Original article

Antiosteoporotic activity of *Salvadora persica* sticks extract in an estrogen deficient model of osteoporosis

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Objectives: The effect of *Salvadora persica* sticks on prevention of tooth decay is well established, but the effect of *S. persica* stick extract (SPE) on the prevention/treatment of osteoporosis has not been studied. The purpose of this study is to provide baseline information of the effectiveness of SPE on ovariectomized (OVX) rat model of osteoporosis.

Methods: SPE was administered at 50, 150, and 300 mg/d orally to OVX rats for 16 weeks. Serum osteocalcin, alkaline phosphatase, calcium, and phosphorus, and urinary deoxypyridinoline, calcium, and phosphorus were measured. Bone mineral density (BMD), 3-point bending test, and histomorphometric characteristics of the femoral bone were also examined.

Results: SPE at doses of 150 and 300 mg/d, but not 50 mg/d, significantly prevented bone loss in OVX rats as proved by decreased biochemical markers of bone resorption and increased BMD and biomechanical indices of the femoral bone.

Conclusions: This study confirms a dose-dependent protective action of SPE on rat OVX model of osteoporosis. This effect needs further investigation at the molecular and clinical levels to provide a natural and cost-effective alternative for the management of postmenopausal osteoporosis.

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1. Introduction

Osteoporosis represents a major health problem especially considering the geriatric populations. Globally, osteoporosis is the cause of more than 8.9 million fractures every year, with estimated one osteoporotic fracture every 3 seconds [1,2]. The estimation of worldwide incidence of hip fractures is expected to rise by 310% and 240% in both men and women respectively in the next 30 years [3]. In Europe, osteoporosis causes disability that is comparable or greater than that caused by a variety of other diseases such as cancers, bronchial asthma, heart diseases, and rheumatoid arthritis [1]. In Egypt, calculations show that significant fraction of postmenopausal women have osteopenia (53.9%) and osteoporosis (28.4%), while 21.9% of males between 20 and 89 years old have osteoporosis [4]. Epidemiological data on the prevalence of osteoporotic fractures in Egyptian people is lacking. However, a case of osteoporotic hip fracture in an ancient Egypt female skeleton dated since the XIIth Dynasty (1990–1786 B.C.) was reported [5].

The pharmacological prevention and treatment of osteoporosis has advanced considerably over the past few years. However, the current treatments have certain restrictions including efficacy and long-term safety concerns. Estrogen replacement therapy has several nonskeletal adverse consequences including breast cancer, heart disease and stroke [6,7]. Osteonecrosis of the jaw and atypical fractures occurring in patients on long-term bisphosphonate treatment have picked up parcel of consideration, with suppression of bone turnover being the most likely clarification [8–10]. Newer anabolic agents such parathyroid hormone analogues are also available for treatment of osteoporosis. However, cost, the need for daily injections and concerns about osteosarcoma limit its widespread and prolonged use [11]. Although some herbs might offer protection against bone loss due to their estrogen-like effects but also may convey a portion of an indistinguishable dangers from supplemental estrogen [12,13]. Consequently, the search for natural, cheap, effective and safe agents to improve upon the existing therapies still represents a definite need.

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“Miswak” is an Arabic name of tooth-brushing sticks harvested from *Salvadora persica* L. of the family Salvadoraceae, an evergreen shrub grown in tropical and subtropical regions. The roots, stems and branches of this plant have been linked for long time in Islamic and pre-Islamic cultures with oral hygiene, prevention of tooth caries and enamel remineralization [14,15]. The simple get to and low cost of miswak has made it an extremely savvy caries control method in different communities [16,17].

Different extracts of individual parts of *S. persica* plant were chemically analyzed. *S. persica* sticks were found to contain ~1.0 μg/g of total fluoride and could release substantial amounts of calcium and phosphorus into water [18]. Repeated brushing with *S. persica* sticks was found to discharge fresh saponins and salivadornines, which have caries combating properties [19]. Calcium saturation of saliva due to the habitual use of miswak was also found to enhance remineralization of dental enamel [20]. However, in spite of well-established effect of *S. persica* sticks on the oral health and prevention of tooth decay, to date, there has been no baseline information regarding the effectiveness of this agent on the prevention and/or treatment of osteoporosis in human or laboratory animals. Also, none is known whether habitual users of miswak, in tooth brushing, have lower rates of osteoporosis. Therefore, the purpose of this piece of research is to provide baseline information of the effectiveness of *S. persica* stick extract on a model of osteoporosis induced by estrogen deficiency. We reasoned that the broad spectrum effects of *S. persica* on the dental enamel structure and function might allow this agent to play an important role in the prevention of osteoporosis and ameliorating bone loss in ovariectomized (OVX) rats.

2. Methods

2.1. Preparation of the extract

Commercially available *S. persica* sticks (miswak) were authenticated at the Department of Botany, Mansoura Faculty of Agriculture. Two hundred grams (200 g) of ground stems was soaked in 1000 mL of distilled water (pH, 6.8) at 4°C for 48 hours. After filtration, the resultant extract was concentrated and lyophilized under reduced pressure below 4°C to give a crude extract (1.0 g of lyophilized material equivalent to 11.4 g of the powdered stems). The lyophilized *S. persica* extract (SPE) was kept frozen and administered by morning gavage at doses of 50, 150, and 300 mg/kg dissolved in 0.5-mL distilled water/100 g of body weight.

2.2. Animals and treatments

Forty-eight, 3-month-old female Sprague-Dawley rats (200–220 g) obtained from the animal house of Mansoura Faculty of Medicine were housed in a naturally controlled lab upon arrival and acclimatized for 5 days. The rats were either OVX (n = 40) or sham-operated (Sham; n = 8). Surgical procedures were done under sodium pentobarbital anesthesia (50 mg/kg intraperitoneally). Four weeks after surgery, the OVX rats were randomly divided into 5 groups (8 rats per group): OVX vehicle control; OVX + 17β-estradiol (E2; 25 μg/kg/d), OVX + SPE (SPE50; 50 mg/kg/d); OVX + SPE (SPE150; 150 mg/kg/d), and OVX + SPE (SPE300; 300 mg/kg/d). Animals in the OVX and sham control groups were given the same volume of vehicle. Vehicle, E2, and SPE were given every morning by oral gavage starting from the 4th week after surgery and lasted for 16 weeks. Rats were pair-fed to the average food of sham group and had free access to distilled water. Diets were similar in macronutrients, calcium (0.9%), and phosphate (0.7%) contents. The body weights were measured every one week.

Each rat was individually housed in a metabolic cage 24 hours before sacrifice and supplied with deionized water only. Urine samples were collected and acidified with 6 mol/L HCl (0.03 vol/vol). The animals were then anesthetized with diethylether and underwent laparotomy. Blood was collected from the abdominal aorta and serum was harvested by centrifugation. Urine and serum samples were stored at −20°C for biochemical assays. The uterus was dissected off each rat and immediately weighed. The uterine index is calculated as the uterine weight/body weight before sacrifice. Femurs were dissected, wrapped, and stored at −20°C until biomechanical and structural analysis. All experiments were conducted according to the Guide to the Care and Use of Laboratory Animals, NIH publication (1996).

2.3. Biochemical markers of bone turnover

Assay for serum calcium (SCa), serum phosphorus (SP), and bone-specific alkaline phosphatase (ALP) activity was done using commercially available kits (Boehringer Mannheim GmbH, Mannheim, Germany). Assay for the urine calcium (UCa) and urine phosphorus (UP) was done as for the serum samples. Urine creatinine (UCr) was estimated colorimetrically using commercial kit (Boehringer Mannheim GmbH). Serum osteocalcin (OC) was estimated by one step sandwich enzyme linked immunosorbent assay using streptavidin technology (Boehringer Mannheim GmbH). The urinary deoxypyridinoline (DPD) concentration was estimated by a competitive enzyme immunoassay in a microassay stripwell method (Quidel, Mountain View, CA, USA) according to the manufacturer instructions, and the data were corrected for UCr concentration for variation in urine volume.

2.4. Assessment of bone density

To measure bone changes due to OVX and treatments, rats were scanned before surgery (baseline) and at the end of experiments with dual-energy X-ray absorptiometry operated with appropriate small animal assessment software (GE Lunar Prodigy, GE Healthcare, Chicago, IL, USA). The femur was scanned at 1 mm/s speed and 0.5 × 0.5-mm resolution. The mean of 3 repeated measurements was used in order to eliminate repositioning errors. The bone mineral density (BMD) was calculated as milligrams per square centimeter (mg/cm²).

2.5. Biomechanical testing

Biomechanical strength was assessed in the left femurs by the 3-point bending test using a materials testing machine (MTS Corp., Minneapolis, MN, USA). Using a digital caliper, the length of each femur was measured and its midpoint was marked before executing the 3-point test. Bone samples were positioned in a similar orientation on 2 support points 19 mm apart, and the force was applied by a crosshead to the femoral midshaft moving at 1 mm/min constant speed. The central load and displacement were recorded until fracture occurred. From the load-deflection curve, the maximum load expressed in Newtons (N), stiffness (N/mm), energy absorption (N.mm), maximum stress (megapascals or MPa) and Young’s modulus (MPa) were calculated as previously described by Turner and Burr [21].

2.6. Digital histomorphometric analysis

After cleaning and fixation in 10% buffered formalin, the right femurs were decalcified in 10% formic acid for 10 days. The decalcified bones were dehydrated in series of ethanol gradients and processed in paraffin. Sections of 5-μm thickness from the distal femur 1 mm away from the midpoint of the growth plate-
metaphyseal junction were stained with hematoxylin and eosin and acquired using Olympus digital camera set on Olympus microscope with 1/2 x photo adaptor and 40 x objectives. Bone morphometric indices including trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp), were analyzed on Intel Core i5-based PC using VideoTest-Morphology software (St.-Petersburg, Russia) with dedicated built-in routine for area and calibrated distance measurement. Five random fields from each section were analyzed and the operator performing the image analysis was blinded to the treatments associated with each sample.

2.7. Statistical analysis

Data were presented as mean ± standard error of the mean. Statistical analyses were performed as appropriate by using the Student t-test or the one-way analysis of variance followed by Dunnett post hoc test. P-values of 0.05 or less were considered significant.

3. Results

3.1. Body and uterus weights

In this study, all rat groups had a similar mean basal body weight. The body weight of control OVX rats were significantly higher than the sham group after 4 weeks of surgery (P < 0.01), and continued throughout the study. The uterus weights in the OVX group were significantly lower than the sham group (P < 0.01), indicating the success of the surgical procedure. E2 administration restored both body and uterine weights of OVX rats to the sham level after 4 weeks of treatment and lasted for the duration of the study (P < 0.01). None of the three doses of SPE exerted significant effects on body or uterine weights of the OVX rats (Fig. 1).

3.2. Biochemical markers of bone turnover

Measurement of SCa and SP concentration did not show significant changes following OVX or drug treatments, while there was marked increase of UCa and UP excretion in the OVX group (P < 0.01 for both). The serum OC and bone specific ALP, 2 putative markers of bone formation, and the bone resorption marker DPD/Cr ratio, were significantly higher in the OVX group compared with the sham operated group (P < 0.01 for all).

Sixteen weeks of treatment with E2 apparently restored all these biomarkers in the OVX rats to almost the sham-control level (P < 0.01). Similarly, SPE300 significantly attenuated the increases of UCa and UP excretion, urinary DPD/Cr levels, serum ALP and OC levels caused by OVX (P < 0.05). A smaller, but significant, reduction of these parameters, but not UP, with SPE150 was also noted (P < 0.05); while SPE in a dose of 50 mg/kg/d did not affect any of these biochemical markers (Table 1).

3.3. Bone density assessment

As shown in Fig. 2, the BMD of left femoral bone was significantly reduced in the OVX group compared with the sham group (P < 0.01). The BMD was significantly higher in the E2 (P < 0.01), SPE150 (P < 0.05), and SPE300 (P < 0.01) groups compared to the OVX group.

3.4. Biomechanical testing

As shown in Table 2, estrogen deficiency resulted in a significant reduction in the biomechanical indices of the left femoral bone of the OVX rats compared with the sham group. There was significant reduction in maximum load (P < 0.01), the energy to failure (toughness; P < 0.01), stiffness (P < 0.05), maximum stress (P < 0.05), and the Young’s modulus (P < 0.01). The reduction of all these indices was restored to almost the sham level with E2 treatment. Also, treatments for 16 weeks with SPE 300 mg/kg/d significantly restored the deterioration convicted by OVX in all these indices (P < 0.05). The dose of SPE 150 mg/kg/d significantly restored the maximum load, energy to failure, and the Young’s modulus (P < 0.05), while SPE in a dose of 50 mg/kg/d was not able to show significant improvement of any of these biomechanical indices (Table 2). Linear regression analysis showed a significant positive correlation (r = 0.69, P < 0.01) between the left femoral maximum stress and BMD.

3.5. Digital histomorphometric analysis

Microscopic examination of the stained femoral bone sections showed normal bony architecture of the sham control rats. OVX rats had significantly deteriorated architecture of the trabecular bone as confirmed by the decline of BV/TV (P < 0.001), Tb.N (P < 0.01), and Tb.Th (P < 0.05) when compared with the sham rats. In contrast, separation between trabeculae (Tb.Sp) was significantly enlarged in the OVX group compared to sham group (P < 0.01). Treating OVX

![Fig. 1. Effects of 16-week treatment with Salvadora persica extract (SPE) or E2 on body and uterus weight (wt) of ovariectomized (OVX) rats. (A) The body weight of the animals was recorded weekly during the experimental period. (B) The uterus index was represented as uterus weight divided by body weight before sacrifice. Values are presented as mean ± standard error of the mean (n = 8 rats per group, analysis of variance). *P < 0.01 vs. OVX, **P < 0.05, ***P < 0.01 vs. sham.](image-url)
Effect of 16-week treatment with E2 and SPE on serum and urine biomarkers of ovariectomized (OVX) rats.

Table 1

| Parameter                  | Group         | Sham          | E2            | SPE50          | SPE150         | SPE300         |
|----------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Percentage changes relative to Sham (%) |               |               |               |               |               |               |
| Bone mineral density (BMD) |               |               |               |               |               |               |
| Energy, N x mm             |               |               |               |               |               |               |
| Stiffness, N/mm            |               |               |               |               |               |               |
| Maximum stress, MPa        |               |               |               |               |               |               |
| Young's modulus, MPa       |               |               |               |               |               |               |

Values are presented as mean ± standard error of the mean (n = 8 rats per group, analysis of variance).

| Parameter                  | Group         | Sham          | E2            | SPE50          | SPE150         | SPE300         |
|----------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Percentage changes relative to Sham (%) |               |               |               |               |               |               |
| Bone mineral density (BMD) |               |               |               |               |               |               |
| Energy, N x mm             |               |               |               |               |               |               |
| Stiffness, N/mm            |               |               |               |               |               |               |
| Maximum stress, MPa        |               |               |               |               |               |               |
| Young's modulus, MPa       |               |               |               |               |               |               |

Values are presented as mean ± standard error of the mean (n = 8 rats per group, analysis of variance).

4. Discussion

The dissolution and swallowing of active ingredients present in S. persica sticks (miswak) in saliva during tooth brushing might have effect on skeletal bone turnover; this is the idea behind this study. We chose the rat OVX model of osteoporosis because it has the same pathological characteristics of postmenopausal osteoporosis in human [22] and we chose the oral route for SPE administration to mimic the habitual use of miswak in tooth brushing, as its constituents are dissolved in saliva; which is composed of 80% water [23]. E2 was involved in this study as a reference drug for the antiosteoporotic action on this model.

Osteoporosis is the most widely recognized metabolic disease of bone characterized by dysregulated bone formation and resorption which prompt expanded danger of bone fractures. The present report is the first to demonstrate a dose-dependent protective effect of SPE against the reduction of bone build and strength in rat OVX model of osteoporosis. The dose-related effect of SPE was confirmed by measuring serum and urine bone turnover markers, and mechanical properties, BMD and histomorphometric characteristics of the femoral bone.

Previous reports showed that estrogen deficiency caused by OVX significantly increased body weight and decreased uterine weight in rats [24,25], the 2 consequences which were entirely prevented by E2 administration. The mechanisms by which E2 restores body and uterine weights in OVX rats were also elucidated [24,25]. In the present study, none of SPE doses affected the body weight of OVX rats or exerted uterotrophic interest suggesting that SPE, at the dosages used, did not employ E2-like action on the control of body weight and/or uterine tissue development; moreover, in agreement with this observation, previous studies showed that SPE is devoid of any phytoestrogenic compounds [26].

To gain further insight into the mechanism by which SPE exerts bone-protective effect, the serum OC and ALP, 2 putative markers of bone formation, and the urinary DPD, an indicator of bone resorption were measured. In the present study, SPE significantly and dose-dependently attenuated the OVX-induced rise in bone formation.

![Table 2](image)

Table 2

| Parameter                  | Group         | Sham          | E2            | SPE50          | SPE150         | SPE300         |
|----------------------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Maximum load, N            |               |               |               |               |               |               |
| Energy, N x mm             |               |               |               |               |               |               |
| Stiffness, N/mm            |               |               |               |               |               |               |
| Maximum stress, MPa        |               |               |               |               |               |               |
| Young's modulus, MPa       |               |               |               |               |               |               |

Values are presented as mean ± standard error of the mean (n = 8 rats per group, analysis of variance).

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turnover markers as evidenced by decreased serum OC and bone specific ALP levels, and the urinary DPD/Cr ratio. In addition, SPE dose-dependently prevented the urinary loss of Ca and P indicating that SPE down-regulated the rate of bone resorption.

The global quality of bone is judged by characteristics such as dimensions, mineral densities and microarchitecture, all of which are essentially influenced by the rate of bone turnover [27]. Assessing bone parameters such as biomechanical indices and bone density, as well as examining trabecular bone microarchitecture such as trabecular thickness, and trabecular separation may enhance our capacity to estimate the overall quality of bone [27,28]. The data presented herein are consistent with these established in the literature of that estrogen deficiency leads to a significant reduction of BMD and decline of bone mass with resulting decrease in bone strength and increased susceptibility to fractures [29]. In this study, treatment with SPE at doses of 150 and 300 mg/kg/d for 16 weeks significantly improved BMD and prevented the decrease in mechanical indices of the femoral bone such as the maximum stress, energy to failure and Young’s modulus in OVX rats as shown from the 3-point bending test.

The effect of the SPE on femoral histomorphometric indices was also evaluated in stained sections of the distal femur 1 mm far from the midpoint of the growth plate-metaphyseal junction because in this region, the value of the BV/TV declines more rapidly during the first 30 days post OVX [30]. The results obtained by the use of a specialized image analysis software from rats treated with 150 and 300 mg/kg/d of SPE demonstrated that it prevented the corruption, and restored the OVX-induced depreciation of bone microstructure as evidenced by increase in the trabecular bone volume, trabecular number and thickness, and reduction of trabecular separation as per the E2 treatment in this model.

At this moment we can confirm a dose-dependent anti-osteoporotic activity of SPE in rat OVX model of osteoporosis based on valid data obtained from serum markers of bone turnover and structural analysis of the femoral bone, this effect might be due to the content of S. persica stems of high amounts of fluorides, silica, calcium, phosphorus, sulfur, together with many sterols, phytochemicals, and heavy metals [14,31]; all of which are released in water and can interact with bone cells and/or cytokines controlling bone regeneration and remodeling. Although the effects of SPE on various aspects of oral health have been widely investigated [15], there are no available studies on the effect of SPE on the skeletal system and/or bone cells, and nothing is known on the modulation by SPE of bone mineral homeostasis and/or osteoblastic/osteoclastic function; however, the scant available data from in vitro studies showed that both ethanol and hexane extract of SP preserved the viability of cultured human skin fibroblast cells [32] and lower concentrations of the water extract of SP increased the proliferation of cultured human dental pulp stem cells, while high concentrations were toxic to them [33]. Could the concentrations of SPE used in this study induce similar proliferative effect on rat osteoblasts or toxic effect on osteoclasts, or interact with cytokines responsible for bone remodeling or regeneration remains to be investigated.

5. Conclusions

The results presented herein confirm at least, partial, dose-dependent protective action of SPE on rat OVX model of osteoporosis as proved by valid data obtained from serum markers of bone turnover, biomechanical properties, and structural analysis of the femoral bone. At this point, we cannot speculate which molecule/ingredient is responsible for this effect, and whether more than one ingredient cooperates to exert it, but at least the bone-protective potential seems unlikely to be due to an E2-like action. More investigation is needed at the mechanistic and clinical levels where the cost-effectiveness of osteoporosis treatment in elderly population and post-menopausal women at high fracture risk warrants consideration. We believe that SPE has ability for further advancement as a natural alternative for the prevention of post-menopausal osteoporosis.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

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