}	33.80±2.75^{ab}	28.54±2.68^{abc}	24.74±2.50^{abcd}
OCN (ng mL^{-1})	10.50±1.60	36.01±2.97^{a}	21.66±2.60^{ab}	17.10±1.94^{abc}	12.05±1.34^{abcd}
RANKL(pg mL^{-1})	41.97±3.62	216.42±6.88^{a}	72.50±4.19^{ab}	63.57±3.09^{abc}	52.03±3.37^{abcd}

Data represented as Mean±SE of 8 rats/group. a: Significant change at $p<0.05$ when it compared to the Sham group; b: Significant change at $p<0.05$ contra OVX group; c: Significant change at $p<0.05$ against the SOE group; d: Significant change at $p<0.05$ against the E2 group. CTX-1: carboxy-terminal cross-linked telopeptide of type 1 collagen; PINP: Procollagen type 1 N-terminal Propeptide; BALP: Bone-specific alkaline Phosphatase; OC: Osteocalcin; RANKL; Receptor activator of NF-κB ligand. Sham: Sham control; OVX: Ovariectomy; SOE: *Salvia officinalis* extract; E2: 17β-estradiol

of the OVX group showed decreased continuity and destruction in the bone matrix compared to the sham control group. Besides, marked bone resorption, with thinning of alveolar bone trabeculae and increased osteoclastic activity were observed in this group (Fig. 1b). Moreover, the bone section of the OVX+SOE treated group revealed newly formed bone, with alternating resting and reversal lines demarcating the fusion among old and new bone. However, minimal osteoclasts were still evident within the malformed lacunae, with an irregular outline of the alveolar bone (Fig. 1c). Furthermore, OVX+E_2 treated group showed also minimal osteoclasts were still evident within the malformed lacunae, with an irregular outline of the alveolar bone compared with OVX untreated group (Fig. 1d). OVX+SOE+E_2 treated group showed more bone formation with regular bone surface. The bony trabeculae were similar to that of normal intact bone lined by resting osteoblasts deprived of osteoclasts (Fig. 1e).

Immunohistochemical investigations: To demonstrate the microscopic finding of the increased inflammatory response, the antibody specific for activated TNF- α immune-staining in

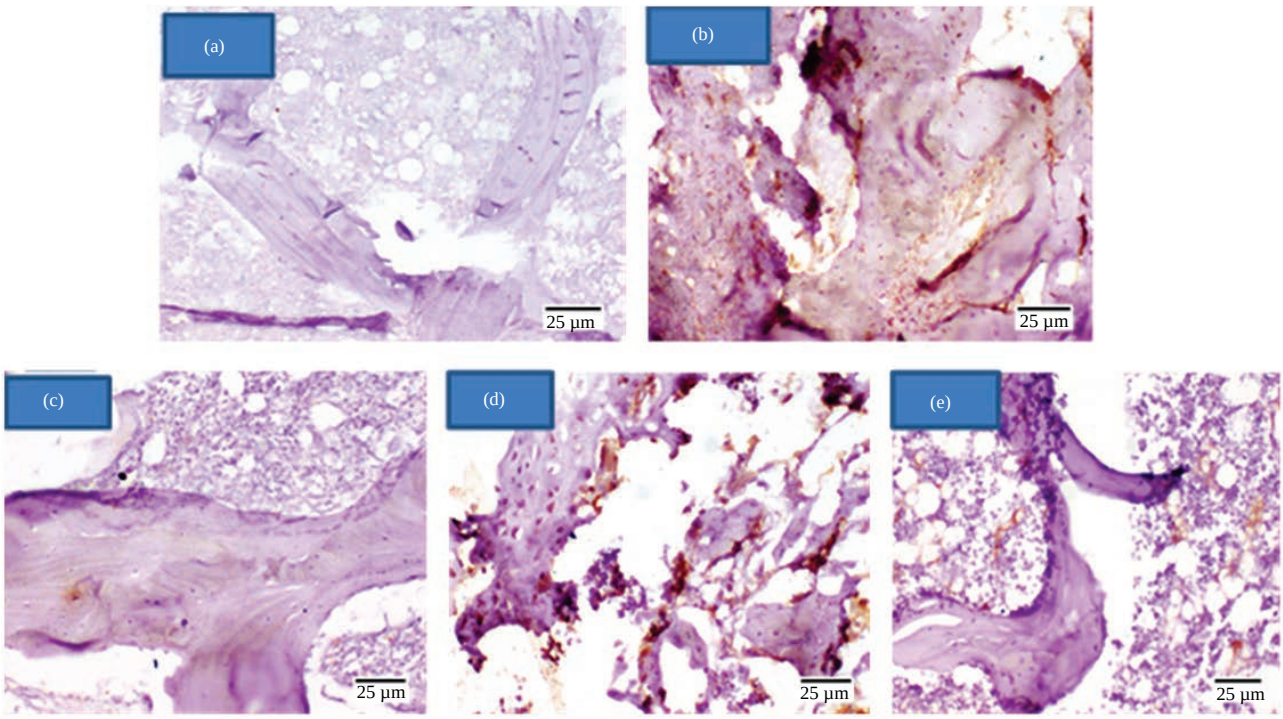

Fig. 2(a-e): Immunohistochemical determination of the antibody specific for activated TNF-a in bone tissue

(A) The Sham group showed a negative reaction. (B) The OVX group showed extensive diffuse expression of TNF-a in the trabecular bone and bone marrow. (C) The SOE treated group showed a markedly reduced expression that was prominent in the osteocytes. (D) The E_2 treated group exhibited relatively moderate expression of TNF-a positive cells. (E) The SOE+E_2 group showed the maximum reduction of TNFa expression that was restricted in few bone marrow cells

bone tissue after different treatments (Fig. 2) showed negative results of the Sham group (Fig. 2a). Whereas, in the OVX group there was a significant increase in the immune reactive cells of TNF-α present in the cytoplasm with morphology consistent with inflammation as compared to the Sham control group (Fig. 2b). On the other hand, the groups of OVX treated with SOE or E_2 showed moderate expression of TNF-α in bone tissue (Fig. 2c-d). While the combination treatment with SOE and E_2 showed a tiny expression than the OVX group (Fig. 2e).

DISCUSSION

Osteoporosis is a public health problem that is characterized by weakened bone formation rendering the bone more vulnerable to fractures[1]. Therefore, it is crucial to identify novel therapies that can enhance osteoporotic fracture healing. So, the present work was conducted to investigate the potential therapeutic effects of SOE and E_2 alone or in combination against ovariectomy-induced osteoporosis in female rats. Ovariectomy has shown that, as with postmenopausal women, it raises the risk of osteoporosis. Ovariectomized rats, however, developed bone changes such as those seen in osteoporotic women, as shown in ovariectomized rats by bone Ca, P, parathyroid hormone and vitamin D levels[34]. In the present study, the model of ovariectomy caused significant reductions in the serum levels of Ca, P, Vit D and E_2 whereas, it revealed a significant increase in the Parathyroid Hormone (PTH) level as compared to the sham control group. These findings are consistent with those stated by Elbahnasawy *et al.*[35] that following ovariectomy, estrogen deficiency is marked by decreased intestinal calcium absorption and can lead to the associated loss of bone. Besides, decreased calcium absorption has been variously attributed to decreased circulating 1,25-dihydroxy vitamin D levels and gastrointestinal resistance to 1,25-dihydroxy vitamin D action[36]. This stimulates (PTH) secretion, which contributes to the increase in bone remodeling, especially osteoclastic bone resorption, which increases bone loss and, consequently, fracture risk[37,38].

Moreover, the results of the current study clearly showed that an experimental model of ovariectomized rats was found to induce an elevation in all bone resorption and formation markers namely C-terminal telopeptide type 1 (CTX-1), procollagen type I intact N-terminal propeptide (PINP), Bone Alkaline Phosphatase (BALP) and osteocalcin (OCN) as well as RANKL levels versus the sham control group. It is well known

that in rats, OVX induces postmenopausal osteoporosis along with an obvious increase in bone resorption, increased bone fragility and substantial trabecular bone loss due to estrogen deficiency, thereby raising bone turnover. This involves an increased bone turnover rate with more resorption than formation and greater cancellous loss than cortical bone loss[39]. Moreover, Zhang *et al.*[40] detected that CTX-1 is increased after OVX and consequently increases the total activity of osteoclast. These results are consistent with the present findings where we found that higher concentrations of plasma CTX-1 in the OVX group than in the sham-operated group, which indicated the increased bone resorption[41].

Bone formation markers, on the other hand, represent the behavior of osteoblasts, including type I collagen N-propeptide (PINP), alkaline phosphatase bone isoform (BALP) and osteocalcin (OC). Both osteoblasts and fibroblasts are formed by them[42]. In other research, elevations in bone formation markers were observed in OVX rats relative to sham-operated animals[43]. Interestingly, it was suggested that ovariectomy was shown to increase the levels of BALP and OCN representing the initial bone turnover (resorption and formation)[44]. The number of bone resorption sites and those of bone formation sites are increased during ovariectomy or estrogen deficiency. At the early stage of each bone formation period, BALP is then released into the blood, so an increase in its production in the ovariectomized population is highly suggestive of increased bone turnover[45]. Our data is consistent with this finding and supported by another previous study that reported that ovariectomized bone loss is categorized by higher bone turnover rates, represented by the higher bone ALP, serum OCN and urinary CTX levels, suggesting an increase in osteoblastic and osteoclastic activity, resulting in an overall net bone loss[46].

Moreover, OVX is considered as a state of low-grade inflammation that leads to the elevation of inflammatory cytokines including TNFα, RANKL and IL-1 that contribute to increased bone loss. A bone-remodeling process occurs by the concerted action of bone-forming osteoblast cells and bone-resorbing osteoclast cells. Moreover, Osteoblasts and osteoclasts are associated with the expression of bone-resorbing cytokines. RANKL belongs to the superfamily of TNF. It is a significant factor in osteoclastic development[47]. It stimulates osteoclast differentiation and activity by increasing the production of macrophage-colony stimulating factor and receptor activator of nuclear factor-κB ligand (RANKL)[48]. In our study, the increased RANKL level in OVX rats is due to estrogen deficiency that correlated to an increase in bone turnover rate and high expression of bone-resorbing cytokines such as RANKL, which accelerate the process of bone resorption through enhances osteoclast differentiation and function and consequent bone loss[49].

Interestingly, the results of our study documented that the administration of SOE to OVX rats induced an increment in levels of serum Ca, P, vitamin D and E_2 accompanying by a decline in PTH hormone. These results in agreement with other studies, where it has been observed that administration of SOE in the form of sage tea restored the decreased levels of serum Ca and P and significantly reduced serum PTH as well as protected the bone[50]. Also, the present study showed that SOE treatment markedly succeeded to reduce the levels of all investigated bone turnover markers including CTX-1, PINP, BALP and OCN as well as RANKL levels as compared to OVX groups. This could attribute to Phytoestrogenic compounds present in the SOE that has a structural similarity to estrogen conformation and binding capabilities to estrogen receptors, which may therefore reduce the bone resorption and formation markers, in addition to polyphenolic compounds that have high antioxidant effects in protecting cells against inflammation-related bone damage and this was evidenced through significant reduction occurred in BALP and RANKL levels[51]. These findings are under those of Elkomy and Fahmy[52] who reported SOE strongly inhibits bone resorption through its possessing high content of biologically active essential oils such as Rosmarinic Acid (RA) and other polyphenolic compounds.

The data of the present experiments demonstrated that with 17β-estradiol treatment the serum levels of Ca, P, vitamin D and E_2 were significantly incremented and the level of PTH was significantly reduced as compared to the sham control group. Our previous studies have shown that estrogen replacement therapy is the most potent inhibitor of bone resorption and the most widely recommended method to reduce the rate of postmenopausal bone loss[53]. It regulates bone homeostasis through regulatory effects on the immune system and oxidative stress and direct effects on bone cells. Furthermore, estradiol could produce beneficial skeletal effects through reducing activation of bone metabolic units[54]. Besides, enhance the survival of osteoblasts via local cytokines or other growth factors, as well as improves the efficiency of gastrointestinal calcium absorption and renal calcium conservation[55].

Our results showed that E_2 treatment led to significant reductions in the levels of CTX-1, PINP, BALP and OCN mirrored by down-regulation in the RANKL level as compared to the OVX group. This concurs with the results of Müller *et al.*[56], who proved that estrogen replacement therapy is a viable tool to protect bone from postmenopausal

osteoporosis. Estrogen inhibits osteoclastogenesis through the down-regulation of inflammatory cytokines from T and B lymphocytes[57]. Also, B cells generate RANKL, which is a possible osteoclast formation regulator. Under conditions of estrogen deficiency, B cells increase RANKL secretion, which binds to osteoclast precursors, causing their differentiation[58]. In this exploration, combined administration of SOE with E_2 exhibited a favorable anti-osteoporotic effect against ovariectomy-induced osteoporosis in female rats through improvements in all serum levels of Ca, P, vitamin D, E_2, PTH hormone and all bone turnover markers through suppression of inflammation via downregulation of RANKL levels. In line with these findings, previous reports have shown that Hormone Replacement Therapy (HRT), as estrogens alone or in combination with gestagens, was not only shown to alleviate postmenopausal complaints but also exerted a positive effect on bone mineral density. Therefore, great interest emerged in using this treatment also for preventing or reducing the progress of osteoporosis[59].

Furthermore, previous studies suggest that treatment with hormones extracted from plants called phytoestrogens tends to have fewer or no adverse side effects and to have a lower risk of cancer than treatment with E_2 alone. The food consumption of fruit and legumes containing plant hormones or the administration of the respective active pharmaceutical ingredients was therefore considered to be a replacement for the missing endogenous development of E_2[60]. The *Salvia* sp. Ethanol extract has been reported to inhibit bone loss in ovariectomized rats by suppressing osteoclastogenesis through inhibition of osteoclast differentiation induced by RANKL, by suppressing phosphorylation of MAPK and Akt and expression of osteoclast marker genes[61]. The data is in agreement with this idea since the significant improvement observed in all investigated bone markers and RANKL levels in the SOE+E_2 group indicating synergistic effects of SOE treatment in inhibiting bone resorption on ovariectomized rats suffering from the deficiency of estrogen hence preventing osteoporosis.

Our results also suggested that the combination treatment with SOE and E_2 contributes to the enhancement of the level of estrogen and consequently inhibit resorption in the bone through the phytoestrogens and phenolic compounds present in SOE which gave additional support to the earlier suggestions that phytoestrogens can bind with nuclear estrogen receptors and are strikingly similar also in chemical structure to the mammalian estrogen, estradiol. Besides, hormone replacement therapy with estrogen eradicated bone loss associated with ovariectomy[62,63].

In this study, OVX rats displayed several histological bone changes manifested by a significant reduction in the size and shape of trabecular bone and destruction in bone matrix compared to sham control rats suggesting osteoporosis, which might be due to lack of estrogen. In line with these results, previous studies showed that the decline of estradiol level in the model of bilateral ovariectomy is a key mechanism leading to increased body mass, decreased bone mass and induced oxidative stress[64]. Oxidative stress including Reactive Oxygen Species (ROS) is involved in estrogen deficiency-induced bone loss through increased osteoclast activity leading to an imbalance between the formation and resorption of the bones[23]. Moreover, Manifestations of osteoporosis were observed in the form of degeneration of bone cells with the appearance of empty lacunae, multiple erosion cavities, decreased collagen fibers and a rarefied bone matrix[65].

Furthermore, SOE or E_2 treatment exhibited moderate signs of recovery in some areas of bone tissue, especially in trabecular bone[21]. The restored homeostasis in bone tissue was confirmed by combined treatment with SOE and E_2 which displayed significantly greatest healing effects in bone tissue and these effects were proved through attenuation in osteoclast formation via a reduction in the inflammatory mediators RANKL levels and improve the observed morphological alterations in the trabecular bone microarchitecture through promoted new bone formation via normal restoration of serum bone formation markers PINP, BALP, OCN[59]. The findings of our study show that the combination of *Salvia officinalis* extract and 17β-estradiol hormone therapy suppressed ovariectomy-induced osteoporosis and had a significant impact on bone metabolism with no adverse side effects other than the separate application and further future animal studies are required to discover more therapeutic properties of *Salvia officinalis* extract in the prevention of osteoporosis.

CONCLUSION

In conclusion, this study concluded that OVX-induced osteoporosis in female rats was significantly attenuated by either SOE or E_2 therapy. The combined SOE and E_2 had a stronger antiosteoporotic effect than separate use and this was evident by improving bone resorption markers, enhancing bone formation markers and suppressing inflammation as well as improving immunohistochemical markers, as confirmed by histological study. Therefore, the present results have suggested that administration of SOE in

combination with E_2 are competent inhibitors of bone loss in OVX rats and may be a promising therapeutic tool for the treatment of ovariectomy-associated osteoporosis.

SIGNIFICANCE STATEMENT

The current study showed that the model of ovariectomy caused bone loss in female rats. Also, it provides strong evidence for the role of *Salvia officinalis* extracts in retarding bone loss through its antiosteoporotic properties. The current study was therefore conducted to investigate the possible mechanism and curative impact of SOE and E_2 on bone loss and remodeling in female rats with estrogen deficiency induced by ovariectomy. Additionally, their combined potential therapeutic activity against ovariectomy in female rats was also, investigated.

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