



The anti-osteoporotic effect of Moringa oleifera leaves extract on glucocorticoids-induced jawbone osteoporosis in Albino rats

Efeito anti-osteoporótico dos extratos de folhas de Moringa oleifera em osteoporose maxilar induzida por glicocorticóides em ratos albinos

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ABSTRACT

Objective: Glucocorticoids induced osteoporosis and its related fragility fractures represent a costly human and socioeconomic load worldwide. All the current pharmacological therapies possess multiple adverse effects and high cost. Thus, the present study aimed to evaluate the bone healing ability of Moringa oleifera (MO) on glucocorticoids induced osteoporosis in the jawbone of Albino rats. **Material and Methods:** Osteoporosis was prompted by a daily intraperitoneal injection of 200 μ g/ 100 g dexamethasone for 30 days. Next, the animals were randomly divided into 2 groups; osteoporotic and MO treated group. The treated group received a daily oral dose of 200mg/kg of MO. Rats from the MO group were sacrificed after 4 weeks from the beginning of treatment, and the same sacrifice date was used for the osteoporotic group. Bone regeneration was evaluated by dual energy x-ray absorptiometry (DEXA), real time polymerase chain reaction (RT-PCR), histopathological and histomorphometric examination. **Results:** After the sacrifice, the DEXA analysis revealed a significant upregulation in the BMD in the MO treated group ($p < 0.001$). The RT-PCR test showed a significant decline in RANKL gene expression and a significant rise in OPG gene expression in the MO group ($p < 0.001$, $p = 0.002$, respectively). The histopathological examination of the MO group displayed a marked healing of the jawbone micro-anatomy. The histomorphometric analysis also showed that the bone area percentage increased significantly in the MO group ($p < 0.05$). **Conclusion:** A cheap, easy to get, yet a powerful plant like MO leaves, can be considered an effective treatment for osteoporosis.

KEYWORDS

Bone regeneration; Glucocorticoids; Moringa oleifera; Osteoporosis.

RESUMO

Objetivos: A osteoporose induzida por glicocorticóides e suas fraturas por fragilidade relacionadas representam um custo humano caro e carga socioeconômica em todo o mundo. Todas as terapias farmacológicas atuais possuem múltiplos efeitos adversos e alto custo. Assim, o presente estudo teve como objetivo avaliar a capacidade de cicatrização óssea de Moringa oleifera (MO) em osteoporose induzida na mandíbula de ratos albinos. **Material e Métodos:** A osteoporose foi induzida por uma injeção intraperitoneal diária de 200 μ g / 100 g de dexametasona por 30 dias. A seguir, os animais foram divididos aleatoriamente em 2 grupos; grupo tratado com osteoporose e MO. O grupo tratado recebeu uma diária dose oral de 200 mg / kg de MO. Os ratos do grupo MO foram eutanasiados após 4 semanas do início do tratamento, e a mesma data de eutanásia foi usada para o grupo osteoporótico. A regeneração óssea foi avaliada por espectrometria de raio-x de energia dupla (DEXA), reação em cadeia da polimerase em tempo real (RT-PCR), análise histopatológica e histomorfométrica. **Resultados:** Após a eutanásia, a análise DEXA revelou uma regulação positiva significativa na DMO no grupo tratado com MO ($p < 0,001$). O teste RT-PCR mostrou um declínio significativo na expressão do gene RANKL

e um aumento significativo na expressão do gene OPG no grupo MO ($p < 0,001$, $p = 0,002$, respectivamente). O exame histopatológico do grupo MO revelou uma cicatrização acentuada da microanatomia do maxilar. A análise histomorfométrica também mostrou aumento significativo na porcentagem de área óssea no grupo MO ($p < 0,05$). **Conclusão:** A MO é uma planta barata, de fácil obtenção, e suas folhas ainda podem ser consideradas poderosas como tratamento eficaz para a osteoporose.

PALAVRAS-CHAVE

Regeneração óssea; Glicocorticoides; *Moringa oleifera*; Osteoporose.

INTRODUCTION

Osteoporosis is a widespread systemic skeletal disease with the key feature of disturbed bone microarchitecture, resulting in high risk of bony fractures. Osteoporosis affects 1 in 4 women and more than 1 in 8 men over 50 years [1,2]. It was stated that the osteoporotic skeletal mass deterioration can be accompanied with an elevated oral bone loss. This subsequent low jaw bone density leads to increased alveolar bone porosity and changed trabecular pattern leading to faster alveolar bone resorption. Consequentially, the host system is progressively susceptible to infectious destruction of the periodontal tissues [3,4].

Glucocorticoids (GCs) are the main iatrogenic cause for secondary osteoporosis, where the fracture risk increases by as much as 75% within the first 3 months of therapy [5]. GCs led to the increased production of macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-beta (NF- κ B) ligand RANKL and the decreased production of osteoprotegerin (OPG) by osteoblastic cells and osteocytes, subsequently increasing the number and activity of osteoclasts, that is why they have direct effects on bone resorption [6].

The long term use of pharmacological drugs in treatment of osteoporosis was proved to have several disadvantages. Bisphosphonates adverse effects range from upper gastrointestinal symptoms and musculoskeletal pain to atypical femur fractures and osteonecrosis of the jaw. Skin and urinary infections are seen with denosumab drug. An increased risk of strokes was noted with raloxifene which is a selective estrogen receptor modulator [7]. That's why shifting to a more natural bone healing approach was mandatory, especially with the presence of a powerful botanical extract like the *Moringa oleifera*.

Moringa oleifera Lam (MO) is a local medicinal Indian herb, mostly found in the subtropical and tropical regions. It comprises numerous nutrients such as calcium, vitamin D, magnesium and phosphorus that are correlated to a rise in bone density and have a serious role in calcium absorption and bone health. MO leaves are its most nutritious part. They act as a major source for B-vitamins, vitamin-C, pro-vitamin A as beta carotene, manganese, vitamin K and proteins [8-10].

Moringa leaf extract has an osteoblastic potential and indirect osteo-inductive properties because of its natural contents, being rich in flavonoids, saponins, tannins and phytoestrogens [11,12]. These flavonoids induce bone mineralization and bone nodule formation. In addition, they contain kaempferol and quercetin. Kaempferol was found to hinder the osteoclastic resorption and induce the osteoblastic differentiation and cells mineralization [13,14].

Moreover, it was reported that the ethanolic extract of MO phytoestrogens content may have a protective action against ovarian hormone insufficiency related bone resorption. These phytoestrogens are similar to endogenous estrogens, estradiol. Therefore, they are considered as natural selective estrogen receptor modulators, as steroidal estrogens which have a positive stimulatory effect on osteoblast activity and a protective role in postmenopausal women [15].

In this context, this study aimed to assess the bone healing regenerative potential of MO leaf extract in treatment of osteoporosis through its oral intake.

MATERIAL AND METHODS

Ethical statement

This study was performed at the animal house, Faculty of Medicine, Cairo University after

the acceptance of the Institutional Animal Care and Use Committee (IACUC), Cairo University (CU-III-F-73-17).

Moringa oleifera (MO) ethanolic extract preparation

MO leaves were collected from the farm of Egyptian Scientific Society of Moringa. The collected leaves were air-dried, powdered and kept for extraction. 200 g of the dry powdered MO leaves were extracted with 1L of 70% ethanol and shaken every 8 h for 24 h. After that, the hydro-alcoholic extract was filtered using a cotton funnel. This step was repeated four times. The extract was concentrated using a rotator evaporator under reduced pressure. The concentrated extract was lyophilized and kept at -20°C [16].

Animals

Eight Albino rats weighing 150-200 gm were used in the experiment. They were placed in separate cages and kept under good ventilation. Rats were allowed to have free access to water and food *ad-libitum*. Rats were randomly divided into 2 groups of 4 rats each, as follows:

Osteoporotic group: included 4 glucocorticoid-induced osteoporotic rats. Rats received distilled water as a physiological solution via oral gavage to experience the same daily stress.

MO group: included 4 glucocorticoid-induced osteoporotic rats. Osteoporotic rats were given their regular rat chow and a daily dose of 200mg/kg of *Moringa oleifera* extract via oral gavage [17].

Study design

The rats were subjected to an intraperitoneal injection of 200 µg/ 100 g dexamethasone once a day for 30 days for osteoporosis induction [18]. Jawbone BMD was measured to confirm that osteoporosis was effectively induced. Rats from the MO group were sacrificed after 4 weeks from

beginning of treatment, with the exact sacrifice date for the osteoporotic untreated group. The lower jaw dissection and processing was done for the following:

Dual-Energy X-ray Absorptiometry (DEXA) Analysis

The Mandibular jaws BMD was evaluated using DEXA. Norland XR-46 DXA scanner (Norland Corp. Fort Atkinson, WI, USA) supplied with suitable software for bone evaluation was used. The scanning resolution was 0.5 x 0.5 mm and the speed was 60 mm/sec. Examination was performed on the picture of the rat's jaw bone on the screen utilizing a region of interest (ROI) at the alveolar bone supporting the socket of the mandibular first molar. Results were shown automatically in gm/cm² [19].

Real time PCR

The RT-PCR determined the total RNA containing genes. RNAs were isolated by QIAzol (QIAGEN). The cDNA was formed by the cDNA synthesis kit (Applied bio system) and RT-PCR was done by RTPCR kit (Applied bio system) on Step One Plus instrument (Applied Biosystems) using standardized protocols. The RQ of each target gene was measured by calculating the $\Delta\Delta Ct$. The calculation of the RQ for the genes of interest was performed by $2^{-\Delta\Delta Ct}$ normalization to the housekeeping gene *GADPH*. Primers for *RANKL*, *OPG* and *GADPH* are listed in Table I.

Histopathological Examination

The samples used were decalcified for 4-5 weeks, then dehydrated in ascending grades of alcohol, cleared in Xylol and inserted in paraffin blocks. Serial sections of 5-6 µm thickness were cut, placed on glass slides and stained with Hematoxylin and Eosin (H&E) for routine histopathological examination.

Table I Primers' sequence specific for each gene

Gene symbol	Primer sequence from 5'- 3'
<i>RANKL</i>	F: ACC AGC ATC AAA ATC CCA AG
	R: TTT GAA AGC CCC AAA GTA CG
<i>OPG</i>	F: GTT CTT GCA CAG CTT CAC CA
	R: AAA CAG CCC AGT GAC CAT TC
<i>GAPDH</i>	F: GACGGCCGCATCTTCTTGA
	R: CACACCGACCTTCACCATTT

Histomorphometric Analysis

The bone area percentage in the area underneath the first molar for each sample was evaluated. Data was obtained using Leica Qwin 500 image analyzer computer system (England). The area and area percentage of bone trabeculae were calculated by an objective lens of magnification 20x (total magnification of 200). Five fields were calculated for each sample. The bone area percentage was calculated in relation to a standardized measuring frame possessing an area of 118476.6 μ m².

Statistical methods

Data analysis was done with SPSS v24 software for Windows (SPSS Inc., Chicago, IL, USA). Results were expressed as mean \pm standard deviation for all variables. Pairwise comparisons between groups were performed using Student T-test. P-values less than 0.05 were considered statistically significant.

RESULTS

DEXA results

There was a significant upregulation in the BMD in the MO treated group compared to the osteoporotic group ($p < 0.001$) (Table II).

RT-PCR results

A significant decline in RANKL gene expression in the MO treated group was found compared to the osteoporotic group ($p < 0.001$), while there was a significant upregulation in the OPG gene expression in the MO treated group compared to the osteoporotic group (p value = 0.002) (Table II).

RANKL/OPG ratio among different studied groups

There was a significant decline in RANKL/OPG ratio in the MO treated group compared to the osteoporotic group (p value < 0.001) (Table II)

Histopathological results

The histopathological examination of the alveolar bone structure of the osteoporotic group displayed clear signs of degeneration. The bone marrow cavities grew wide and became filled with multiple chronic inflammatory cells and extravasated RBCs. The bone trabeculae became very thin, mal-organized and surrounded by multiple marrow spaces. In addition, areas of fatty degeneration filled with adipose tissue were observed in one of the marrow cavities. Some empty lacunae, multiple osteoporotic bony clefts and many areas in the marrow spaces with a diminished osteoblastic lining were also detected. All these findings denoted cellular degeneration (Figure 1).

The histopathological examination of the alveolar bone structure of the MO treated group showed a great evidence of bone healing. The marrow cavities got narrower and regained their osteoblastic lining. They became surrounded by more organized lamellar bone architecture. A fewer number of bony clefts was detected. The osteocytes were back to their normal size and shape, filling their lacunae throughout the bone trabeculae. The marrow cavities also showed increased cellularity where megakaryocytes and multiple granulocytes were observed. Bone resting lines, multinucleated osteoclasts and reversal scalloped lines with areas of newly formed bone matrix (osteoid) were also noted and denoted the active progressing bone remodeling and healing

Table II - DEXA, RT-PCR and histomorphometric results

Test	Osteoporotic group	Moringa oleifera group	P value
DEXA level	0.03 \pm 0.01	0.1 \pm 0.02*	$P < 0.001$
RANKL gene expression	2.6 \pm 0.19	0.75 \pm 0.21*	$P < 0.001$
OPG gene expression	0.38 \pm 0.08	1.16 \pm 0.45*	$P = 0.002$
RANKL/OPG ratio	6.98 \pm 1.25	0.78 \pm 0.45*	$P < 0.001$
Histomorphometric analysis	29.08 \pm 2.5	64.44 \pm 4.9*	$P < 0.05$

Data are expressed as mean \pm SD; *Denotes statistical significance at p value < 0.05 .

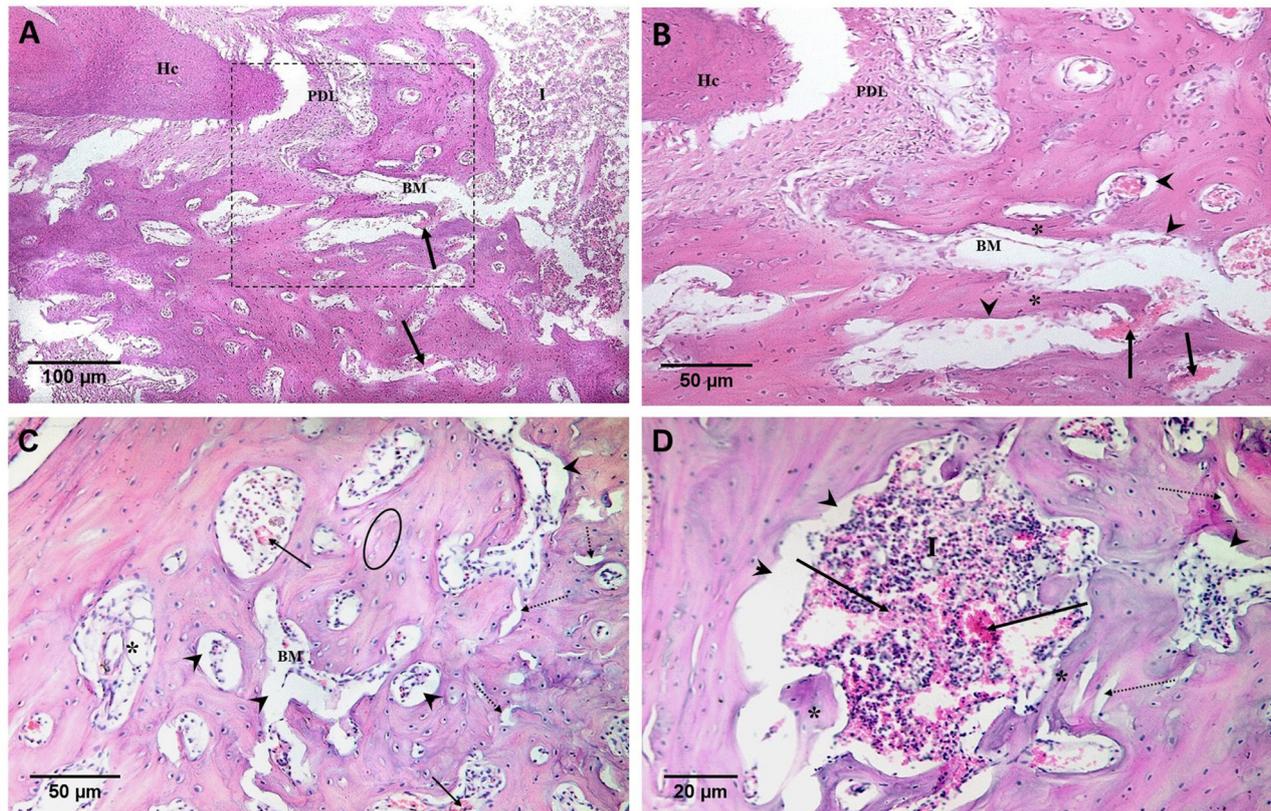


Figure 1 - A photomicrograph of the osteoporotic group showing (A) multiple widened bone marrow cavities (BM), a large marrow cavity filled with chronic inflammatory cells (I), hypercementosis (Hc) and degenerated periodontal ligaments (PDL) (H&E, original Mag. X100, scale bar=100µm); (B) a higher magnification of the previous figure, showing wide bone marrow cavities (BM) with areas of extravasated RBCs (arrows), areas with diminished osteoblastic lining (arrow heads), areas of thin bone trabeculae (asterisks), hypercementosis (Hc) and degenerated periodontal ligaments (PDL) (H&E, original Mag. X200, scale bar=50 µm); (C) widened bone marrow cavities (BM), some with a reduced osteoblastic lining (arrow heads), areas of extravasated RBCs (arrows), empty lacunae (oval), multiple bony clefts (dotted arrows) and fatty degeneration (asterisk) (H&E, original Mag. X200, scale bar=50 µm); (D) a very wide marrow space containing multiple extravasated RBCs (arrows), chronic inflammatory cells (I) with a contracted osteoblastic lining (arrow heads) surrounded by thin bone lamellae (asterisks) and some bony clefts (dotted arrows) (H&E, original Mag. X400, scale bar=20 µm).

process. A network of vascular channels was observed. Also, a vascular canal turned into a secondary osteon, where the vascular canal was surrounded by centrally deposited lamellar bone with a reversal line (Figure 2).

Histomorphometric results

Comparing between MO treated group and the osteoporotic group, revealed that the bone area percentage values were statistically significantly different ($p < 0.05$) (Table II).

DISCUSSION

Synthetic GCs are commonly used in treating multiple diseases. Although they have great therapeutic effects, they unavoidably produce many side effects by long-term use. GCs-induced osteoporosis is a grave side effect and have become the main reason behind secondary osteoporosis

in adults [20]. All the available osteoporosis pharmacological treatments carry a huge burden of multiple side effects and high cost [7].

In view of these facts, this study aimed to evaluate the bone healing ability of MO on GCs induced jawbone osteoporosis, and since animal models are vital for the testing of new treatments, the rat animal model was chosen for this study in order to easily develop a quick osteoporotic model [21].

In this study, osteoporosis was induced by a daily intraperitoneal injection of 200 µg/100 g dexamethasone for 30 days [18]. GCs-induced osteoporosis is identified by reduced bone formation, with an additional initial transient rise in bone resorption. The initial remodeling rate increase is escorted by decreased bone formation at the individual bone multicellular unit (BMU) level. The combination of the elevated bone turnover and the undesirable remodeling balance

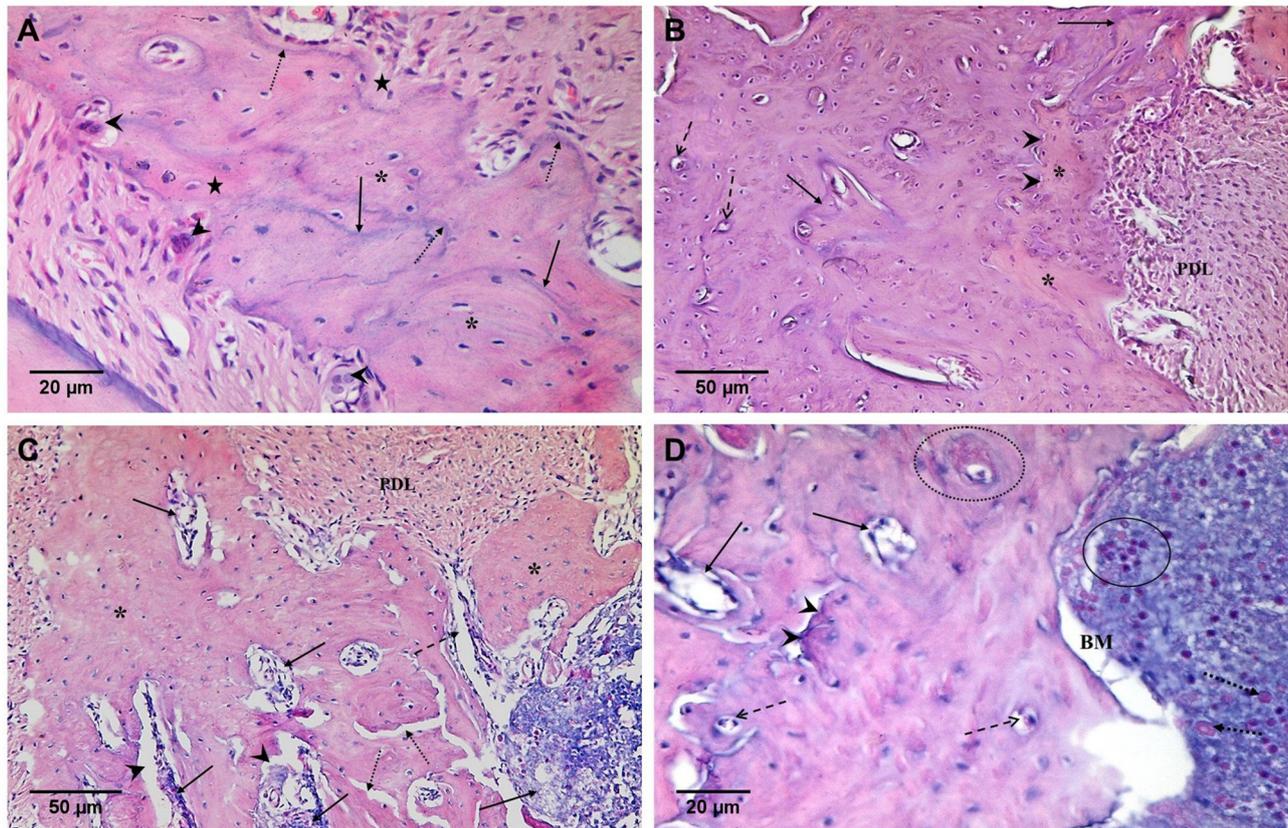


Figure 2 - A photomicrograph of the MO treated group showing (A) osteoclasts (arrow heads), bone resting lines (arrows), bone reversal lines (dotted arrows), osteoid formation (stars) and normal osteocytes in their lacunae (asterisks) (H&E, original Mag. X400, scale bar=20 µm); (B) bone vascular channels (dashed arrows), resting lines (arrows), reversal lines (arrow heads), newly formed bone matrix (asterisks) and normal periodontal ligaments (PDL) (H&E, original Mag. X200, scale bar=50 µm); (C) normal periodontal ligaments (PDL) connected to a nutrient canal (dashed arrow), bone marrow cavities with noticeably increased cellularity (arrows), with few areas with diminished osteoblastic lining (arrow heads), normal areas of bone trabeculae with normal osteocytes in their lacunae (asterisks) and fewer bone clefts (dotted arrows) (H&E, original Mag. X200, scale bar=50 µm); (D) a high magnification of a large cellular bone marrow cavity (BM) with multiple granulocytes (circle), megakaryocytes (dotted arrows), smaller bone marrow cavities with normal osteoblastic lining (arrows), reversal lines (arrow heads), vascular spaces (dashed arrows) and a secondary osteon (dotted circle) (H&E, original Mag. X400, scale bar=20 µm).

causes a speedy bone loss. Afterwards, the declined bone formation, both at the tissue and BMU level, dominates causing a low turnover state [22].

In the present work, osteoporosis was examined in the mandibular jaw bone of an adult rat. It was proved that the mandible presents a considerably delayed and less intense response concerning the BMD loss compared to the femur bone in an osteoporotic rat model. It was also stated that the interradicular septum of the first molar was the commonly affected region, revealing the microarchitectural changes. That is why clinicians should be mindful that patients presenting with osteoporotic changes in the mandible are predicted to display severely advanced BMD reduction in other bone areas [23].

In the current study, bone weakening and deterioration were clearly noticed in the osteoporotic group through the decline of BMD. Similar results were shown by Govindarajan et

al where the BMD was significantly lower in the ovariectomised (OVX) rats compared to the control ones [24].

In the present work, the *RANKL* gene expression was significantly elevated in the osteoporotic group, while the *OPG* gene expression was significantly decreased. Similarly Mo et al observed down-regulated *OPG* expression and up-regulated *RANKL* expression aroused by dexamethasone compared to healthy rats [25].

The histopathological findings of the osteoporotic group showed obvious bone deterioration. This comes in agreement with Mustafa et al where (OVX) osteoporotic rats revealed noticeable osteoporotic alterations in bone trabecular architecture [26]. In the current study, marrow cavities were filled with inflammatory cells. This is in agreement with Rauner et al who stated that cells of the immune system, such as T cells, B cells, macrophages or

dendritic cells, become activated and produce inflammatory cytokines, which are crucial mediators in osteoimmunology and many of these cytokines cause osteoclasts stimulation [27]. Activated T cells act as major stimulators of osteoclastogenesis by increasing the bone-resorbing cytokines production, especially TNF- α and RANKL. Hence, activated T cells are proposed to have a chief role in osteoporosis [28].

Overall, the activation of osteoclasts via these cytokines leads to exaggerated systemic and localized bone loss. Therefore, the excessive production of the bone-resorbing cytokines significantly explains the incidence of osteoporosis in conjunction with chronic inflammatory reactions. Remarkably, these cytokines are also of pathogenic importance in 'primary' forms of osteoporosis, like the age-related and postmenopausal osteoporosis [29].

The histomorphometric analysis in this study confirmed the histopathological architectural changes in the osteoporotic group, where the bone area percent values showed a great decline after the sacrifice. Kozai et al study went accordingly, where the steroid treatment significantly lowered the cortical thickness and bone volume of the rats' mandible compared to healthy animals [30].

In this study, the osteoporotic rats were given their regular rat chow and a daily oral dose of 200mg/kg body weight of MO extract for 30 days [17]. Concerning the BMD, there was a significant elevation in BMD in MO treated group compared to the osteoporotic group. Moreover, the BMD in MO treated group was nearly similar to that of healthy rats as reported by Ezzat et al and Haggag et al [18,31]. Habib and Al-Moalem had similar results where BMD significantly increased in a glucocorticoid model of osteoporotic rats fed on dried moringa leaves. This could be attributed to the minerals found in MO, which possess both a curative and preventive role against osteoporosis [32].

The RT-PCR analysis for the RANKL gene expression showed a significant decrease, while the OPG gene expression showed a remarkable increase in the MO treated group compared to osteoporotic group. In addition, gene expression levels of RANKL and OPG in MO treated group were close to those of healthy rats as stated by Meijie et al and Ma et al [33,34]. Adhikary et al had supporting results where dietary flavonoid

kaempferol inhibited glucocorticoid induced bone loss. The oral uptake of kaempferol enhanced bone strength, BMD and bone formation-related genes like OPG and decreased the bone resorption-related gene RANKL in kaempferol treated group [35]. These findings went in accordance with our results as the MO leaves extract were proved to possess an effective kaempferol content. The main flavonoids found in MO leaves ethanolic extract are the kaempferol and quercetin. Kaempferol is not only a powerful antioxidant and promotor of cancer cell apoptosis but it also prevents DNA damage [36].

The histopathological findings of the MO treated group revealed a noticeable improvement in bone microarchitecture resembling normal histology of mandibular alveolar bone in rats as described by Ezzat et al, Montaser et al and Sherif et al [18,37,38]. This comes in accordance with Patel et al who studied the MO leaf extract treatment at 200mg/kg bodyweight on (OVX) rats for 30 days. They proved that bone formation process was increased by enhancement of the osteoblastic activity, which led to the improvement of ALP activity in MO treated groups. MO accelerated mineralization of the organic matrix, thus speeding up the bone healing [17].

Moreover, the bone marrow cavities showed an increased cellularity. This makes the MO leaves a significant source of regular cell reinforcements. This increased cellularity also indicated a regenerative response in the bone marrow [39]. In addition, some megakaryocytes were observed in the bone marrow. They are rare cells found in the bone marrow, responsible for the daily production of platelets into the bloodstream. They also act as regulators of bone marrow homeostasis [40].

Concerning the histomorphometric analysis, the bone area percent values increased in the MO treated group and approached the normal values in healthy rats as reported by Ezzat et al and Montaser et al [18,37]. Ei Behairy et al also had supporting results where the histomorphometric analysis showed the highest mean value of bone area percent in the MO leaf powder aqueous extract injected at extraction site in dogs [41].

These osteoinductive properties of MO leaf extract may be attributed to its flavonoids content which stimulate the osteoblast proliferation and differentiation. Furthermore, tannins contained in MO leaf extract could also inhibit osteoclasts differentiation. Moreover, the MO phytoestrogens

have a positive effect on the bone with stimulatory effects on osteoblast activity through estrogen-mediated action [15].

CONCLUSION

Although MO is a cheap and easy to get plant, yet it was proved in the current study to have a powerful anti-osteoporotic effect, remarkable bone healing regenerative potential and a great safety profile, thus it could be used in treatment of osteoporosis through its oral intake.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

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Regulatory statement

This study was conducted in accordance with all the guidelines and policies of the Institutional Animal Care and Use Committee (IACUC), Cairo University. The approval code for this study is: (CU-III-F-73-17).

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