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The Ameliorating Effects of *Salvia hispanica* Seeds Ethanolic Extract on Methylprednisolone-Induced Osteoporosis in Female Rabbits

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A B S T R A C T

This study aimed to determine the effect of an ethanolic extract of *Salvia hispanica* (Sh) seeds on osteoporosis induced by methylprednisolone (MP) in female rabbits by assessing the bone mineral density (BMD), serum levels of calcium, phosphorus, vitamin D, and parathyroid hormone, as well as histopathological changes in the femur bone. Fifty female rabbits (*Lepus cuniculus*) averaging 1800±125 g and 8±1.4 months old were used in this study. They were randomly allocated to five groups of 10 rabbits each. The negative control group received only subcutaneous normal saline (1 mL/kg BW). The positive control group received MP subcutaneously at a dose of 0.2 mg/kg BW per day for 30 days, followed by oral normal saline (1 mL/kg BW) per day for two months. Group 3 received MP followed by oral administration of Sh seed ethanolic extract at a dose of 600 mg/kg BW per day for two months. Group 4 received MP followed by oral administration of alendronate (Ale) at a dose of 3.6 mg/kg weekly for two months. Group 5 received MP followed by Ale and then Sh seed ethanolic extract. The study continued until one week after the cessation of the treatments. The results showed that the BMD and serum concentrations of calcium, phosphorus, and vitamin D significantly decreased ($P<0.05$), while serum concentrations of parathyroid hormone significantly increased ($P<0.05$) in the positive control and alendronate groups compared with the negative control group after two months of treatment and after one week of withdrawal. However, the administration of Sh methanolic extract to the animals in the three treatment groups ameliorated these parameters and reverted them to normal values. Histopathological analysis of the femur bone head revealed abnormal bone morphology in the positive control group, while Sh extract treatment preserved the normal histology in the other groups. These findings concluded to that Sh seeds ethanolic extract has the potential to prevent bone loss induced by chronic glucocorticoid therapy and may have significant implications for the treatment of osteoporosis.

Keywords: *Salvia hispanica* seeds, ethanolic extract, bone, female rabbits, osteoporosis

INTRODUCTION

Glucocorticoids are important for the treatment of chronic, non-infectious, inflammatory, and rheumatic diseases, as well as organ transplantation, due to their immunosuppressive and anti-inflammatory actions (1). However, one of the most serious side effects of glucocorticoid therapy is osteoporosis, which is related to long-term use (2, 3). Bone loss occurs when calcium (Ca) transport is prevented, leading to secondary hyperparathyroidism, hypogonadism, and impaired osteoblast activity (4, 5). Bone loss can be reduced by

following a healthy diet, weight-bearing exercise, Ca and vitamin D supplementation (6), and bisphosphonate (alendronate) therapy if necessary (7, 8). However, bisphosphonate medication has unfavorable side effects such as jaw and long bone osteonecrosis, which limits its clinical application (9-13). Therefore, there is a need to develop drugs for the treatment of osteoporosis that are less likely to cause side effects**.**

Chia seed (*Salvia hispanica*), a member of the Laminaceae family, is a rich source of omega-3 (n-3) fatty acid and antioxidants such as polyphenols, chlorogenic and caffeic acids, myricetin, quercetin, and kaempferol (14-17). These seeds contain high quantities of dietary fiber, minerals, vitamins, and proteins (18-20), as well as bioactive components such as tocopherols and phenolic compounds (21, 22). The present study aims to evaluate the effects of ethanolic extract of chia seeds on osteoporosis in female rabbits.

MATERIALS AND METHODS

Ethics

The experimental design, and procedures used were reviewed and approved in accordance with animal welfare ethical standards by the Scientific Committee of the Department of Physiology, Biochemistry and Pharmacology, College of Veterinary Medicine, University of Baghdad, in its session held on April 7, 2021, and the local Committee for Animal Care and Use at the College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq.

Collection of Seeds and Extraction

Seeds of *Salvia hispanica* L (chia) were bought from local markets in Basra -Iraq. Then cleaned and grounded by an electrical grinder. A Soxhlet apparatus and 70% ethanol were used to extract 50 grams of seeds at a temperature of 45 °C. The dry powders (100 g) were placed on a piece of a thimble and set in the extraction apparatus until a clear and colorless solvent appeared. The extract was then filtered and dried under vacuum for 4 h. by using a rotary evaporator at 40 °C, and 150 rotates per minute. Later, the crude extract of seeds was concentrated in a glass petri dish by placing it in an incubator at 40 °C until a semi-solid mass, and thick appeared. All dried extract was collected and then kept in a dark sterile glass container in the refrigerator at 4 °C (23).

Minerals Tests of *Salvia hispanica* L Seeds

To determine the mineral content of chia seeds, seeds were grinded, and weighed, 0.2 g of the seeds powder and dissolved in 5 mL of H_2SO_4 solution for digestion. Then left it for a day, then warmed up to a temperature 60 °C for 30 min, and then left to cool and 3 mL from the acid mixture consisting of 96% H_2SO_4 and $HClO_4$ (Fluka, Switzerland) was added then warmed up to a temperature 80 °C, until it turned to a bright color. The results were then diluted with distilled water to a volume of 50 mL. The concentrations of calcium (Ca), magnesium (Mg), potassium (K), iron (Fe), sodium (Na), zinc (Zn), copper (Cu) and manganese (Mn) were determined using Shimadzu 7000 atomic absorption spectrophotometer (Germany). Total phosphorus (P) was determined after ascorbic acid digestion of the samples. The absorbance was measured by spectrophotometer at 700 nm (24).

Experimental Animals

Fifty female local rabbits (Lepus cuniculus) with an average body weight of 1800±125 g and an age of 8±1.4 months were used in this study. The animals were housed in the animal house at the College of Veterinary Medicine, University of Baghdad, in individual stainless-steel cages under standard environmental conditions (temperature: 23±2 °C, humidity: 50±5%, 12-hour light/dark cycle). They were maintained on unrestricted supplies of food consisting of green leaves, fodder, and water *ad libitum*.

The rabbits were randomly divided into five equal groups of ten as follows: the control negative group (C^{-ve}) received subcutaneously 1 mL/kg normal saline daily for 90 days; the control positive group (C^{+ve}) received subcutaneously methylprednisolone (MP) of 0.2 mg/kg for 30 days (25), then received orally 1 mL/kg normal saline per day for 60 days; Group 3 (MP+Sh) received subcutaneously MP of 0.2 mg/kg for 30 days then received 600 mg/kg *Salvia hispanica* (Sh) seed ethanolic extract orally per day for 60 days; Group 4 (MP+Ale) received subcutaneously MP of 0.2 mg/kg for 30 days then was treated with alendronate (Ale) 3.6 mg/kg BW weekly orally for 60 days; and Group 5 (MP+Ale+Sh) received subcutaneously MP of 0.2 mg/kg for 30 days then was treated with Ale 3.6 mg/kg BW orally weekly and 600 mg/kg Sh seed ethanolic extract orally per day for 60 days. Half of the rabbits in each group were sacrificed after three months of treatment, and the other half after one week of treatment withdrawal.

Dual-energy X-ray absorptiometry (DXA)

Rabbits were anaesthetized using mixture of ketamine (Alfasan, Holland, 35 mg/kg BW) and xylazine (VMD Livestock Pharma, Belgium, 5 mg/kg BW) intramuscularly. All scans were performed using DXA (Hologic QDR-1000 System, Hologic Inc., Waltham, USA) after one month, two months, three months, and one week of withdrawal of treatment. The high-resolution scan was performed to evaluate the bone mineral density (BMD) at the femur of rabbits.

Collection of Blood Samples

After three months of the experiment and one week of treatment withdrawal, blood samples of 10 mL were collected from each rabbit using the heart puncture technique and a disposable 10 mL syringe. Serum was obtained by depositing blood into an anticoagulant-free

tube and centrifuging it for 10 min at a speed of 3,000 rpm. The serum was then separated into Eppendorf tubes and stored at -20 °C until analysis. Parameters such as calcium (Ca) and phosphorus (P) were measured using a special kit (BioSystem, Spain) according to the method described by (26). Vitamin D was measured according to (27), and parathyroid hormone (PTH) was measured using a special kit (Monobind Inc., USA) according to the method described by (28).

Histological Examination

After three months of the experiment and one week of treatment withdrawal, the right femur bones were extracted from each group, and the surrounding soft tissue was removed. The bones were fixed in 10% buffered formalin for 48-72 h. After fixation, the bones were decalcified in EDTA solution, with the solution changed twice or three times a week. The EDTA solution was prepared according to the method described by (29) and consisted of 50 g disodium salt, 350 mL distilled water, and NaOH. The decalcified bones were then washed in distilled water to remove residual EDTA. The bones were dehydrated in a graded series of ethanol solutions (70%, 80%, 95%, and 100%) for 2 h each and then cleared in xylene (Sigma-Aldrich) for 2 h. The bones were then embedded in paraffin wax (Sigma-Aldrich) and sectioned at a thickness of 5 µm using a microtome. The sections were mounted on glass slides and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). Microscopy (Olympus BX51) was used to examine the histopathological changes.

Statistical Analysis

IBM Statistical Package for the Social Sciences (SPSS, Version 24) was used for the data analysis. If one-way analysis of variance (ANOVA) yielded statistically significant means, then the least-significant-difference (LSD) post hoc test at $P \leq 0.05$ was used to differentiate statistically significant means (31).

RESULTS

Minerals Content of *Salvia hispanica* Seeds

Table 1 shows the concentration of minerals in the *Salvia hispanica* seeds ethanolic extract. The minerals analysis revealed the availability of several minerals such as Ca, Mg, K, Fe, Zn, Cu, Mn, and P.

Table 1. Minerals available in *Salvia hispanica* seeds ethanolic extract

Minerals	Concentration (µg/g)
Ca	905.875
Mg	795.125
K	25.1850
Fe	9.17250
Zn	18.5000
Cu	2.97250
Mn	6.94500
P	462.500

Femur BMD

After one month of treatment, the group treated with methylprednisolone alone (C^{+ve}) showed a significant decrease ($P<0.05$) in BMD compared to the group with no treatment (C^{-ve}). There were no significant changes ($P>0.05$) between the C^{+ve} group and the group treated with alendronate alone (MP+Ale). The group treated with Salvia hispanica (MP+Sh) showed non-significant differences ($P>0.05$) compared to the C^{-ve} group. However, the BMD of the group treated with methylprednisolone, alendronate, and *Salvia hispanica* (MP+Ale+Sh) showed a significant increase ($P<0.05$) compared to the C^{+ve} group but still significantly less than the C^{-ve} group**.** After two months of treatment, there was a significant decline ($P<0.05$) in BMD in the C^{+ve} group compared to the C^{-ve} group and other treated groups. The group treated with *Salvia hispanica* (MP+Sh) showed a significant increase ($P<0.05$) in BMD compared to the C^{-ve} group and other treated groups. There was no significant difference ($P>0.05$) in BMD between the MP+Ale+Sh and MP+Ale groups compared to the C^{+ve} group**.** After one week of treatment withdrawal, there was a significant decrease ($P<0.05$) in BMD in the C^{+ve} group compared to the C^{-ve} group and other groups. The group treated with *Salvia hispanica* (MP+Sh) showed a significant increase ($P<0.05$) in BMD compared to the C^{-ve} group. However, the BMD of the MP+Ale+Sh and MP+Ale groups showed a significant increase ($P<0.05$) compared to the C^{+ve} group (Table 2).

Table 2. Effect of *Salvia hispanica* L seeds ethanolic extract on femur bone mineral density (BMD) in female rabbits

	BMD (g/cm^3)		
Groups	One Month	Two Months	One Week Withdrawal
C^{-ve}	0.37±0.01 ^{a}	0.41±0.03 ^{b}	0.41±0.03 ^{b}
C^{+ve}	0.22±0.02 ^{c}	0.18±0.01 ^{d}	0.17±0.01 ^{e}
MP+Sh	0.38±0.01 ^{a}	0.46±0.02 ^{a}	0.47±0.01 ^{a}
MP+Ale	0.24±0.02 ^{c}	0.31±0.02 ^{c}	0.29±0.08 ^{d}
MP+Ale+Sh	0.28±0.01 ^{b}	0.32±0.01 ^{c}	0.31±0.07 ^{cd}
LSD	0.038	0.025	0.033

C^{-ve}: control negative group, animals received subcutaneously 1 mL/kg normal saline daily for 90 days. C^{+ve}: control positive group, animals received subcutaneously methylprednisolone (MP) at 0.2 mg/kg for 30 days and then received orally 1 mL/kg BW normal saline per day for 60 days. MP+Sh group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then received 600 mg/kg BW *Salvia hispanica* (Sh) seed ethanolic extract orally per day for 60 days. MP+Ale group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then were treated with alendronate (Ale) 3.6 mg/kg BW weekly orally for 60 days. MP+Ale+Sh group: animals received subcutaneously MP of 0.2 mg/kg for 30 days then were treated with Ale 3.6 mg/kg BW orally weekly and 600 mg/kg Sh seed ethanolic extract orally per day for 60 days

Serum Ca and P

The obtained results in Table 3 revealed a significant decline ($P<0.05$) in serum Ca and P, after two months and after one week of treatment withdrawal, in the C^{+ve} group compared with the C^{-ve} group, so there were no significant changes ($P>0.05$) between C^{+ve} group, MP+Ale, and MP+Ale+Sh groups, while there was no significant difference($P>0.05$) observed in serum Ca between MP+Sh group and C^{-ve} group.

Table 3. Effect of *Salvia hispanica* L seeds ethanolic extract on serum Ca and P levels in female rabbits

Groups	Ca (mg/mL)		P (mg/mL)	
	Two Months	Withdrawal	Two Months	Withdrawal
C $^{-ve}$	14.3±0.49 ^{a}	14.3±0.47 ^{a}	7.95±1.15 ^{a}	7.96±1.13 ^{a}
C $^{+ve}$	10.2±0.24 ^{d}	9.62±0.11 ^{b}	4.33±0.58 ^{b}	4.11±0.65 ^{b}
MP+Sh	14.5±0.49 ^{a}	15.2±1.36 ^{a}	6.08±0.99 ^{a}	7.22±0.13 ^{a}
MP+Ale	11.5±1.69 ^{cd}	9.98±0.59 ^{b}	4.70±1.46 ^{b}	4.18±1.37 ^{b}
MP+Ale+Sh	12.6±0.80 ^{bd}	11.4±1.06 ^{b}	4.48±0.39 ^{b}	4.40±0.21 ^{b}
LSD	2.29	1.85	2.47	1.89

C^{-ve}: control negative group, animals received subcutaneously 1 mL/kg normal saline daily for 90 days. C^{+ve}: control positive group, animals received subcutaneously methylprednisolone (MP) at 0.2 mg/kg for 30 days and then received orally 1 mL/kg BW normal saline per day for 60 days. MP+Sh group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then received 600 mg/kg BW *Salvia hispanica* (Sh) seed ethanolic extract orally per day for 60 days. MP+Ale group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then were treated with alendronate (Ale) 3.6 mg/kg BW weekly orally for 60 days. MP+Ale+Sh group: animals received subcutaneously MP of 0.2 mg/kg for 30 days then were treated with Ale 3.6 mg/kg BW orally weekly and 600 mg/kg Sh seed ethanolic extract orally per day for 60 days

Vit D and PTH

The results presented in Table 4 show that after two months of treatment, the C^{+ve} group had significantly lower levels of vitamin D (15.2±0.11 ng/mL) compared to the C^{-ve} group (27.8±0.07 ng/mL) (P<0.05). However, there was no significant difference in vitamin D levels between the MP+Sh group and the C^{-ve} group, and no significant difference between the MP+Ale group and the MP+Ale+Sh group. After one week of treatment withdrawal, the C^{+ve} group had significantly lower levels of vitamin D (15.0±0.12 ng/mL) compared to the C^{-ve} group (28.2±0.21 ng/mL) (P<0.05). Meanwhile, the MP+Sh group had significantly higher levels of vitamin D (27.7±3.80 ng/mL) compared to the C^{+ve} group (P<0.05). There were no significant changes (P>0.05) in vitamin D levels between the C^{+ve}, MP+Ale and MP+Ale+Sh groups. The results for PTH after two months of treatment and after one week of treatment withdrawal showed a significant rise (P<0.05) in the C^{+ve} group compared to the C^{-ve} group. There were no significant changes (P>0.05) between the *Salvia hispanica* (MP+Sh) group and the C^{-ve} group. Additionally, no significant change (P>0.05) was shown in serum PTH levels between the MP+Ale and MP+Ale+Sh groups after two months. However, after one week of treatment withdrawal, there were no significant changes (P>0.05) between the C^{+ve} group and the MP+Ale group.

Histopathological Changes

The femoral bone section of female rabbits in the C^{-ve} group showed normal micro-architecture of the bone (Figure 1). In contrast, the bone section of the C^{+ve} (Figure 2) showed abnormal histological features, including lacunae without osteocytes, erosion cavities with osteoclasts, and thinning of trabeculae in cortical bone. However, the bone section of the MP+Sh group (Figure 3) revealed well-formed compact bone with characteristic lamellae, marked normal Haversian canals, numerous mature osteoblasts, well-differentiated osteocytes in their lacunae, trabeculae bone characterized by a large empty cavity between the mature trabecular bone formation filled with bone marrow, showing branching and anastomosing trabeculae of normal thickness and shape. The female rabbits treated with alendronate in the MP+Ale group (Figure 4) showed some osteocytes inside their apparently large lacunae, while other lacunae appeared without osteocytes. Large-sized Haversian canals and non-differentiated osteoblasts were observed, and trabeculae bone formation was characterized by a thin wall. Bone specula did not anastomose with others and had a bland end. In Figure 5 (Ale+Sh group), cortical bone showed some osteocytes inside lacunae while other lacunae appeared without osteocytes. Trabeculae bone showed numerous immature osteocytes, a thin wall, and a large empty cavity space filled with bone marrow. After a week of treatment withdrawal, the bone section from the MP+Sh group (Figure 6) revealed that the compact bone's normal histology was substantially comparable to that of the C^{-ve} group. In contrast, the cortical bone in the MP+Ale group (Figure 7) showed irregularity of the general architecture, erosion cavities, and immature osteocytes. Trabeculae bone formation was characterized by thin walls, immature osteocytes, and largely empty space filled with bone marrow. The section of bone in the MP+Ale+Sh group (Figure 8) showed presence of immature osteocytes, non-differentiated osteoblasts proliferation, trabeculae bone, bone specula's not anastomosing with others, and largely empty space filled with bone marrow.

Table 4. Effect of *Salvia hispanica* L seeds ethanolic extract on Vit. D and parathyroid hormone (PTH) in female rabbits

Groups	Vit. D(ng/mL)		PTH (ng/mL)	
	Two Months	Withdrawal	Two Months	Withdrawal
C $^{-ve}$	27.8±0.07 ^{a}	28.2±0.21 ^{a}	23.4±0.07 ^{c}	23.4±0.05 ^{c}
C $^{+ve}$	15.2±0.11 ^{c}	15.0±0.12 ^{b}	55.2±0.15 ^{a}	56.3±1.22 ^{a}
MP+Sh	27.4±3.73 ^{a}	27.7± 3.80 ^{a}	24.2±1.18 ^{c}	23.9±0.21 ^{c}
MP+Ale	17.2±0.55 ^{c}	16.1±0.22 ^{b}	43.9±16.1 ^{b}	45.8±16.5 ^{a}
MP+Ale+Sh	20.1±3.64 ^{b}	17.4±0.90 ^{b}	36.0±8.22 ^{b}	37.3±5.31 ^{b}
LSD	9.77	5.29	19.1	19.4

C^{-ve}: control negative group, animals received subcutaneously 1 mL/kg normal saline daily for 90 days. C^{+ve}: control positive group, animals received subcutaneously methylprednisolone (MP) at 0.2 mg/kg for 30 days and then received orally 1 mL/kg BW normal saline per day for 60 days. MP+Sh group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then received 600 mg/kg BW *Salvia hispanica* (Sh) seed ethanolic extract orally per day for 60 days. MP+Ale group: animals received subcutaneously MP at 0.2 mg/kg for 30 days then were treated with alendronate (Ale) 3.6 mg/kg BW weekly orally for 60 days. MP+Ale+Sh group: animals received subcutaneously MP of 0.2 mg/kg for 30 days then were treated with Ale 3.6 mg/kg BW orally weekly and 600 mg/kg Sh seed ethanolic extract orally per day for 60 days

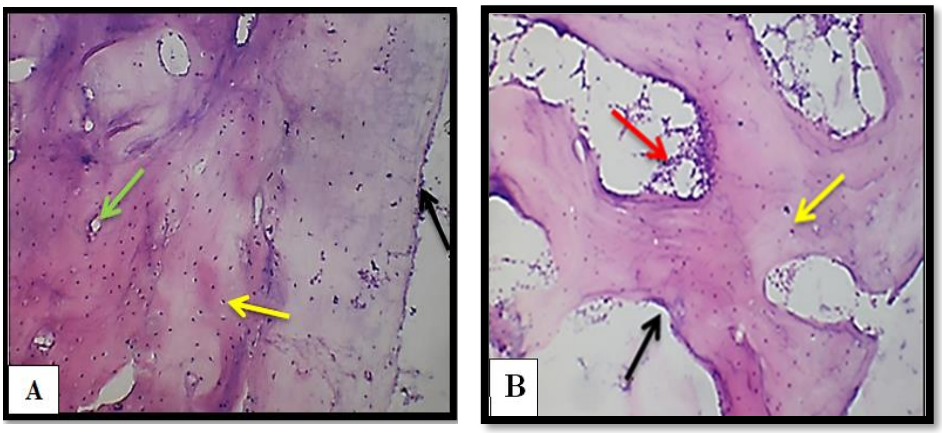

Figure 1. Photomicrograph of bone from the negative control group (C^{-ve}) showing the normal micro-architecture of the bone in female adult rabbits. **(A)** The cortical bone has a normal shape with osteocytes inside their lacunae (yellow arrow). Haversian canals are observed (green arrow), and the endosteum is lined by osteoblast cells (black arrows). **(B)** Trabeculae bone consists of osteocytes cells inside the lacunae with large empty cavity between the mature trabecular bone formation filled with bone marrow (red arrow). H&E, 10×

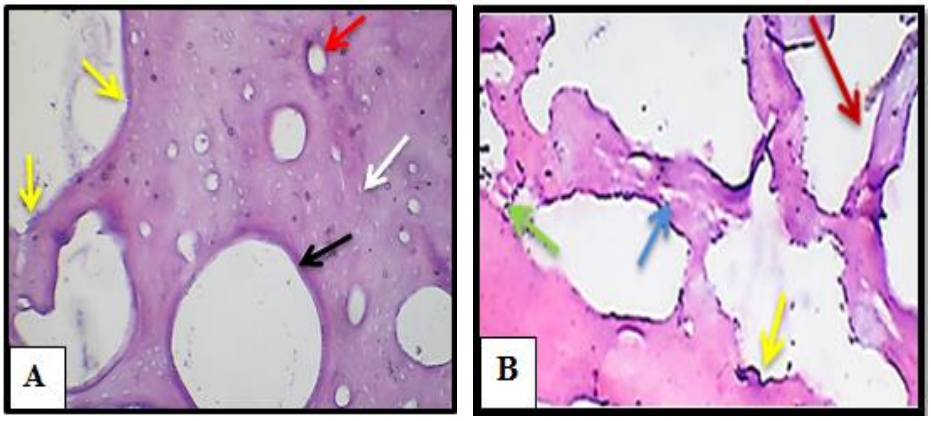

Figure 2. Photomicrograph of bone from the positve control group (C^{+ve}) in female adult rabbits. **(A)** The cortical bone shows lacunae without osteocytes (white arrow), large cavities (black arrow), erosion cavity containing osteoclasts (yellow arrow), and widening in the Haversian canal (red arrow). **(B)** Trabeculae bone formation is characterized by thin walls (brown arrow), apparent thinned-out trabeculae (blue arrow), bone speculae not anastomosing with others and having a bland end (green arrow). H&E, 10×

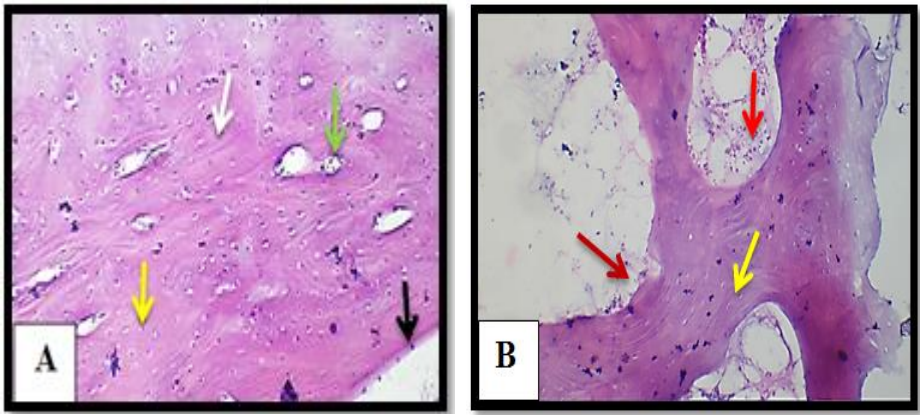

Figure 3. Photomicrograph of bone section from *Salvia hispanica* seeds ethanolic extract-treated group (MP+Sh) in female adult rabbits. **(A)** The cortical bone reveals well-formed compact bone with characteristic lamellae (white arrow), marked normal Haversian canals (green arrow), numerous mature osteoblasts (black arrow), and well-differentiated osteocytes in their lacunae (yellow arrow) **(B)** Trabeculae bone consists of osteocytes cells inside the lacunae, with large empty cavity between the mature trabecular bone formation filled with bone marrow, showing branching and anastomosing trabeculae of normal thickness and shape (brown arrow). H&E, 10×

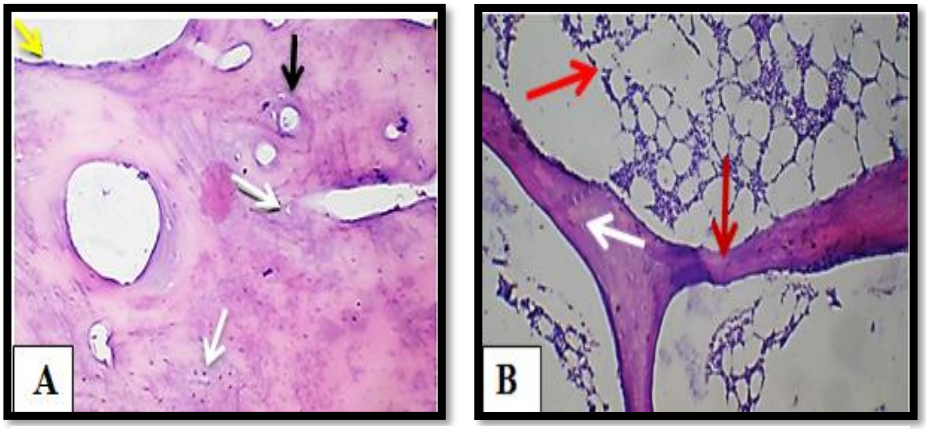

Figure 4. Photomicrograph of a bone tissue section from alendronate-treated group (MP+Ale) of female adult rabbits. **(A)** Cortical bone with some osteocytes inside their apparently large lacunae, while other lacunae appear without osteocytes (white arrow). Haversian canals are also visible (black arrow), as well as non-differentiated osteoblasts (yellow arrow). **(B)** Trabeculae bone formation is characterized by thin walls (brown arrow), immature osteocytes, and large empty spaces filled with bone marrow (red arrow). H&E, 10×

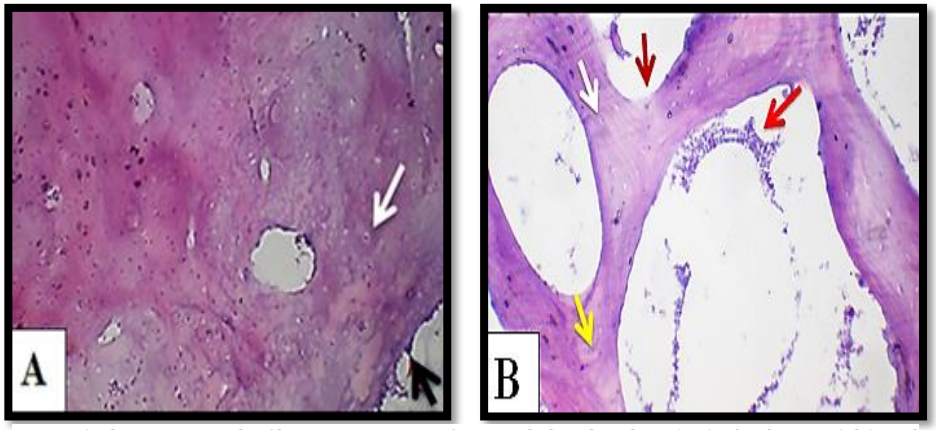

Figure 5. Photomicrograph of bone tissue section from methylprednisolone (MP), alendronate (Ale), and *Salvia hispanica* seed ethanolic extract (Sh) treated group in female adult rabbits. **(A)** Cortical bone with some osteocytes inside lacunae while other lacunae appear without osteocytes (white arrow) and showed few surface osteoblasts (black arrow). **(B)** Trabeculae bone shows recently formed lamellar structure of bone (yellow arrow), the lamellar bone is increasing and organizing to compact the tissue, numerous immature osteocytes (white arrow), thin wall of trabeculae bone (brown arrow), and large empty space that filled with bone marrow. H&E, 10×.

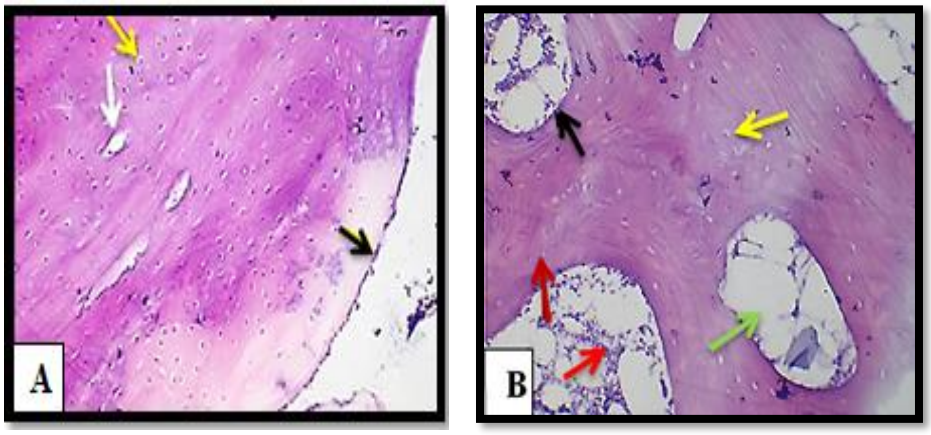

Figure 6. Photomicrograph of bone section from a *Salvia hispanica* seeds ethanolic extract treated (MP+Sh) group after one week withdrawal in female adult rabbits. **(A)** Cortical bone shows the normal histology, numerous mature osteocytes (yellow arrow) in their lacunae and large numbers of osteoblasts presented on the edges of bone trabeculae (black arrow)and showed normal Haversian canals (white arrow). **(B)** Trabeculae bone consists of osteocytes cells inside the lacunae, with large empty cavity between the mature trabecular bone formation filled with bone marrow(red arrow), showing branching and anastomosing trabeculae of normal thickness and shape (brown arrow), many trabeculae bone formation well developed with compromised space (green arrow). H&E, 10×

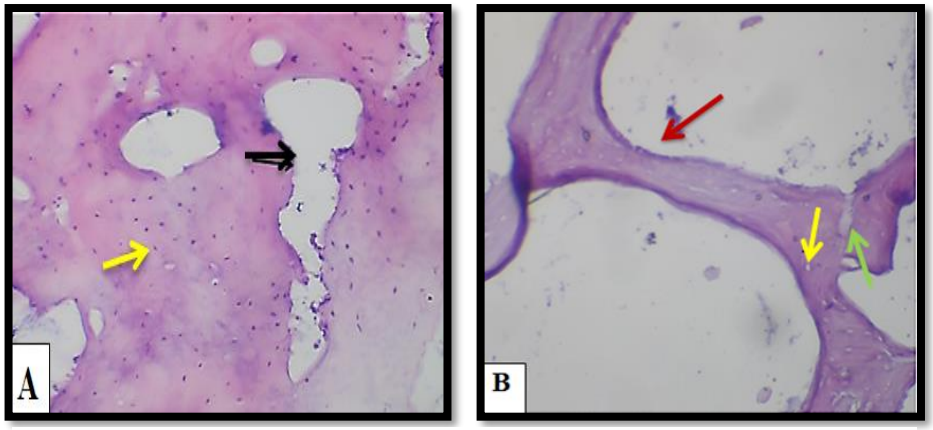

Figure 7. Photomicrograph of bone tissue section from alendronate alone treated (MP+Ale) group, after one week withdrawal, **(A)** Cortical bone shows irregularity of the general architecture, erosion cavities (black arrow) and immature osteocytes (yellow arrow). **(B)** Trabeculae bone formation is characterized by thin wall (brown arrow), bone specula's not anastomosis with others and have bland end (green arrow). H&E, 10×

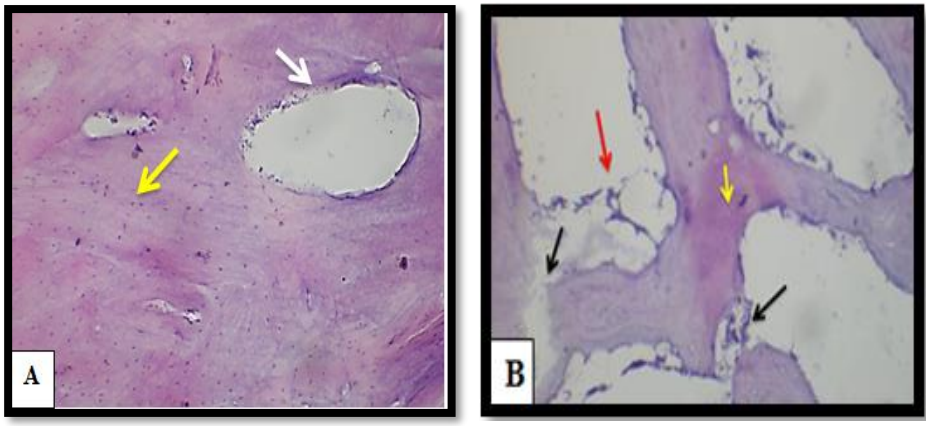

Figure 8. Photomicrograph of bone tissue section from a methylprednisolone (MP), alendronate (Ale), and Salvia hispanica seed ethanolic extract (Sh) treated group after one week withdrawal in female adult rabbits. (A) Cortical bone shows the presence of immature osteocytes (yellow arrow) and non-differentiated osteoblasts proliferation (white arrow). (B) Trabeculae bone shows bone speculae not anastomosing with others and having a bland end (black arrow), immature osteocytes, and large empty space filled with bone marrow (red arrow). H&E, 10×

DISCUSSION

To the best of our knowledge, this is the first study to investigate *Salvia hispanica* as protection against methylprednisolone-induced osteoporosis in female adult rabbits. In this study, BMD may give a more accurate representation of BMD. Glucocorticoids are commonly associated with reduced BMD, osteoblast numbers, and bone formation rates (32). It has been demonstrated that administering glucocorticoids increases bone resorption, lowers the body's total bone mineral content, and results in decreased bone mineral density (33). It appears that glucocorticoids impede osteoblast formation by preventing the release of cellular growth factors such as insulin-like growth factor (IGF) and transforming growth factor (TGF), which has a profound effect on mineral metabolism and skeletal function and can result in the development of osteoporosis (34).

The mineral analysis of *Salvia hispanica* seeds ethanolic extract revealed the availability of several minerals such as Ca, Mg, K, Fe, Zn, Cu, Mn, and P. The extract was found to be a good source of several important minerals, including Ca, Mg, and P. These minerals are known to play important roles in bone health and may contribute to the beneficial effects of Salvia hispanica extract on bone density observed in our study. The extract may also have potential applications in the food and pharmaceutical industries as a natural source of these minerals.

Salvia hispanica extract was used as a treatment, BMD levels were effectively recovered, indicating that the extract raised bone mass in those animals and likely decreased osteoblast apoptosis, the α-linolenic (ALA, ω-3 fatty acid) is rich chia seeds may have caused to the raised of the weight of the musculoskeletal system and the changes seen with the DXA analysis of the bone structures (35). Due to the presence of kaempferol in chia seeds, which is a natural flavonoid that significantly increased BMD and enhanced bone quality, osteoblasts were prompted to mineralize while osteoclasts were inhibited from resorbing bone (36). According to another study, Kaempferol may have an

osteogenic effects (37), and anti-osteoclastogenic (38), agent by acting on both osteoblasts and osteoclasts and activation the estrogen receptor signaling pathway (39).

Treatment with glucocorticoids led to a significant decline in serum Ca levels. This could be due to the fact that glucocorticoids enhance the excretion of Ca through the urine, inhibit the reabsorption of Ca in the renal tubules, which leads to hypocalcemia, and decrease the absorption of Ca through the intestines (40, 41). The levels of P in serum were also significantly altered in the methylprednisolone treated group. The reduction in the levels of P might be due to enhanced renal excretion and alterations in their transport across the brush border membrane (42). Treatment with chia produced an appreciable increase in the levels of Ca and P. Chia seeds are rich in Ca, Mg, P, and protein. The elevated serum Ca may be a result of these nutrients, which are all essential for bone health (43). *Salvia hispanica* L. richness and variety in phenolic acid derivatives and flavonoids (44), might also affect the metabolism of Ca. Plant polyphenols are effective at reducing oxidative stress, which acts by differentiation, the activity of osteoclasts, and enhancing bone resorption, (45). The high Ca and P content of chia seeds, which has been found to have beneficial effects on bone and restored the lowered levels of bone Ca and P to normal values, is most likely the cause of the seeds' positive effect on bone density (46, 47). The presence of genistein and daidzein in chia seed extract increased the amount of Ca and P and bone tissue (48, 49).

Vit. D metabolism is affected by glucocorticoids, which reduces the production of active vit. D (1, 25 dihydroxycholecalciferol) and impairs its biological activity in tissues. Glucocorticoids may directly oppose the peripheral effects of vit. D by lowering intestinal Ca absorption and promoting renal Ca excretion, resulting in a negative Ca balance (50, 51). An et al. (2012) (52) showed that ω-3 PUFAs α-linolenic acid (ALA) and ω-6 PUFA (linoleic acid) improve the level of 1,25-dihydroxyvitamin D level. According to one hypothesis, elevated 1,25-dihydroxyvitamin D (1,25(OH)2D) concentrations resulted from the omega-3 PUFAs and omega-6 PUFAs contained in chia seeds activating the enzyme 1-hydroxylase (53).

It is well known that PTH has anabolic effects on bone by enhancing osteoblast proliferation, differentiation, and survival of bone forming osteoblasts, this concurrently induces osteoclast differentiation, which promotes bone resorption (54). When blood Ca levels are low, PTH is released to restore Ca from bone into the blood, which leads to bone resorption process (55). Secondary hyperparathyroidism is typically caused by glucocorticoid-induced negative Ca balance (56). On the other hand, it appears that glucocorticoids also directly stimulate PTH secretion (57). As consequence, PTH promotes bone resorption indirectly by raising the expression of the (RANKL) RANK ligand/(RANK) receptor activator of nuclear factor kappa B pathway, this, in the end, indicates an activating effect on osteoclasts and bone resorption (58). In accordance with Varela-López et al. (2017) who found that *Salvia hispanica* seed extract administration alters PTH levels (59) and that ω-3 PUFAs may protect against bone loss by regulating systemic calcitrophic hormones like PTH.

The head of the femur was selected in this study to evaluate the changes in cancellous bone trabeculae because it is the area most sensitive to the effects of glucocorticoid treatment on glucocorticoid-induced osteoporosis (60, 61). The histopathological findings of the femoral bone of the C^{+ve} group showed loss of normal architecture, large cavities, erosion cavity containing osteoclasts, and widening in the Haversian canal due to reduction in the boney material (62). Treatment with *Salvia hispanica* seeds ethanolic extract, may prevent the side effects of methylprednisolone. It is believed that this extract inhibited the action of osteoclasts and promoted the activity of osteoblasts, thereby suppressing bone resorption and enhancing bone formation (63). Chia seeds provide large content of α-linoleic acid with a variety of potential health benefits. Even though few studies have examined the consumption of ω-3 fatty acid-rich plant foods like chia omega-3. However, improved Ca absorption by altering the lipid composition of the intestinal cell membrane, intestinal Ca loss, and increased bone mineral content (64, 65). Additionally, the ethanolic extract of *Salvia hispanica* seeds is rich in copper, an essential co-factor of the lysyl oxidase enzyme, which is involved in the cross-linking of the extracellular matrix proteins, collagen, and elastin, and is obviously necessary for maintaining bone integrity. This demonstrates the chia seeds anabolic effects on bone (66).

The results of this research clearly showed that treatment with ethanolic extract of *Salvia hispanica* seeds produces anti-osteoporosis effects against methylprednisolone-induced osteoporosis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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التأثيرات المحسنة للمستخلص الإيثانولي لبذور الشيا على فقدان العظام في إناث الأرانب التي تعاني من هشاشة العظام

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الخلاصة

في هذه الدراسة تم تحديد تأثير المستخلص الإيثانولي لبذور الشيا على هشاشة العظام الناجم عن الميثيل بريدنيزولون في إناث الأرانب من خلال دراسة الكثافة المعدنية للعظام ، قياس تركيز الكالسيوم والفسفور في الدم ، فيتامين د، هرمون الغدة جارالدرقية والتغيرات النسجية المرضية لعظم الفخذ. تم استخدام خمسون انثى ارنب بوزن ١٥٠٠- ٢٠٠٠ غم وبعمر ثمانية اشهر ، قسمت الى خمسة مجاميع كل مجموعة تحتوي على عشرة ارانب ، المجموعة الاولى (السيطرة السالبة): جرعت ١مل من المحلول الفسلجي الطبيعي لمدة ثلاثة اشهر ، المجموعة الثانية (مجموعة الميثيل بريدنيزولون) مجموعة السيطرة الموجبة: حقنت دواء الميثيل بريدنيزولون بجرعة ٠,٢ ملغم/ كغم من وزن الجسم تحت الجلد لمدة شهر واحد ثم جرعت ١مل من المحلول الفسلجي الطبيعي لمدة شهرين، المجموعة الثالثة (مجموعة الميثيل بريدنيزولون + مستخلص بذور الشيا): حقنت دواء الميثيل بريدنيزولون بجرعة ٠,٢ ملغم/ كغم من وزن الجسم تحت الجلد لمدة شهر يوم ثم جرعت ٦٠٠ ملغم/ كغم من وزن الجسم من مستخلص بذور الشيا لمدة شهرين ،المجموعة الرابعة (مجموعة الميثيل بريدنيزولون + دواء الالندرونيت): حقنت دواء الميثيل بريدنيزولون بجرعة ٠,٢ ملغم/ كغم من وزن الجسم تحت الجلد لمدة شهر ثم جرعت دواء الالندرونيت اسبوعيا بجرعة ٣,٦ ملغم/ كغم من وزن الجسم لمدة شهرين، المجموعة الخامسة (مجموعة الميثيل بريدنيزولون + دواء الالندرونيت + المستخلص الايثانولي لبذور الشيا): حقنت دواء الميثيل بريدنيزولون بجرعة ٠,٢ ملغم/ كغم من وزن الجسم تحت الجلد لمدة شهر ثم جرعت اسبوعيا دواء الالندرونيت بجرعة ٣,٦ ملغم/ كغم من وزن الجسم ثم جرعت ب٦٠٠ ملغم/ كغم من وزن الجسم مستخلص بذور الشيا لمدة شهرين. واستمرت هذه الدراسة حتى بعد اسبوع واحد من التوقف عن العلاج . تم اجراء قياس امتصاص الاشعة السينية الثنائي لتقييم الكثافة المعدنية للعظام بعد شهر وبعد شهرين وبعد ثلاثة اشهر من التجربة وبعد اسبوع واحد من التوقف عن العلاج. اظهرت النتائج ان هنالك انخفاض معنوي($P<0.05$) في الكثافة المعدنية للعظام ، تركيز الكالسيوم والفسفور في الدم, فيتامين د الى جانب ذلك هنالك زيادة معنوية ($P<0.05$) في هرمون الغدة جار الدرقية في اناث الارانب بعد شهرين من العلاج وبعد اسبوع واحد من سحب الدواء في المجموعة الثانية(مجموعة الميثيل بريدنيزولون) ومجموعة (الميثيل بريدنيزولون ودواء الالندرونيت) ، بينما عندما عولجت الحيوانات بالمستخلص الإيثانولي لبذور الشيا في المجموعة الثالثة أظهرت تحسنًا في المعايير المذكورة أعلاه ، حيث عادت إلى قيمها الطبيعية وحققت نتائج ممتازة حتى أسبوع واحد من التوقف عن العلاج . اظهرت التغيرات النسجية لعظم الفخذ في مجموعة السيطرة الموجبة ان المظهر النسيجي للعظم غير طبيعي, في حين أن العلاج بالمستخلص الإيثانولي لبذور الشيا في المجموعة الثالثة كان فعالاً في إيقاف التغيرات النسيجية المرضية وعودة العمارة الطبيعية مقارنة بمجموعة السيطرة. نستنتج من ذلك أن المستخلص الايثانولي لبذور الشيا يحتوي على مواد فعالة في معالجة هشاشة العظام، وعليه فقد كانَ له تأثير أقوى من دواء الالندرونيت.

الكلمات المفتاحية: بذورالشيا، المستخلص الإيثانولي ، العظام، اناث الأرانب، هشاشة العظام