MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Therapeutic impact of Extra Virgin Olive Oil on Bone Health in Ovariectomized-induced osteoporosis in rats

Eman Saber Abdelkhalek Ali[1*]. El-Sayed El-Shafaey[2]. Magda Mahmoud Arafa Elkomy[1].

[2]*Department of Surgery, Anesthesiology and Radiology -Faculty of Veterinary Medicine-Mansoura University*

[1]*Zoology department-Faculty of Science-Mansoura University*

[*]***Correspondence to:*** *sabereman972@gmail.com, 01029855366*

Received:2/10/2023
Accepted: 8/11/2023

Osteoporosis is a global health problem that commonly occurs in elderly people, especially postmenopausal women. Unfortunately, the current strategies for osteoporosis treatment are frequently hampered by severe side effects, especially after long-term use. Therefore, this study is aimed to evaluate the potentially effective impact of extra virgin olive oil on postmenopausal osteoporosis treatment based on an ovariectomized (OVX) rat model. In this study, 24 female Wistar rats were used; the rats were divided into four equal groups: control group, rats given olive oil, OVX-group, and OVX rats given olive oil. At the end of the experimental period, bone mineral density (BMD) was measured by DEXA scan. Additionally, serum and bone level of different parameters were assessed. Our results revealed that olive-oil administration had a beneficial therapeutic effect in osteoporosis treatment, and there was a significant amelioration in different estimated parameters. This was demonstrated by preventing the changes in both bone remodeling and BMD. In addition to improving the hormonal changes, oxidant/antioxidant imbalance, and inflammatory cytokines abnormal levels associated with OVX-induced osteoporosis, Further, olive oil treatment showed a marked improvement in the histological architecture, including improvement of the cancellous and cortical bone appearance with less widened bone marrow spaces, and the endosteal surface appeared smooth and lined with osteoblast cells. It is concluded that olive oil utilization is efficacious in reducing the impact of ovariectomy-induced osteoporosis in rats. Additionally, it has the potential to serve as a viable remedy for postmenopausal osteoporosis, devoid of any detrimental side effects.

Keywords: Osteoporosis, Rats, Ovariectomy, Postmenopausal, Olive-oil.

Introduction

Osteoporosis is a common metabolic bone disease characterized by low bone mineral density (BMD) and the deterioration of bone microarchitecture [1]. It is worth noting that while there are various causes of osteoporosis, the most prevalent one is the bone loss that is linked to lack of estrogen that occurs subsequent to menopause[2].

Indeed, osteoporotic fracture is a severe clinical consequence of osteoporosis, resulting in high morbidity, mortality, and healthcare costs. It is estimated that osteoporosis can cause approximately 9.0 million fractures annually and affect around 200 million individuals worldwide, with 34% of women over the age of 50 being affected [3, 4]. Therefore, the prevention and treatment of osteoporosis are crucial to avoid osteoporotic fractures [5].

However, many pharmacotherapies have been explored as potential treatments for postmenopausal osteoporosis, not all patients exhibit a positive response to these synthetic medications, and some even experience severe side effects [6]. Hence, the search for alternative agents that are both effective and safe for preventing and treating osteoporosis in postmenopausal women remains a significant concern [7].

Many studies have revealed insights into the search for natural compounds that possess anti-osteoporotic properties and cause minimal side effects [8]. Medicinal plants offer safe alternatives to current therapies. Olive oil is a crucial element of the Mediterranean diet, which is associated with various health benefits. Research has demonstrated that olive oil can enhance different aspects of human health and has protective effects against heart disease, cancer, neurodegeneration, diabetes, and aging [9, 10]. It has additionally been documented that olive oil facilitates bone mineralization and development and may contribute to maintaining bone density. This is achieved through mechanisms involving increased bone formation, inhibition of bone resorption, and potentially reducing oxidative stress and inflammation [11].

Indeed, the Ovariectomy model is widely employed for investigating phenomena related to postmenopausal osteoporosis caused by lac of estrogen. It has been firmly recorded that ovariectomy induce the depletion of bone mass and an elevation in bone turnover in rats. Therefore, rat models of osteoporosis that undergo ovariectomy can effectively replicate conditions in postmenopausal women and are appropriate for evaluating potential treatments aimed at preventing or treating osteoporosis [12]. Therefore our current study aims to examine the impact of olive oil consumption on postmenopausal osteoporosis using an OVX rat model.

Material and methods

Chemicals

Extra virgin olive oil was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals or kits used in the experiment were of the highest grade available.

Experimental animals and study design

Twenty-four female Wistar rats, weighing 160±10g, were utilized in this experiment. The rats were obtained from the animal house of the Biological Products and Vaccines (VACSERA, Cairo, Egypt) and were housed in sterilized stainless-steel cages under controlled environmental conditions of 25°C and a 12-hour light/dark cycle. The rats were allowed to acclimate for one week prior to the start of the experiment and were supplied with a standard diet and unlimited access to water. The osteoporosis model was induced by performing bilateral ovariectomy, following the established protocol described by [13]. Subsequently, the experimental animals were divided into four equitably sized groups, with each group consisting of 6 rats. Group I served as the control group, consisting of normal rats that supplied with the standard diet without any additional treatment throughout the study duration. Group II, the olive-oil group, involved normal rats that received an orally administered supplement of olive oil (1ml/100g b.w.). Group III, the OVX-group, comprised of OVX rats that received a standard diet without any additional treatment. Lastly, Group IV, the OVX+olive oil group, included OVX rats that received a standard diet supplemented with oral olive oil (1ml/100g b.w.) [14]. All experimental procedures were conducted in accordance with the regulations frothed by the Animal Care and Use Committee of Mansoura University and aligned with the National Institute of Laboratory Animal Resources [15] The study was also approved by the local experimental animal ethics committee.

Blood and tissue sampling

At the end of the designated period of experimentation, 12 hrs fasted rats were euthanized under anesthesia using ketamine HCL. Blood samples were obtained in non-heparinized centrifuge tubes and then centrifuged at 3000 rpm for 10 minutes to separate the sera. Each serum sample was then labeled in Eppendorf's tubes and stored at -20°C for subsequent biochemical analysis.

After the collection of blood samples, the rats underwent dissection. Both the left and right femurs were promptly extracted and rinsed with a chilled saline solution. Subsequently, the left femur was weighed and homogenized in an ice-cold saline solution utilizing a Potter-Elvehjem type homogenizer. The resulting homogenate was then subjected to centrifugation at a force of 860 Xg for a duration of 20 minutes. Following centrifugation, the obtained supernatants were preserved at a temperature of -20°C for subsequent analysis. In the meantime, the right femur was employed for the determination of BMD.

Estimated parameters

Determination of bone mineral density

The measurement of the BMD of the rat's femurs was conducted in a manner that ensured objectivity, utilizing the Lunar Prodigy Advance by dual energy X-ray absorptiometry (DEXA), which was provided by GE Healthcare (Chicago, IL, USA), specifically in the small-subjects mode. The scans were performed with a pixel size of 0.125 x 0.25 mm, a line spacing of 0.0254 cm, and a point resolution of 0.0127 cm. All samples were measured three times and the mean values were calculated. BMD was represented in g/cm^2 [16].

Determination of serum total protein and creatinine levels

Levels of serum total protein (TP) and creatinine (Cr) were assessed calorimetrically using a colorimetric assay kit purchased from Spinreact (Girona, Spain), Cat.No:1001290 and 1001110, respectively.

Determination of minerals metabolism markers and enzymes

Levels of both serum and bone calcium (Ca) and phosphorus (P) were assessed using a colorimetric assay kit purchased from Spinreact (Girona, Spain), Cat.No:1001061 and MD1001155. The serum activity of alkaline phosphatase (ALP) was assessed using a kinetic photometric assay kit purchased from Spinreact (Girona, Spain), Cat.No:41245. The bone activity was determined using an enzyme-linked immunosorbent assay (ELISA) technique with the Rat bone alkaline phosphatase (BALP) ELISA kit obtained from MyBioSource company (California, USA), Cat.No: MBS164916, as per the manufacturer's instructions.

Determination of some hormones in serum

Serum levels of both parathyroid hormone (PTH) and estradiol (E2) hormone were assessed using the ELISA technique. The PTH measurements were conducted using the Scantibodies ELISA kit (Cat.No: 3KG151) obtained from Santee, CA, USA. Likewise, for E2, the BioVendor RAT estradiol ELISA kit (Cat.No: RTC009R) was utilized.

Determination of proinflammatory cytokines

Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), were measured using ELISA kits from R&D system (R&D systems, Manneapolis, USA; Cat.No: 45-TNFRT-E01.1) and RayBiotech, Inc Company (Cat.No: ELR-IL1beta-001C).

Determination of oxidative stress marker in bone

The determination of the bone malondialdehyde (MDA) level, which serves as the final outcome of lipid peroxidation, was conducted through the utilization of an ELISA kit (specifically, the Rat malondialdehyde, MDA ELISA kit from the life science market) identified as Cat No: CELI-66086r.

Determination of antioxidant markers in bone

The content of reduced glutathione (GSH) and activity of superoxide dismutase (SOD) and catalase (CAT) in bone was determined using an ELISA kit in accordance with the manufacturer's instructions. For GSH, ELISA kit obtained from Cusabio Biotech (Wuhan, China), Cat.No: CSB-E12144r was used. For SOD, ELISA kit obtained from Cusabio Biotech (Wuhan, China), Cat.No: CSB-E08555r was used. For CAT, ELISA kit obtained from MyBioSoruce company, Cat.No: MBS2600683 was used.

Histopathological examination

Serial sections from the rats' femurs were fixed in 10% neutral buffer formalin for 48 hours before undergoing decalcification in a 10% EDTA solution with daily exchanges for 6 weeks. The decalcified samples were then dehydrated in increasing concentrations of ethyl alcohol, cleared in xylene, and finally embedded in liquid paraffin wax. These decalcified sections from all groups were sliced at a thickness of 4 µm and stained using hematoxylin and eosin (H&E) for histopathological examinations as per the protocol provided by [17].

Statistical analysis

All statistical analysis was assessed using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, California, USA) and a statistical software package (SPSS 15.0 for Microsoft Windows, SPSS Inc.). The results were reported as the mean ± standard deviation

(SD) (n=6). Statistical comparisons were performed using one-way analysis of variance (ANOVA). Probability values of (P<0.05) were considered statistically significant.

Results

Effect of olive oil on BMD and biochemical parameters

As results, OVX rats exhibited a notable reduction in BMD levels in contrast to control group. Additionally, OVX rats was associated with significant reduction in serum TP level. While they were associated with a high significant elevation (P<0.0001) in serum Cr level in relation to control group (0.91±0.05 vs .0.70±0.05 mg/dL; respectively).

On the contrary, the application of olive oil yielded a notable increase (P<0.001) in BMD level by 90.9% compared to OVX group. Also, olive oil administration to OVX group resulted in a significant rise (P <0.05) in serum TP levels by 15.6% and a significant (P <0.0001) decrease in Cr level by 19.8% in contrast to OVX group as shown in **Table 1**.

Further, OVX group exhibited a notable reduction (P<0.0001) in both serum and bone level of Ca and P in comparison to control group. The OVX group was also exhibited a significant (P<0.0001) elevation in ALP activities in both serum and bone. While olive oil treatment to OVX group was resulted in a significant elevate in both bone and serum level of Ca and P in relation to OVX group. Additionally, it also resulted in a significant reduction (P <0.0001) in ALP activities by 41.6% and 39.9% in both bone and serum, respectively in comparison to OVX group as shown in **Figure 1A-F**.

Effect of olive oil on hormonal parameters

Levels of serum parathyroid hormone (PTH) and estradiol (E2) hormone were determined among different groups and the results were demonstrated in **Figure 2A-B**. As a consequence, there was no statistically significant difference (P >0.05) observed between both the olive oil group and control group in PTH level but there was a notable elevate in E2 levels (P<0.05). However as anticipated OVX group was associated with high PTH level and reduced E2 levels compared to control group with a significant difference. Contrariwise, olive oil supplementation was found to attenuate levels of such hormones. As serum PTH level was significantly decreased (by 44.7 %), serum E2 level increased (by 41.1%) in OVX+olive oil group in contrast to OVX group.

Effect of olive oil on proinflammatory cytokines

Levels of serum TNF-α and IL-1β were determined among different groups and the results were demonstrated in **Figure 2C-D**. As a result, there was no statistical significant difference (P >0.05) between olive oil group and control group in TNF-α levels. On contrary, the TNF-α levels were significantly increased in OVX group by 116.7% compared to control group. But fortunately, olive-OVX rats showed attenuation in TNF-α levels. As olive-OVX rats was found to have a significant reduction in TNF-α levels by 30.8% in comparison to OVX group.

Regarding IL-1β levels, there was also no significant difference (P >0.05) between control group and olive oil group. On the contrary, there was a significant increase in IL-1β level in the serum of OVX rats (0.21±0.01pg/mL) in relation to control group (0.13±0.01pg/mL). While olive-OVX rats showed a significant (P < 0.001) decrease in IL-1β level by 23.8% compared to OVX group which may suggest the anti-inflammatory effect of olive oil in OVX-induced osteoporosis.

Effect of olive oil on oxidative stress and antioxidant biomarkers

Levels of bone MDA was determined among different groups and the results were demonstrated in **Table 2**. As a consequence, there was no difference (P >0.05) between both olive oil and control groups. On the contrary, there was a significant increase (P<0.0001) in MDA level in OVX rats (0.34±0.08 nmoL/mL) in relation to control group (0.17±0.01 nmoL/g). However, olive oil treatment to OVX group significantly (P<0.0001) reduces bone MDA level (0.19±0.02 nmoL/mL) as compared to OVX group.

Bone content of GSH and activities of SOD and CAT enzymes were determined among different groups and the results were demonstrated in **Table 2**. As a consequence, there was no difference between both olive oil

and control groups in bone GSH content. On contrary, the OVX group was observed to be linked with a significant decrease (P<0.0001) in GSH content when compared to control group. Whereas olive-OVX treated rats exhibited a notable elevation in GSH content than OVX rats by 81.3% change.

Also, in regard to SOD activity, there was no significant difference (P >0.05) between control group and olive oil group. While the OVX group was associated with a significant (P<0.0001) decrease in SOD activities in relation to control group. Olive oil treatment was found to enhance the activity of this enzyme in olive-OVX group compared to OVX group (0.30±0.01 *vs.* 0.19±0.03 U/L, P<0.0001).

Regarding CAT bone activity, there was a significant difference (P <0.05) between both the olive oil and control groups. On contrary, like other antioxidant enzymes, OVX rats were associated with significant (P<0.001) decrease in CAT activities by 46.2% in relative to control group (0.14±0.04 *vs.* 0.26±0.04 ng/mL). Whereas olive oil treatment increase CAT activity by 14.3% when compared to OVX group.

Effect of olive oil on histopathological architecture

The results of Photomicrograph of the femoral bone sections stained with H&E for the experimental groups were presented in **Figure 3**. The analysis yielded findings indicating that the bone examination of sections from the control group exhibited regular cortical and trabecular bone. Additionally, the bone marrow was characterized as of normal cellularity and normal bone marrow spaces were seen between the trabeculae **(Figure 3A)**. Furthermore, sections in the olive group were nearly similar to the control group. It was manifested by normal bone marrow stem cells that appeared as small cells with large basophilic nuclei and scanty cytoplasm with scattered fat globules **(Figure 3B)**.

However, in OVX-rats, the stained sections exhibited a clear histological modification of both trabeculae and cortical bone with widening of bone marrow spaces. These alterations were appeared in the form of bone marrow degeneration and distortion of endosteal surface which displayed erosion without the presence of osteoblast and osteogenic lining **(Figure 3C)**. Interestingly, OVX-induced rats that received the olive oil treatment revealed marked improvement in the histological architecture as compared to those of the OVX-rats. There was significant improvement of the cancellous and cortical bone appearance with less widened bone marrow spaces. The osteocytes were distributed within the bone matrix, and the endosteal surface appeared smooth and lined osteoblast cells **(Figure 3D)**.

Discussion

In the current investigation, we examined the alterations in bone turnover and bone depletion subsequent to ovariectomy. As anticipated, a notable decline in BMD was observed in the OVX-group in relation to the control group, thereby corroborating the manifestation of ovarian deficiency in the OVX model. Numerous studies have presented evidence of bone loss stimulated by insufficient levels of estrogen in rats that underwent ovariectomy. For instance, a 20% reduction in BMD has been reported in the femur of their OVX rats in contrast to sham rats during the period of 4-16 weeks post ovariectomy [18]. Also, it has been noted significantly lower BMD and cortical BMD in the OVX rats [19]. Furthermore, other investigators have reported an average femoral BMD loss of 15% and 20% post ovariectomy [20, 21].

Our findings reveal that the utilization of olive oil resulted in a significant 90.9% increase in bone mineral density (BMD) compared to the OVX group. This is consistent with the findings of [22] who observed an increase in BMD in the lumbar spine and left femur of OVX rats following olive oil supplementation. Similarly, it has been recorded a notable enhancement in BMD in OVX rats after administering virgin olive oil [23]. Also, it has been found that oleuropein, hydroxytyrosol, and olive oil can effectively mitigate trabecular bone loss induced by ovariectomy and promote an increase in BMD [24]. This favorable outcome can be attributed to the presence of phenolic compounds within olive oil, which exhibit antioxidant properties by scavenging free radicals. As a result, bone

cells are shielded from oxidative harm, thereby contributing to the observed effect [25].

In addition, it has been observed that a lack of estrogen may be linked to certain changes in metabolism and alterations in body composition, which can potentially lead to liver disorders [26]. Our research findings demonstrate a noteworthy decrease in TP levels in OVX rats, as previously noted in other studies [27, 28]. However, the administration of olive oil to OVX rats yielded a significant increase in TP levels. This rise in levels may suggest that olive oil has the capacity to stimulate the regeneration of liver tissue, thereby improving protein synthesis in a damaged liver and enhancing the functional status of liver cells [29].

Interestingly, Cr is a byproduct of creatine phosphate metabolism in the muscle. It is produced in the body at a consistent level, which is dependent on the mass of the muscle. Furthermore, it is not influenced by food intake, muscular efforts, or diuresis level. It is worth noting that osteoporosis can cause changes in blood and bone parameters. Cr is considered one of the most notable indicators of bone imbalance and is used for monitoring purposes [30]. In this study, the levels of Cr were found to be significantly elevated in cases of ovariectomy-induced osteoporosis, which aligns with previously reported findings [27, 31]. On the other hand, when olive oil supplementation was given to OVX-rats, there was a significant decrease in Cr levels. Likewise, it has been noted that the consumption of oleuropein appeared to have a significant attenuating effect on Cr levels in comparison to OVX-rats [32].

Additionally, Ca and P have a pivotal role in the formation of the skeletal system by actively participating in skeletal development. Sufficient levels of Ca and P are indispensable in mitigating skeletal deterioration and minimizing the probability of fractures. Earlier scientific investigations have revealed that ovariectomy in rats leads to an imbalanced Ca distribution, which in turn contributes to the development of osteoporosis induced by ovariectomy. Furthermore, multiple studies have provided evidence that there is a notable reduction in the levels of Ca and P in rats that have undergone ovariectomy [23, 33]

Menopause is linked to a reduction in the absorption of calcium in the intestines and an increase in the excretion of calcium by the kidneys. Additionally, estradiol affects the kidneys by increasing the reabsorption of Ca in the renal tubules. Consequently, alterations in estradiol levels are intricately linked to modifications in the expression of diverse proteins that play a crucial role in the reabsorption of Ca within the distal tubules [34]. Similarly, ovariectomy is also associated with imbalances in the rate of absorption and reabsorption of P by the small intestine and kidneys, resulting in a notable reduction in blood P concentrations [35]. Our experimental findings support this perspective, as OVX-rats that showed a significant decrease in both Ca and P levels in the serum and bones ($P<0.0001$).

On the other side, there was a notable increase ($P<0.0001$) in the levels of Ca and P with the application of olive oil treatment. Similarly, various studies have shown that olive oil effectively prevents hypocalcemia caused by ovariectomy in in the OVX+olive rats [23]. Furthermore, the presence of hydroxytyrosol and oleuropein in olive oil enables the deposition of Ca ions in osteoblastic cells and hinders the formation of osteoclasts[36]. Additionally, olive oil aids in the absorption of Ca in the intestines [24, 37]. Furthermore, t functions as a significant reservoir of gamma linolenic acid, which reduces the elimination of Ca, decrease bone reabsorption and affects bone turnover markers, while simultaneously increasing Ca levels in the bone [38]. Moreover, research has indicated that the phenols present in olive oil can effectively controlled the proliferative capacity and cell maturation of osteoblasts through the regulation of Ca ion deposition in the extracellular matrix [25].

Interestingly, ALP is acknowledged as a factor that determines the process of osteoblastic differentiation [24]. The activation of ALP prompts the mineralization of matrix proteins by means of the hydrolysis of pyrophosphate and inorganic phosphate [39]. In the course of this study, the OVX group demonstrated a significant increase in ALP

activities in both serum and bone in comparison to the control group. Consistent with these findings, it was previously documented that ALP levels significantly increased in OVX rats, indicating heightened osteoblastic activity and enhanced bone formation [34]. Additionally, it has been established a correlation between estrogen deficiency and expedited bone remodeling, wherein bone resorption surpassed bone formation [40].

Meanwhile, there was a notable decrease in the level of ALP in our OVX+olive oil rats. These findings align with previous studies that have shown the ability of olive oil to effectively reduce the solubility of serum ALP [41]. It is possible to postulate that the potential inhibition of bone loss by olive oil is attributed to its capacity to diminish the quantity of ALP, thereby impeding the generation of mineralized matrix protein by osteogenesis cells. Nonetheless, additional investigation is imperative in order to comprehensively comprehend this particular mechanism. [41]. Additionally, it has been observed that the phenolic compounds present in olive oil contribute to a significant enhancement in ALP synthesis, thereby promoting osteoblastic differentiation [42].

Furthermore, our findings indicate that the inclusion of olive oil in the diet of rats that have undergone ovariectomy can provide protection against osteoporosis by regulating the levels of PTH and E2 hormones. These results contradict previous studies that showed a significant decrease in serum estradiol levels in the OVX group compared to the sham group[19], and supported the notion that supplementing with olive oil in OVX-rats mitigated the development of osteoporosis caused by ovariectomy. One potential elucidation for this enhancement in skeletal well-being may be linked to the heightened concentrations of E2 and the abundance of mono-unsaturated fatty acids that are found in olive oil [43]. Additionally, it has been suggested that the consumption of extra virgin olive oil polyphenols can regulate the expression of genes associated with estrogen response in the uterus in a manner that mimics the effects of E2. Furthermore, olive oil may contain phytoestrogens, similar to those found in other plants or their extracts, which could potentially have milder effects similar to estradiol-17β benzoate and contribute to the preservation of bone mass during the post-menopausal period by acting as osteoprotective agents [44].

In contrast, the present investigation reveals that OVX rats exhibited a significant correlation with elevated levels of bone MDA, diminished bone GSH content, and reduced bone SOD and CAT activity when compared to the control group. Oxidative stress is a state characterized by an imbalance between the generation of pro-oxidants, oxidizing agents (ROS), and the presence of antioxidant defenses. This perturbation of equilibrium may result in the accumulation of ROS, which can subsequently transform into more potent oxidants, such as hydroxyl radicals, consequently leading to oxidative harm to lipids, proteins, and DNA. Ultimately, this oxidative damage can culminate in cellular demise[45].

Osteoporosis is often accompanied by the presence of oxidation stress and osteoblast apoptosis. This occurrence can be attributed to bone matrix degradation and the stimulation of osteoclast cells [46]. Notably, MDA levels in OVX-rats have been demonstrated to be markedly increased, indicating an increased presence of oxidative stress in these estrogen-deficient rats [23]. Moreover, the regulated cell death process known as ferroptosis can potentially contribute to the onset and progression of osteoporosis. This process is characterized by the generation of ROS, lipid peroxidation, accumulation of lipid hydroperoxides, and iron availability [47, 48].

Under normal physiological conditions, cells have the ability to combat free radical attack or oxidative stress by promoting antioxidant defenses. The body possesses various endogenous defense mechanisms, such as metal chelating proteins and endogenous antioxidant enzymes (GSH, CAT, and SOD) [49]. However, when antioxidants are unable to effectively counteract oxidative stress, diseases related to oxidative stress can manifest, including cardiovascular disease, diabetes, neurological diseases, cancer, and osteoporosis [50]. Previous studies have demonstrated that aged rats and post-menopausal women with increased oxidative stress exhibited reduced

SOD, GSH, and CAT activities [39, 51, 52]. These findings may explain the marked reduction in the activities of different oxidative parameters observed in the current study.

Interestingly, our findings recorded that the addition of olive oil effectively countered the oxidative damage in the OVX rats through the augmentation of the antioxidant defense mechanism and the decrease of lipid peroxidation. In fact, previous researches have been illustrated that the phenolic compounds present in olive oil possess antioxidant properties that can protect lipids and DNA from oxidation [53, 54]. These compounds have also been found to stimulate the transcription of the antioxidant enzyme glutathione peroxidase [55]. Moreover, they possess the capability to engage with biological systems and function as bioactive molecules. It should be noted that they exhibit remarkable efficacy in hindering lipid peroxidation [56] and are thought to exert their impacts via the process of metal chelation and free radical scavenging [57, 58]. Additionally, oleuropein, a compound found in olive oil, has been shown to scavenge free radicals and preserve other dietary antioxidants such as alpha-tocopherol and vitamin C from degradation in the intestines [59]. As a matter of fact, both black Luckes olives and extra virgin olive oil has been discovered to alleviate the heightened oxidative stress triggered by a lack of estrogen. In rats treated with virgin olive oil, markers indicative of oxidative stress exhibited a noteworthy decrease in the cerebral, musculoskeletal, and cardiac structures [60].

Surprisingly, the immune system assumes a pivotal function in the pathophysiology of osteoporosis subsequent to menopause. Consequently, an elevation in pro-inflammatory cytokines such as TNF-α and IL-1β can worsen osteoporosis as they participate in bone turnover and act as potent stimulants of bone resorption [61, 62]. It has been demonstrated that estrogen deficiency resulting from ovariectomy leads to the production of IL-1β and TNF-α by osteoblasts, which in turn activate and differentiate osteoclasts, ultimately increasing bone resorption [14, 63]. Also, numerous studies have Furthermore, many investigations have examined the connections between IL-1β gene polymorphisms and osteoporosis across a wide range of populations [43]. This may serve as a possible explanation for the significant high IL-1β and TNF-α levels observed in the OVX-rats in our investigation.

Meanwhile, administration of olive oil to our OVX-rats yielded a marked reduction decrease in IL-1β and TNF-α level. These results are inconsistent with previously studies that supported the bone-preserving effects of olive oil can be attributed to the suppression of inflammatory cytokines [43]. There exists evidence suggesting that certain phenols found in virgin olive oil possess anti-inflammatory properties [64] and can impact various biological processes like maintenance of DNA repair mechanisms, cell differentiation, and initiation of apoptosis in cancer cells [25, 65]. Further, it was demonstrated that the daily consumption of olive oil for a period of 84 days had a regulating effect on bone loss in OVX-rats, ultimately concluding that it exhibited favorable effects on proinflammatory cytokines, specifically IL-1β [66].

Moreover, the examination of bone resections of vertebrae and femurs indicated a clear reduction in bone mass in OVX rats compared to the sham group [67]. Similarly, our OVX group exhibited noticeable histological changes in trabeculae and cortical bone, accompanied by an enlargement of bone marrow spaces. Conversely, the administration of olive oil resulted in the restoration of these histological alterations in the cancellous and cortical bone. These findings align with previous studies conducted by [24, 60, 66]. Also, [23] noted a significant improvement in cortical and trabecular thickness in OVX rats who received virgin olive oil after ovariectomy.

In conclusion, olive oil successfully ameliorated osteoporosis induced by ovariectomy in rats, displaying potential as a viable option for addressing postmenopausal osteoporosis without the occurrence of unfavorable side effects. More research and well-planned clinical trials are required to validate the utilization of olive supplements in individuals with osteoporosis and ascertain the underlying mechanism of its impact.

Competing interests: None

Table 1. Levels of bone mineral density, serum total protein and serum creatinine in all experimental groups

Variables	Animal groups		Control	Olive oil	OVX	OVX+olive oil
BMD (g/cm^2)	Mean± SD	0.19±0.02	0.20±0.02	0.11±0.03**	0.21±0.01$^{\#\#}$	
	%Of change		+5.3^a	-42.1^a	+10.5^a; +90.9^b	
Serum TP (g/dL)	Mean± SD	7.2±0.4	7.6±0.3	6.4±0.4*	7.4±0.5$^{\#}$	
	%Of change		+5.6^a	-11.1^a	+2.8^a; +15.6^b	
Serum Cr (mg/dL)	Mean± SD	0.70±0.05	0.66±0.04	0.91±0.05***	0.73±0.02$^{\#\#\#}$	
	%Of change		-5.7^a	+30.0^a	+4.3^a; -19.8^b	

Data were expressed as mean± SD, n=6. OVX: ovariectomized; BMD: bone mineral density; TP: total protein; Cr: creatinine.

$^{*}P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.0001 compared to control group.

$^{\#}P$ <0.05, $^{\#\#}P$ <0.001 and $^{\#\#\#}P$ <0.0001 compared to OVX group.

^{a} and ^{b} percentage (%) of change compared to control and OVX groups, respectively.

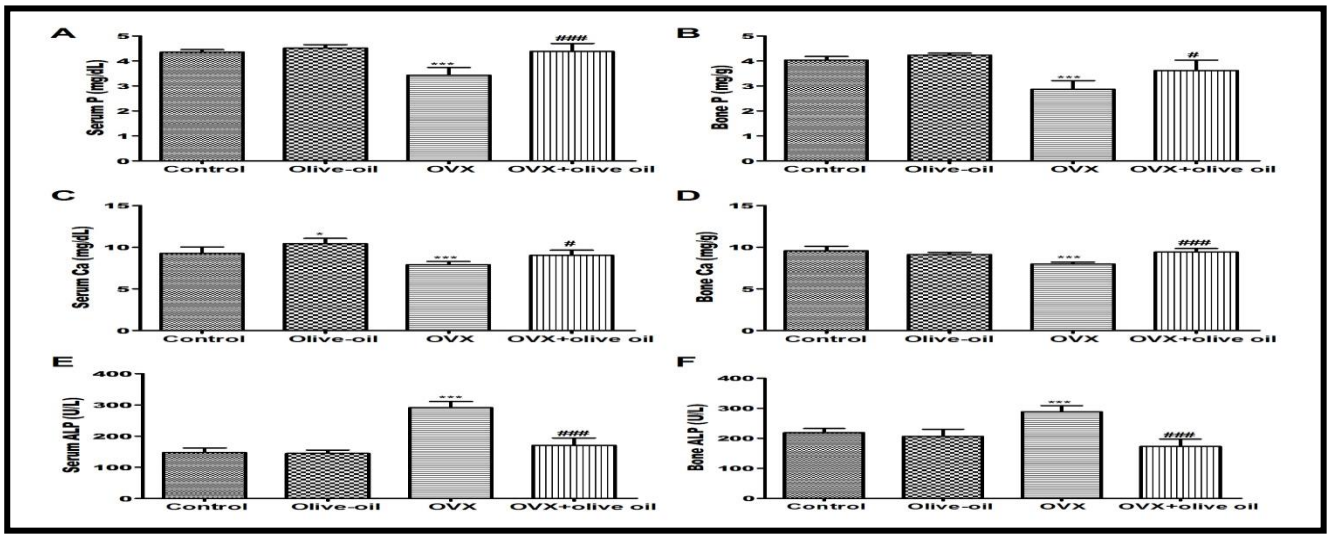

Figure 1. Minerals changes including (**A**): serum P, (**B**): bone P, (**C**): serum Ca, (**D**): Bone Ca, (**E**): serum ALP, (**F**): bone ALP in all experimental groups. Data were expressed as mean± SD, n=6. OVX: ovariectomized, P: phosphorous, Ca: calcium, ALP: alkaline phosphatase.

$^{*}P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.0001 in contrast to control group.

$^{\#}P$ <0.05, $^{\#\#}P$ <0.001 and $^{\#\#\#}P$ <0.0001 in contrast to OVX group.

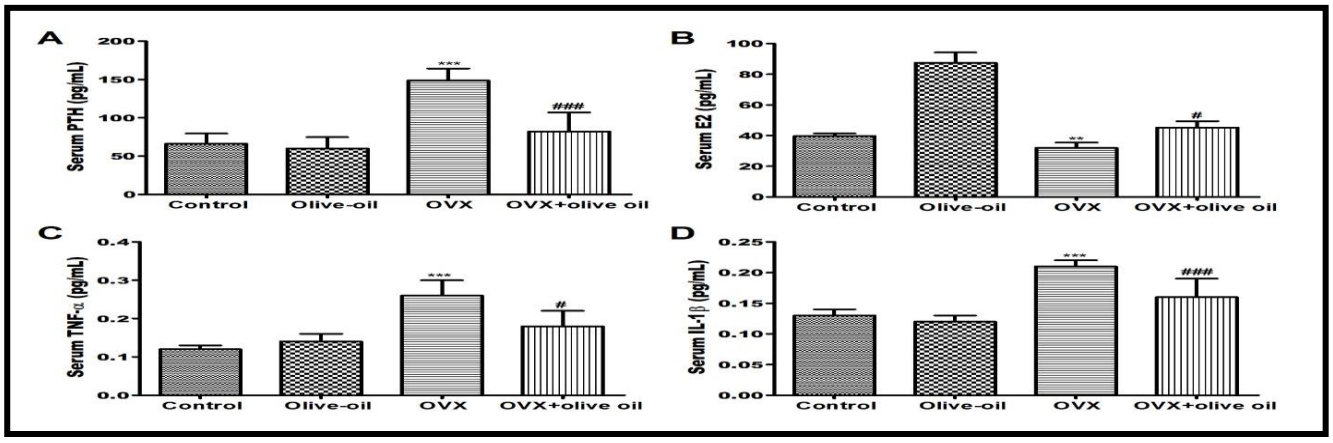

Figure 2. Level of serum (**A**): parathyroid hormone (PTH), (**B**): estradiol (E2) hormone (**C**): tumor necrosis factor-α (TNF-α) and (**D**): interleukin 1 beta (IL-1β) in all experimental groups. Data were expressed as mean± SD, n=6. OVX: ovariectomized.

$^{*}P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.0001 in contrast to control group.

$^{\#}P$ <0.05, $^{\#\#}P$ <0.001 and $^{\#\#\#}P$ <0.0001 in contrast to OVX group

Table 2. Oxidative stress and antioxidant biomarkers in all experimental groups

Variables	Animal groups			Control	Olive oil	OVX	OVX+olive oil
Bone MDA (nmoL/mL)	Mean± SD	0.17±0.01	0.17±0.02	0.34±0.08***	0.19±0.02###		
	%Of change		0.0[a]	+100.0[a]	+11.8[a]; -44.1[b]		
Bone GSH (ng/mL)	Mean± SD	0.28±0.04	0.48±0.03	0.16±0.05***	0.29±0.03###		
	%Of change		+71.4[a]	-42.9[a]	+3.6[a]; +81.3[b]		
Bone SOD (U/mL)	Mean± SD	0.43±0.03	0.45±0.03	0.19±0.03***	0.30±0.01###		
	%Of change		+4.7[a]	-55.8[a]	-30.2[a]; +57.9[b]		
Bone CAT (ng/mL)	Mean± SD	0.26±0.04	0.36±0.08*	0.14±0.04**	0.16±0.01		
	%Of change		+38.5[a]	-46.2[a]	-38.5[a]; +14.3[b]		

Data were expressed as mean± SD, n=6. OVX: ovariectomized; MDA: malondialdehyde; GSH: reduced glutathione; SOD: superoxide dismutase; CAT: catalase.

$^{*}P$ <0.05, $^{**}P$ <0.001 and $^{***}P$ <0.0001 in contrast to control group.

$^{\#}P$ <0.05, $^{\#\#}P$ <0.001 and $^{\#\#\#}P$ <0.0001 in contrast to OVX group.

[a] and [b] percentage (%) of change compared to control and OVX groups, respectively.

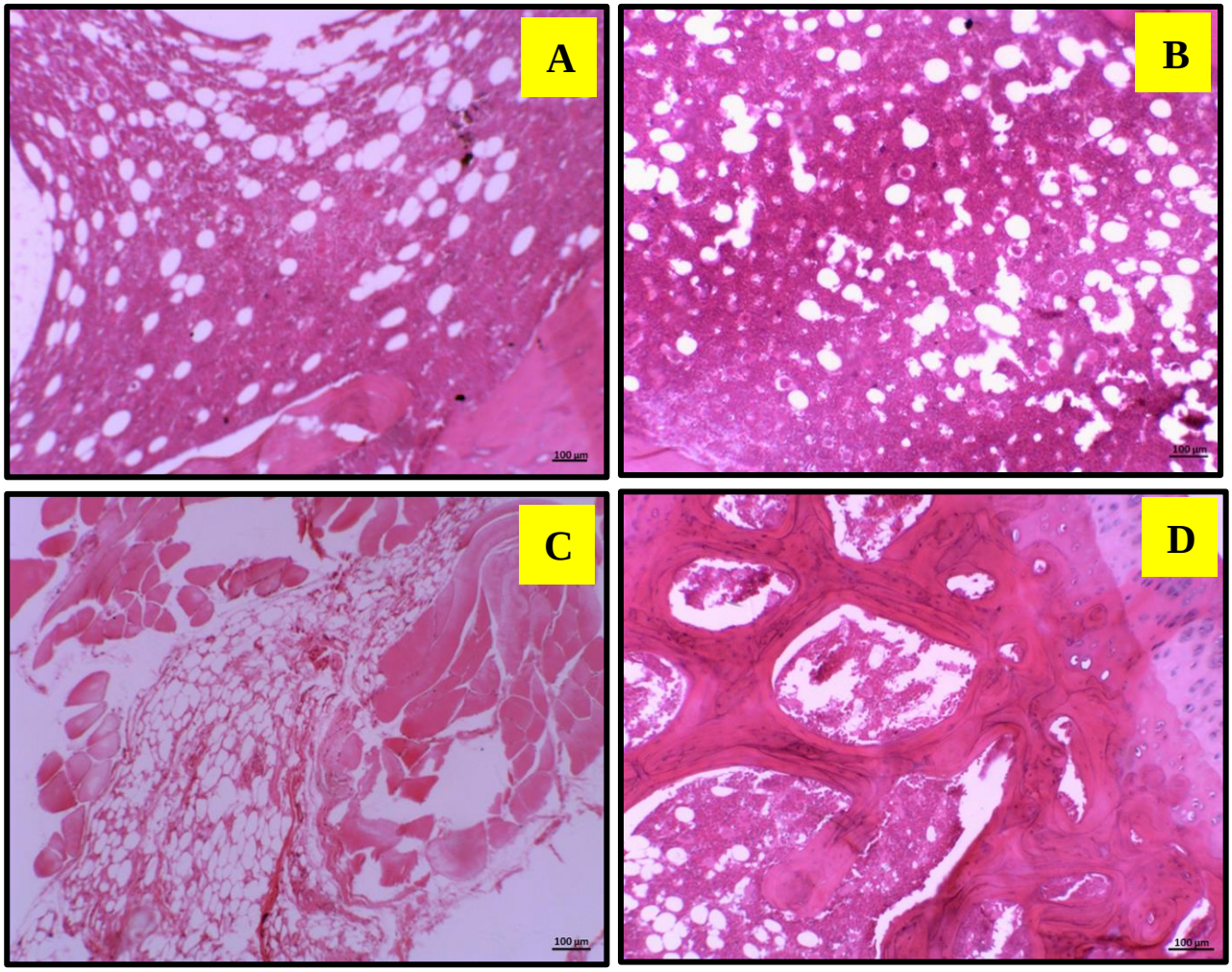

Figure 3. Histopathological examinations of all experiment group. **(A):** normal cortical and trabecular bone, in addition to normal bone marrow, (**B**): normal bone marrow stem cells appear as small cells with large basophilic nuclei and scanty cytoplasm with scattered fat globules, (**C**): histological alteration of trabeculae and cortical bone with bone marrow degeneration and widening of bone marrow spaces, (**D**): normal cortical bone with normal osteoblasts and normal osteoid matrix with normal bony trabeculae (HE, 10x).

References

1. Liang B, Burley G, Lin S, Shi Y-C. (2022). Osteoporosis pathogenesis and treatment: existing and emerging avenues. Cellular & Molecular Biology Letters.**27(1)**:72-97.
2. Salamanna F, Contartese D, Veronesi F, Martini L, Fini M. (2022). Osteoporosis Preclinical Research: A Systematic Review on Comparative Studies Using Ovariectomized Sheep. *International Journal of Molecular Sciences*.**23(16)**:1-18.
3. Salari N, Ghasemi H, Mohammadi L, Behzadi Mh, Rabieenia E, Shohaimi S, et al. (2021). The global prevalence of osteoporosis in the world: a comprehensive systematic review and meta-analysis. *Journal of Orthopaedic Surgery and Research*.**16(1)**:1-20.
4. Shen Y, Huang X, Wu J, Lin X, Zhou X, Zhu Z, et al. (2022). The Global Burden of Osteoporosis, Low Bone Mass, and Its Related Fracture in 204 Countries and Territories, 1990-2019. Frontiers in Endocrinology.**13**: 1-11.
5. Jiang Z, Wang H, Qi G, Jiang C, Chen K, Yan Z. (2022). Iron overload-induced ferroptosis of osteoblasts inhibits osteogenesis and promotes osteoporosis: An in vitro and in vivo study. IUBMB Life.**74(11)**:1052-1069.
6. Hassan N, Gregson CL, Tobias JH. (2021). Anabolic treatments for osteoporosis in postmenopausal women. Faculty reviews.**10**:44-52.
7. Tao ZS, Li TL, Wei S. (2022). Probucol promotes osteoblasts differentiation and prevents osteoporosis development through reducing oxidative stress. Molecular medicine (Cambridge, Mass).**28(1)**:1-14.
8. Suvarna V, Sarkar M, Chaubey P, Khan T, Sherje A, Patel K, et al. (2018). Bone Health and Natural Products- An Insight. Frontiers in pharmacology.**9**:1-12.
9. Casado-Díaz A, Dorado G, Quesada-Gómez JM. (2019). Influence of olive oil and its components on mesenchymal stem cell biology. *World journal of stem cells*.**11(12)**:1045-1064.
10. Farràs M, Almanza-Aguilera E, Hernáez Á, Agustí N, Julve J, Fitó M, et al. (2021). Beneficial effects of olive oil and Mediterranean diet on cancer physio-pathology and incidence. Seminars in cancer biology.**73**:178-195.
11. Díaz-Curiel M, Torrubia B, Martín-Fernández M. (2020). Effects of Virgin Olive Oil on Bone Health in Ovariectomized Rats. Nutrients.**12(5)**:1270-1283.
12. Yousefzadeh N, Kashfi K, Jeddi S, Ghasemi A. (2020). Ovariectomized rat model of osteoporosis: a practical guide. *EXCLI journal*.**19**:89-107.
13. Turner RT, Maran A, Lotinun S, Hefferan T, Evans GL, Zhang M, et al. (2001). Animal models for osteoporosis. Reviews in endocrine & metabolic disorders.**2(1)**:117-127.
14. Brincat S, Borg M, Camilleri G, Calleja-Agius J. (2014). The role of cytokines in postmenopausal osteoporosis. Minerva ginecologica.**66(4)**:391-407.
15. National Research Council. Nutrient requirements of laboratory animals: (1995), 4th ed edn Washington DC: National Academy Press, pp. 56-82.
16. Lei Z, Xiaoying Z, Xingguo L. (2009). Ovariectomy-associated changes in bone mineral density and bone marrow haematopoiesis in rats. International *journal of experimental pathology*.**90(5)**:512-519.
17. Bancroft JD, Gamble M. (2002) Theory and practice of histological techniques. Fifth ed. London (UK), Churchill Livingstone: Elsevier health sciences;.
18. Sequeira L, Nguyen J, Wang L, Nohe A. (2020). A Novel Peptide, CK2. 3, Improved Bone Formation in Ovariectomized Sprague Dawley Rats. *International journal of molecular sciences*.**21(14)**:1-13.
19. Yoon K-H, Cho D-C, Yu S-H, Kim K-T, Jeon Y, Sung J-K. (2012). The change of bone metabolism in ovariectomized rats: analyses of microCT scan and biochemical markers of bone turnover. *Journal of Korean Neurosurgical Society*.**51(6)**:323-327.

20. Yıldırım M, Saral S, Mercantepe T, İskender H, Tümkaya L, Atak M, et al. (2020). White tea reduced bone loss by suppressing the TRAP/CTX pathway in ovariectomy-induced osteoporosis model rats. Cells Tissues Organs.**209(1)**:64-74.
21. Zhou RP, Lin SJ, Wan WB, Zuo HL, Yao FF, Ruan HB, et al. (2016). Chlorogenic acid prevents osteoporosis by Shp2/PI3K/Akt pathway in ovariectomized rats. PLoS One.**11(12)**:1-11.
22. Liu H, Huang H, Li B, Wu D, Wang F, Zheng X, et al. (2014). Olive oil in the prevention and treatment of osteoporosis after artificial menopause. Clinical interventions in aging.**9**:2087-2095.
23. Saleh NK, Saleh HA. (2011). Olive oil effectively mitigates ovariectomy-induced osteoporosis in rats. BMC Complement Altern Med.**11**:1-11.
24. Hagiwara K, Goto T, Araki M, Miyazaki H, Hagiwara H. (2011). Olive polyphenol hydroxytyrosol prevents bone loss. European *journal of pharmacology*.**662(1-3)**:78-84.
25. García-Martínez O, Rivas A, Ramos-Torrecillas J, De Luna-Bertos E, Ruiz C. (2014). The effect of olive oil on osteoporosis prevention. International *Journal of Food Sciences and Nutrition*.**65(7)**:834-840.
26. Chong CLG, Hussan F, Othman F. (2019). Hepatoprotective effects of Morinda citrifolia leaf extract on ovariectomized rats fed with thermoxidized palm oil diet: evidence at histological and ultrastructural level. Oxidative medicine and cellular longevity, 2019: 1-10.
27. Khajuria DK, Razdan R, Mahapatra DR. (2015). Additive effects of zoledronic acid and propranolol on bone density and biochemical markers of bone turnover in osteopenic ovariectomized rats. Revista Brasileira de Reumatologia (English Edition).**55(2)**:103-112.
28. de Quadros VP, Tobar N, Viana LR, dos Santos RW, Kiyataka PHM, Gomes-Marcondes MCC. (2019). The 17β-oestradiol treatment minimizes the adverse effects of protein restriction on bone parameters in ovariectomized Wistar rats. Bone & Joint Research.**8(12)**:573-581.
29. Cornwell DG, Ma J. (2008). Nutritional benefit of olive oil: the biological effects of hydroxytyrosol and its arylating quinone adducts. *Journal of agricultural and food chemistry*.**56(19)**:8774-8786.
30. Patel SS, Molnar MZ, Tayek JA, Ix JH, Noori N, Benner D, et al. (2013). Serum creatinine as a marker of muscle mass in chronic kidney disease: results of a cross-sectional study and review of literature. J Cachexia Sarcopenia Muscle.**4(1)**:19-29.
31. Lazar A, Lăcătuş R, Cristian P, Bel L, Sarpataki O, Papuc I, et al. (2017). Implications of creatinine and alp in the induction of osteoporosis by ovariectomy in female wistar rats. Revista de Chimie, **68**: 1799-1803.
32. Puel C, Mathey J, Agalias A, Kati-coulibaly S, Mardon J, Obled C, et al. (2006). Dose–response study of effect of oleuropein, an olive oil polyphenol, in an ovariectomy/inflammation experimental model of bone loss in the rat. Clinical Nutrition.**25(5)**:859-868.
33. Hassan HA, El Wakf AM, El Gharib NE. (2013). Role of phytoestrogenic oils in alleviating osteoporosis associated with ovariectomy in rats. Cytotechnology.**65(4)**:609-619.
34. Seif AA. (2014). Nigella Sativa reverses osteoporosis in ovariectomized rats. BMC Complementary and Alternative Medicine.**14(1)**:1-8.
35. Xu Z-S, Dai F, Chen J, Lv M, Cheng J-W, Zhang X-M, et al. (2018). Experimental research into the potential therapeutic effect of GYY4137 on Ovariectomy-induced osteoporosis. Cellular & Molecular Biology Letters.**23(1)**:1-13.
36. García-Villalba R, Larrosa M, Possemiers S, Tomás-Barberán F, Espín J. (2014). Bioavailability of phenolics from an oleuropein-rich olive (Olea europaea) leaf extract and its acute effect on plasma antioxidant status: comparison between pre-and postmenopausal women. European *journal of nutrition*.**53(4)**:1015-1027.
37. Campos M, López-Aliaga I, Barrionuevo M, Lisbona F, Coves F. (1989). Nutritive

utilization of calcium in rats: effects of dietary fat components and vitamin D3 on intestinal resected rats. *Journal of nutritional science and vitaminology*.**35(5)**:511-521.
38. Claassen N, Potgieter H, Seppa M, Vermaak W, Coetzer H, Van Papendorp D, et al. (1995). Supplemented gamma-linolenic acid and eicosapentaenoic acid influence bone status in young male rats: effects on free urinary collagen crosslinks, total urinary hydroxyproline, and bone calcium content. Bone.**16(4)**:385-392.
39. Yin H, Shi Z-G, Yu Y-S, Hu J, Wang R, Luan Z-P, et al. (2012). Protection against osteoporosis by statins is linked to a reduction of oxidative stress and restoration of nitric oxide formation in aged and ovariectomized rats. European *Journal of Pharmacology*.**674(2)**:200-206.
40. Grassi F, Fan X, Rahnert J, Weitzmann MN, Pacifici R, Nanes MS, et al. (2006). Bone re/modeling is more dynamic in the endothelial nitric oxide synthase(-/-) mouse. Endocrinology.**147(9)**:4392-4399.
41. Liu H, Huang H, Li B, Wu D, Wang F, Zheng X, et al. (2014). Olive oil in the prevention and treatment of osteoporosis after artificial menopause. Clinical interventions in aging.**9**:2087-2095.
42. Melguizo-Rodríguez L, Manzano-Moreno FJ, De Luna-Bertos E, Rivas A, Ramos-Torrecillas J, Ruiz C, et al. (2018). Effect of olive oil phenolic compounds on osteoblast differentiation. *European Journal of Clinical Investigation*.**48(4)**:1-6.
43. Zheng X, Huang H, Zheng X, Li B. (2016). Olive oil exhibits osteoprotection in ovariectomized rats without estrogenic effects. Exp Ther Med.**11(5)**:1881-1888.
44. Keiler AM, Zierau O, Bernhardt R, Scharnweber D, Lemonakis N, Termetzi A, et al. (2014). Impact of a functionalized olive oil extract on the uterus and the bone in a model of postmenopausal osteoporosis. *European journal of nutrition*.**53**:1073-1081.
45. Domazetovic V, Marcucci G, Iantomasi T, Brandi ML, Vincenzini MT. (2017). Oxidative stress in bone remodeling: role of antioxidants. Clin Cases Miner Bone Metab.**14(2)**:209-216.
46. Sánchez-Rodríguez MA, Ruiz-Ramos M, Correa-Muñoz E, Mendoza-Núñez VM. (2007). Oxidative stress as a risk factor for osteoporosis in elderly Mexicans as characterized by antioxidant enzymes. BMC musculoskeletal disorders.**8(1)**:1-7.
47. Gao Z, Chen Z, Xiong Z, Liu X. (2022). Ferroptosis - A new target of osteoporosis. Experimental Gerontology.165:111836.
48. Xia Y, Zhang H, Wang H, Wang Q, Zhu P, Gu Y, et al. (2022). Identification and validation of ferroptosis key genes in bone mesenchymal stromal cells of primary osteoporosis based on bioinformatics analysis. Front Endocrinol (Lausanne).**13**:1-15.
49. Ighodaro O, Akinloye OA. (2017). First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. Alexandria *Journal of Medicine*.**54**:287-293.
50. Rao AV, Rao LG. (2007). Carotenoids and human health. Pharmacological research.**55(3)**:207-216.
51. Yalin S, Sagir O, Comelekoglu U, Berköz M, Eroglu P. (2012). Strontium ranelate treatment improves oxidative damage in osteoporotic rat model. Pharmacological Reports.**64(2)**:396-402.
52. Ozgocmen S, Kaya H, Fadillioglu E, Aydogan R, Yilmaz Z. (2007). Role of antioxidant systems, lipid peroxidation, and nitric oxide in postmenopausal osteoporosis. Molecular and cellular biochemistry.**295(1-2)**:45-52.
53. Masella R, Vari R, D'Archivio M, Di Benedetto R, Matarrese P, Malorni W, et al. (2004). Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. *The Journal of nutrition*.**134(4)**:785-791.
54. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. (2000). Phenolic compounds and squalene in olive oils: the concentration and antioxidant potential of total phenols, simple phenols,

secoiridoids, lignansand squalene. Food and chemical toxicology.**38(8)**:647-659.
55. Nakbi A, Tayeb W, Dabbou S, Issaoui M, Grissa AK, Attia N, et al. (2010). Dietary olive oil effect on antioxidant status and fatty acid profile in the erythrocyte of 2,4-D- exposed rats. Lipids in Health and Disease.**9(1)**:1-10.
56. Salah N, Miller NJ, Paganga G, Tijburg L, Bolwell GP, Rice-Evans C. (1995). Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. Archives of biochemistry and biophysics.**322(2)**:339-346.
57. Kandaswami C, Middleton E, Jr. (1994). Free radical scavenging and antioxidant activity of plant flavonoids. Advances in experimental medicine and biology.**366**:351-376.
58. Omar SH. (2010). Oleuropein in olive and its pharmacological effects. Scientia pharmaceutica.**78(2)**:133-154.
59. Edgecombe SC, Stretch GL, Hayball PJ. (2000). Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. *The Journal of nutrition.***130(12)**:2996-3002.
60. Tagliaferri C, Davicco M-J, Lebecque P, Georgé S, Amiot M-J, Mercier S, et al. (2014). Olive oil and vitamin D synergistically prevent bone loss in mice. PloS one.**9(12)**:1-19.
61. Knudsen S, Harsløf T, Husted LB, Carstens M, Stenkjær L, Langdahl BL. (2007). The effect of interleukin-1α polymorphisms on bone mineral density and the risk of vertebral fractures. Calcified Tissue International.**80(1)**:21-30.
62. Lechner J, Rudi T, von Baehr V. (2018). Osteoimmunology of tumor necrosis factor-alpha, IL-6, and RANTES/CCL5: a review of known and poorly understood inflammatory patterns in osteonecrosis. Clinical, cosmetic and investigational dentistry.**10:**251-262.
63. Redlich K, Smolen JS. (2012). Inflammatory bone loss: pathogenesis and therapeutic intervention. Nature reviews Drug discovery.**11(3)**:234-250.
64. Cicerale S, Lucas L, Keast R. (2012). Antimicrobial, antioxidant and anti-inflammatory phenolic activities in extra virgin olive oil. Current opinion in biotechnology.**23(2)**:129-135.
65. Cicerale S, Conlan XA, Barnett NW, Keast RS. (2013). Storage of extra virgin olive oil and its effect on the biological activity and concentration of oleocanthal. Food Research International.**50(2)**:597-602.
66. Puel C, Quintin A, Agalias A, Mathey J, Obled C, Mazur A, et al. (2004). Olive oil and its main phenolic micronutrient (oleuropein) prevent inflammation-induced bone loss in the ovariectomised rat. *British journal of nutrition.***92(1)**:119-127.
67. Palumbo C, Ferretti M, Bertoni L, Cavani F, Resca E, Casolari B, et al. (2009). Influence of ferutinin on bone metabolism in ovariectomized rats. I: role in preventing osteoporosis. *Journal of bone and mineral metabolism*.**27**:538-545.