Protective effect of fish liver oil and propolis on anticonvulsant drugs-induced osteoporosis

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Background/aim
Osteoporosis is a major health problem and its prevalence increases the risk of bone fracture. It is classified into primary (postmenopausal or age related) and secondary (related to chronic diseases, drug therapy, or life style). There is accumulating evidence that patients on antiepileptic drugs (AEDs) are at an increasing risk of developing osteoporosis. The present study aimed at investigating the protective effect of dietary natural products, fish liver oil, and propolis on osteoporosis caused by anticonvulsant drugs.

Materials and methods
A total of 105 albino rats were used, divided into seven groups of 15 rats each. Group 1 was used as a control group. In group 2, rats were injected intraperitoneally with pilocarpine (300 mg/kg body weight). The pilocarpine-induced epileptic rats in the other five groups were orally treated with valproate (400 mg/kg body weight), a combination of valproate and fish liver oil (0.4 ml/kg body weight/day), a combination of valproate and propolis (50 mg/kg body weight/day), fish liver oil, and propolis, respectively. At the end of the experiment (6 months treatment), animals were sacrificed, femur shafts were extracted, decalcified, and processed into paraffin blocks for histopathological and image analysis and morphometric studies.

Results
Rats treated with the antiepileptic valproate alone showed a decrease in the thickness of shaft cortical bone, with a marked decrease in the number of osteocytes, increase in Haversian canals, and decrease in bone trabeculae, disruption of normal architecture, and widening of bone marrow spaces compared with the control group.

Conclusion
Treatment with the dietary natural products, fish liver oil, and propolis along with the AED valproate might improve histopathological changes and morphometric parameters in bone associated with AED-induced osteoporosis.

Keywords:
epilepsy, fish liver oil, morphometry, osteocytes, osteoporosis, propolis, rat

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Introduction

Epilepsy is a major public health problem that affects almost 50 million individuals worldwide [1]. Treatment with antiepileptic drugs (AEDs) is generally chronic if not lifelong and may be associated with significant metabolic effects including decreased bone mass and increased fractures [2,3]. The antiepileptic medications encompass a wide range of drugs including phenytoin and benzodiazepines, which are enzyme inducers with a variety of effects, including induction of cytochrome P450 and other enzymes, which may lead to catabolism of vitamin D, with hypocalcemia [4]. Anticonvulsants such as valproic acid, which is a hepatic enzyme inhibitor, is also associated with decreased bone mineral density (BMD) and is believed to act by stimulating osteoclast activity and may cause imbalance between bone formation and resorption, contributing toward bone loss [5].

Osteoporosis is a disease of bone that leads to reduced BMD, disrupted bone microarchitecture, and alteration in the amount and variety of noncollagenous proteins in bone [6].

Valproic acid (VPA) is a broad-spectrum AED that has been used for more than 30 years and is effective in the treatment of different types of partial and generalized epileptic seizure. Sodium valproate is a branched-chain saturated fatty acid (2-propylpentanoic acid) that has been used as an anticonvulsant in the treatment of epilepsy [7]. It is also prescribed to treat bipolar and schizoaffective disorders, social phobias, and neuropathic pain, as well as prophylaxis or treatment of migraine. VPA is a branched-chain carboxylic acid (2-propylpentanoic acid or di-*n*-propylacetic acid) with a chemical structure very similar to that of short-chain fatty acids [8].

Valproate has been reported to induce reversible Fanconi syndrome and to cause renal tubular dysfunction resulting in excess calcium and phosphorus loss [9].

It is well known that cod liver oil contributes toward bone health, preventing rickets in children and osteomalacia in adults [10]. Cod liver oil, fish, and vitamin D-fortified food are the usual dietary sources of vitamin D, which aids building of strong bones in children and prevents osteoporosis in adults [11]. Consumption of n-3 polyunsaturated fatty acids in fish oil markedly modulates immune and inflammatory responses [12].

Sufficient elongated ω-3 oils found in cod liver oil are one of the keys for maintaining and rebuilding bone. In women, higher levels of vitamin D from cod liver oil improve BMD. Vitamin D in cod liver oil promotes absorption of calcium and magnesium, thereby lowering blood pressure [13]. Dietary fish oil enriches phospholipids with ω-3 fatty acids, particularly eicosapentaenoic acid, while decreasing the content of arachidonic acid [14].

Propolis has been used in folk medicine for centuries. It is known that propolis possesses antimicrobial, antioxidant, antiulcer, and antitumor activities. Therefore, propolis has attracted considerable attention in recent years as a useful or a promising substance in medical and cosmetics products. Furthermore, it is now used extensively in food and beverages with the claim that it can maintain or improve human health. The chemical composition of propolis is quite complicated. More than 300 compounds such as polyphenols, phenolic aldehydes, sesquiterpene, quinines, coumarins, amino acids, steroids, and inorganic compounds have been identified in propolis samples. The contents depend on the collection time and the plant source [15].

Inokuchi *et al*. [16] investigated the Brazilian green propolis and reported that it exerts a neuroprotective effect in the retina *in vitro* and *in vivo*. They added that propolis induced inhibition of oxidative stress, which may be responsible for these neuroprotective effects.

Yamaguchi [17] determined the effects of bee pollen and propolis extract (also known as bee glue) on bone components in the femoral tissues of rats. He reported that the water-solubilized propolis extract obtained from the bee pollen of *Cistus ladaniferus* exerted anabolic effects on bone components and caused a significant increase in calcium content in the femoral diaphyseal and metaphyseal tissues of rats *in vivo* and *in vitro*. Propolis and bee propolis contain, pollen, resins, waxes, and large amounts of flavonoids, which are benzo-γ pyrone derivatives found in all photosynthesizing cells.

It appears likely that the beneficial effects of propolis and honey are a result of their flavonoid content [18].

The phenolic ester (caffeic acid phenethyl ester or CAPE) present in propolis is known to have antimitogenic, anticarcinogenic, anti-inflammatory, and immunomodulatory properties. The CAPE completely blocks the activation of nuclear factor κB (NF-κB) transcription factor or RANK by tumor necrosis factor (TNF) in a dose-dependent manner. CAPE prevented the translocation of the P65 subunit of NF-κB to the nucleus and exerted no significant effect on TNF-induced 1κBα degradation, but delayed 1κBα resynthesis [19].

The aim of the present study is to investigate the reversing effects of fish oil and propolis against valproate-induced osteoporosis in the bone of rats.

Materials and methods

A total of 105 male rats were used, divided into seven groups of 15 rats each.

Group 1: this group was used as the control group.
Group 2: rats were injected intraperitoneally with pilocarpine (300 mg/kg body weight).
Group 3: pilocarpine-induced epileptic rats were orally treated with valproate (400 mg/kg body weight).
Group 4: pilocarpine-induced epileptic rats were treated with a combination of valproate and fish liver oil (0.4 ml/kg body weight/day).
Group 5: pilocarpine-induced epileptic rats were treated with a combination of valproate and propolis (50 mg/kg body weight/day).
Group 6: rats were orally treated with fish liver oil.
Group 7: rats were orally treated with propolis.

At planned time points, animals were scarified and the shafts of the femur were removed, immersed in glutaraldehyde, and after 4 h, were decalcified by EDTA solution for 20 days. Paraffin (5 μm) tissue sections from the middle shaft of the femur were cut using a conventional technique. Sections were stained with hematoxylin and eosin [20] and examined by a light microscope.

To measure the gray levels of total proteins in specially stained slides [21], areas of reactivity were marked and the optical density of total proteins was measured using the gray image menu in 10 small measuring frames in each specimen. The image was transformed into a gray image (a grid of pixels), each representing the intensity of brightness at that point by a range of numbers, typically from 0 (black) to 255 (white). This means that when the readings are nearest to the black, the highest contents of total protein are found and when the readings are nearest to the white, small amounts of the protein are found.

The experiment was conducted in accordance with the national regulations of animal welfare and Institutional Animal Ethical Committee (IAEC), National Research Center.

Morphometric analysis

The mean values of areas of reactivity at five fields in different sections from five animals were assessed using a Leica-Qwin 500 Image Analyzer System (Leica Imaging Systems Ltd, Cambridge, UK) in the Pathology Department, National Research Center.

Results

Histological and morphometric results

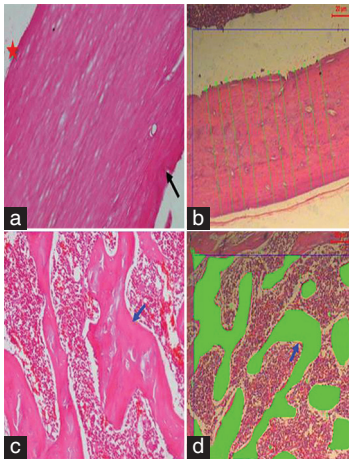
Histological sections from the middle shaft of the femur in rats in the control group showed that bone tissue was externally covered by a layer of a dense connective tissue, the periosteum. A thin layer of cell-rich connective tissue, the endosteum, lined the internal surface of bone facing the bone marrow cavity. Within the bone matrix, osteocytes in their lacunae were detected. Most of the individual lamellae in mature compact bone form concentric layers around large longitudinal canals called Haversian canals (Figs. 1a and b). Bone trabeculae of the cancellous bone of control rats were composed of a network of irregular bone lamellae between which osteocytes reside in their lacunae. Bone marrow spaces were observed between trabeculae (Figs. 1c and d).

No pathological changes were observed in the case of rats treated with fish liver oil and/or propolis.

The group of pilocarpine-induced epilepsy rats showed a slight decrease in the thickness of the compact bone in the shaft (68.57 vs. 74.93 μm) and decreased number of osteocytes (58 vs. 112) compared with the control group. Resorption cavity with granulation tissue within the shaft and calcified cartilage were observed in the shaft of compact bone (Table 1 and Fig. 2a). A slight decrease in the mean area of bone trabeculae in the head of the femur was detected in the same group (632.25 μm2) compared with the control group (674.34 μm2) (Table 1 and Fig. 2b).

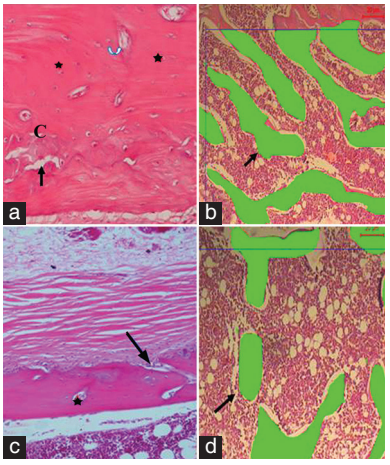
Examination of the middle shaft of the femur of epileptic rats treated with valproate only showed decreased thickness of the compact bone in the shaft (33.29 vs. 74.93 μm) with decreased number of osteocytes (56 vs. 112) compared with the control group. An increase in the mean areas of Haversian canals (28.06 μm2) was observed compared with the

Figure 1



(a) A longitudinal section of the middle shaft of the femur of a control rat showing an outer fibrous layer, periosteum (star), and an inner layer facing the marrow cavity, endosteum (arrow). The bone matrix contains many Haversian canals with blood vessels with concentric lamellae surrounded by osteocytes. (b) A longitudinal section of the middle shaft of femur bone of a control rat showing normal cortical width of the shaft. (c) A longitudinal section in the head of the femur of a control rat showing normal architecture of the trabeculae of the inner cancellous bone (arrow) and bone marrow spaces. (d) The head of the femur of normal rat showing normal thickness of bone trabeculae (arrow). (a, c) Hematoxylin and eosin-stained sections; (b, d) binary image morphometric measurement, × 400.

Figure 2



(a) A longitudinal section of the middle shaft of the femur of a rat treated with pilocarpine showing the resorption cavity (curved arrow) and decreased number of osteocytes (stars). The bone tissue contains calcified cartilage (C) and osteoporotic cavities (black arrow). (b) The head of the femur of a rat treated with pilocarpine showing a slight decrease in the thickness of the bone trabeculae (arrow). (c) A longitudinal section of the middle shaft of the femur of a pilocarpine-induced epileptic rat treated only with valproate, showing a marked decrease in shaft cortical width, erosion cavity at the outer surface (arrow), and decreased number of osteocytes. The bone tissue contains calcified cartilage and resorption cavities (star). (d) The head of the femur of a pilocarpine-induced epileptic rat treated only with valproate showing a marked decrease in the thickness of the bone trabeculae (arrow). (a, c) Hematoxylin and eosin-stained sections; (b, d) binary image morphometric measurement, ×400.

control group (15.66 μm2). Erosion cavities were detected on the outer surface, and calcified cartilage and resorption cavities were observed in the bone tissue. The periosteum showed marked thickening (Table 1 and Fig. 2c). Decreased mean areas of bone trabeculae (589.71 μm2) were observed compared with the control group (674.34 μm2) (Table 1 and Fig. 2d). The bone trabeculae of cancellous bone lost their normal architecture and the periosteum appeared discontinuous. Some bone trabeculae showed an irregular eroded outer surface. They contained calcified cartilage and were separated by bone marrow spaces (Fig. 3).

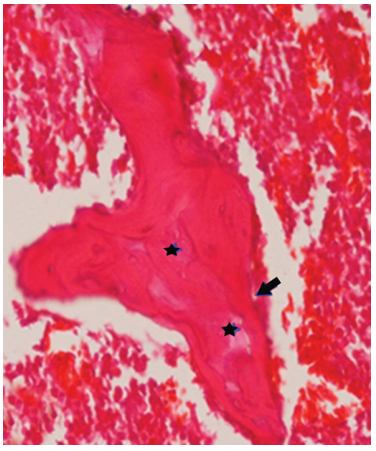
Bone sections in epileptic rats treated with valproate and fish liver oil showed a slight increase in the number of osteocytes (n = 56) and increased thickness of the shaft (63.26 μm) compared with rats treated only with valproate (33.29 μm). Erosion cavities were detected on the endosteal surface and resorption cavities were observed in the bone tissue (Table 1 and Fig. 4a). There was an increase in the (mean area) of bone trabeculae in the head of the femur (776.48 μm2) compared with group treated only with valproate (589.71 μm2) (Table 1 and Fig. 4b).

Examination of bone sections in rats treated with valproate and propolis showed an improvement in the pathological changes in the form of increased number of osteocytes (n = 71) and increased thickness of the shaft cortical bone (68.67 μm) compared with rats treated only with valproate (n = 55 and 33.29 μm, respectively). Erosion cavities were detected on the periosteal surface, and resorption cavities with granulation tissue were observed within the compact bone of the shaft (Table 1 and Fig. 4c). The mean areas of Haversian canals were fewer in the valproate and propolis-treated group (22 μm2) than in the group of rats treated only with valproate (28.06 μm2). Increased mean area of trabeculae in the femur head (880.29 μm2) in the valproate and propolis-treated group compared with rats treated only with valproate (589.71 μm2) was detected (Fig. 4d and Fig. 5)

Table 1 Mean area of cortical bone thickness (shaft), mean Haversian canal, trabecular thickness, and number of osteocytes of different groups

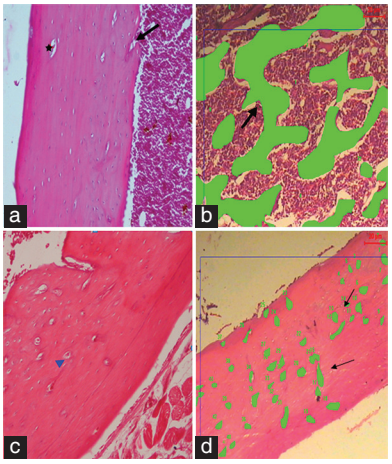
Groups	Parameter			
	Shaft thickness mean (μm) (n = 5)	Haversian canal's area mean (μm2) (n = 5)	Head trabeculae area mean (μm2) (n = 5)	Number of osteocytes
Control	74.93	15.66	674.34	112
Pilocarpin	68.57	18.3	632.25	58
Valproate	33.29	28.06	589.71	55
Valproate+fish liver oil	63.26	27.97	776.48	56
Valproate+propolis	68.67	22	880.29	71
Fish liver oil	68.85	19.53	976.71	83
Propolis	68.67	29.47	963.73	88

Figure 3



A longitudinal section of the head of femur bone of a pilocarpine-induced epileptic rat treated only with valproate showing that the trabeculae of cancellous bone lost their normal architecture. They have an eroded irregular surface (arrow) and areas of calcified cartilage inside (stars). The periosteum appears discontinuous. Hematoxylin and eosin-stained sections, ×400.

Figure 4



(a) A longitudinal section of the middle shaft of the femur of a pilocarpine-induced epileptic rat treated with valproate and fish liver oil showing increased mean thickness of the shaft compared with group treated only with valproate. There was an erosion cavity at the endosteal surface (arrow). A resorption cavity in bone tissue (star) was also observed. (b) The head of the femur of a rat of the same group showing increased thickness of the bone trabeculae (arrow) compared with the group of rats treated only with valproate. (c) A longitudinal section of the middle shaft of the femur of a pilocarpine-induced epileptic rat treated with valproate and propolis showing an increase in shaft cortical width, increased number of osteocytes (arrowhead) – that is, recovery of bone tissue. (d) The head of the femur of a rat of the same group showing a decrease in the mean area of the Haversian canal (arrows) compared with group treated only with valproate. (a, c) Hematoxylin and eosin-stained sections; (b, d) binary image morphometric measurement, ×400.

Histochemical results

Sections of bone in the control group showed moderate protein content in the periosteum and endosteum (Fig. 6a). The bone of pilocarpine-treated rats showed a decrease in protein content compared with the control group (Fig. 6b). Bone sections in the group treated only with valproate showed an increase in the protein content compared with the control group (Fig. 6c, Table 2 and Fig. 8).

The bone of rats treated with valproate and fish oil showed an increase in the protein content (Fig. 7b) compared with the group treated only with valproate (Fig. 7a). An increase in protein content was observed in rats treated with valproate and propolis (Fig. 7c, Table 2 and Fig. 8).

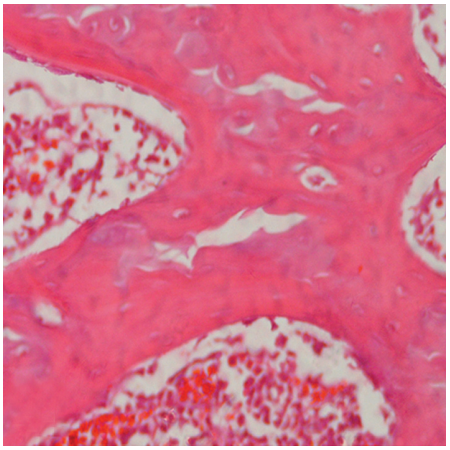
Discussion

Epilepsy is a major public health problem that affects almost 50 million individuals worldwide. It is a common problem in children and therapy may continue throughout the patient's life. VPA is well known for its broad-spectrum antiepileptic activity and is gaining popularity as a part of antipsychotic therapy [22]. It significantly inhibits the carnitine uptake by the brushborder membrane of mouse kidney and intestinal cells [23]. VPA therapy was associated with elevation in liver function tests and fatal hepatotoxicity because of inhibition of beta oxidation of fatty acid and VPA metabolites [24]. Some authors suggested that valproate directly inhibits glycogen synthase kinase-3B, which protects cells from endoplasmic reticulum stress-induced lipid accumulation [25]. In rat liver, administration of VPA induces mega mitochondria and microvesicular steatosis [26].

In bone tissue, both the periosteum and the endosteum have osteogenic potency. Following injury, cells in these layers may differentiate into osteoblasts (bone-forming cells), which become involved in the repair damage of the bone. Osteoporosis and related fractures represent major public health problems that are expected to increase with age. It is characterized by decreased bone mass and deterioration of bone tissue [27].

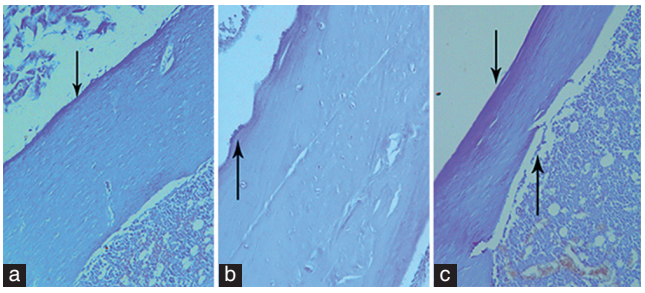
In the present study, the treatment of rats with valproate only at the dose level of 400 mg/kg/day for 6 months induced decreased thickness of shaft cortical bone, increased mean area of Haversian canals, erosive cavities on the outer surface, and marked decrease in the number of osteocytes. Trabeculae of cancellous bone became thinner with increased trabecular separation. The results of the present work are in agreement with those of Takahashi and Onodera [28], who reported that treatment of rats with valproate only at a dose level of 400 mg/kg for 5 weeks induced decreased cancellous

Figure 5



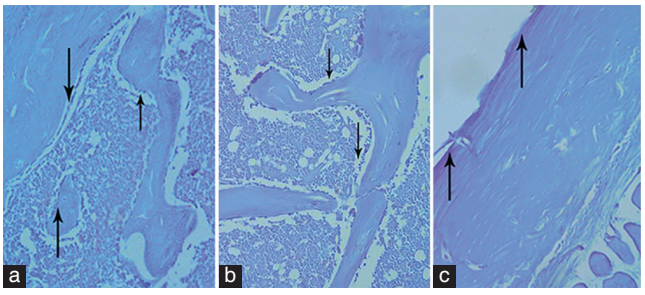
A longitudinal section of the head of the femur of a pilocarpine-induced epileptic rat treated with valproate and propolis showing increased thickness of bone trabeculae. Hematoxylin and eosin-stained sections, ×400.

Figure 6



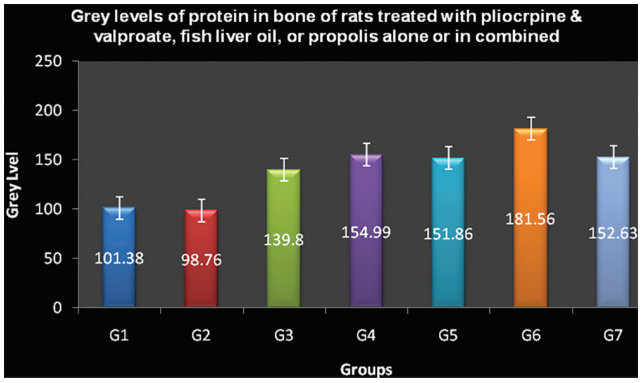
Section of the femur bone of rats showing greenish blue protein content in periosteum and endosteum (a) control; (b) section of the middle shaft of the femur bone of a rat treated with pilocarpine showing a decrease in protein compared with the control group; (c) section of the middle shaft of the femur bone of a pilocarpine-induced epileptic rat treated only with valproate showing an increase in the protein content. Bromophenol blue stain, ×400. The arrow in this figure points to the periosteum in sections (a & b) and to periosteum and endosteum in section (c) with their protein contents.

Figure 7



(a) Section of the middle shaft of the femur bone of a pilocarpine-induced epileptic rat treated only with valproate; (b) section of the middle shaft of the femur bone of a pilocarpine-induced epileptic rat treated with valproate and fish liver oil showing an increase in the protein content compared with the previous group; (c) section of the middle shaft of the femur bone of a pilocarpine-induced epileptic rat treated with valproate and propolis showing an increase in the protein content compared with the group treated only with valproate. Bromophenol blue stain, ×400.

Figure 8



Gray levels of protein in bone of rats treated with pilocarpine and valproate, fish liver oil, and propolis individually or in combination.

Table 2 Gray levels of protein in bone of rats treated with pilocarpine, valproate, fish liver oil, and propolis individually or in combination

Groups	Gray level of protein
Control group	101.38 ± 0.22
Pilocarpine (300 mg/kg b.w.) group	98.76 ± 0.24
Valproate (400 mg/kg b.w.) group	139.80 ± 0.29
Pilocarpine, valproate, and fish liver oil (0.4 ml/kg b.w.) group	154.992 ± 0.181
Pilocarpine, valproate, and propolis (50 mg/kg b.w.) group	151.867 ± 0.718
Fish liver oil (0.4 ml/kg b.w.) group	181.563 ± 0.664
Propolis (50 mg/kg b.w.) group	152.632 ± 2.413

All data are presented as means ± SE; b.w., body weight.

bone mass and dilated Haversian canals with clearly bordered osteoid. According to Petty *et al.* [29], chronic AED therapy has been associated with vitamin D deficiency, low bone mass, increased fracture risk, and altered bone turnover. Also, Onodera *et al.* [30] reported that decreased activity of acid phosphatase and alkaline phosphatase in the serum suggested that valproate accelerated both bone resorption and formation. These findings are in agreement with those of Christiansen [31], Seeman [32], and Wang *et al.* [33], who reported that osteoporosis occurs because of increased bone resorption and less bone formation, resulting in decreased average trabecular thickness and cortical width, with a concomitant increase in cortical porosity. Senn *et al.* [34] reported that there were significant differences in trabecular bone parameters between control and valproate-treated animals. Trabecular bone volume of valproate-treated C3H/HeJ mice reduced by 19.6% ($P < 0.05$), trabecular number reduced by 14.3% ($P < 0.05$), and trabecular separation increased by 19.9% ($P < 0.05$). Mildly elevated numbers of osteoclast and osteoblast were found.

The effect of treatment of mice with valproate on trabecular bone was more marked with increased fragility of trabecular structures without an effect on trabecular diameter. This effect could be related to increased osteoclast activity. In the animals studied, it is possible that the osteoclasts present were more active and that bone resorption increased shortly after commencement of valproate treatment [35]. Also, decreased bone volume could have arisen either from increased bone resorption or a decrease in the bone formation process, which are regulated primarily by osteoclasts and oseoblasts, respectively [36,37].

Reduced bone formation plays a central role in the pathogenesis of bone loss and bone fragility [38]. These findings are in agreement with those of Boluk *et al.* [39] and Vestergaard [40] as they reported that valproate induced a reduction in skeletal growth and bone mass formation and decreased BMD in epileptic patients.

Treatment of male Wistar rats with valproate at a dose of 200 mg/kg caused decreased BMD of tibial metaphysis and diaphysis. There was also decreased trabecular bone volume, but the symptoms of rickets were not observed [28].

The use of anticonvulsants such as VPA, which is a hepatic enzyme inhibitor, is also associated with decreased BMD and is considered to act by stimulating osteoclast activity and may cause imbalance between bone formation and resorption, contributing toward bone loss [41]. However, previous studies have suggested that even in the absence of vitamin D deficiency, some AEDs (such as valproate and lamotrigine) that do not induce liver P450 enzymes may also lead to osteopenic changes and a decrease in the BMD [42].

Long-term use of carbamazepine (CBZ) or VPA is associated with bone metabolism abnormalities, which include reduced BMD and decreased bone turnover (mainly decreased bone formation). Long-term antiepileptic therapy is an important factor for impaired bone health in epileptic children, and low calcium intake and high BMI could be two aggravating factors [43].

The mechanism of valproate-induced bone effect has been explained by Hawkins and Brewer [44], who postulated that valproate-induced effect on bone may be mediated by renal tubular dysfunction, which leads to reduced normal activation of vitamin D in the kidney and decreased calcium absorption from the gut, resulting in impairment of bone mineralization.

High pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen; (ICTP) (an increased bone resorption marker) was correlated positively with ionized calcium. Sato *et al.* [5] have indicated that the

resorption may be more than formation, thus leading to a reduction in bone density. An optimal diet for preventing or treating osteoporosis includes consuming an adequate number of calories as well as optimal amount of calcium and vitamin D, which are essential to help maintain proper bone formation and density [5].

In the present work, the treatment of rats with valproate and fish liver oil led to some improvement in the pathological changes in the form of increased thickness of the shaft cortical width and increased thickness of bone trabeculae in the femur head compared with rats treated only with valproate. These results can be explained by Elwakkad *et al.* [45] as they reported that the increase in bone formation markers and decrease in bone resorption markers may be because of a decrease in bone turnover, which was caused by hyperparathyroidism caused by valproate (increased calcium excretion and decreased absorption). Fish liver oil causes increased calcium absorption and a decrease in its excretion, and downregulation of the parathyroid hormone. Griel *et al.* [46] reported that ω-3 fatty acids cause a decrease in TNF-α and reduce bone resorption.

According to Watkins *et al.* [13,47], fish oil, which is rich in n-3 polyunsaturated fatty acids, stimulates bone formation in growing rats by downregulating osteoclastogenesis or upregulating osteoblastogenesis associated with CBFa1 (an osteoblast-specific transcription factor essential for the development of active osteoblast). ω-3 fatty acids modulate cyclooxygenase-2 protein expression, reduce prostaglandin E2, and increase osteoblastic bone formation markers [48]. A diet rich in long-chain-n-3-fatty acids reduces bone resorption by suppressing PGE2 that stimulate osteoclast activity, resulting in secondary osteoporosis [13,47]. Sun *et al.* [49] postulated that fish oil docosahesaenoic acid and eicosapentaenoic acid inhibit calcium excretion, and increase its absorption, causing inhibition of osteoporosis.

The administration of fish liver oil orally at a dose 0.04 mg/kg/day with valproate 400 mg/kg/day for 6 months caused a significant increase in osteoprotegrin (OPG) in comparison with the rat group treated only with valproate and the control group, with a decrease in RANKL compared with the treated group [45]

In the present study, administration of propolis orally at a dose of 50 mg/kg daily with valproate 400 mg/kg daily for 6 months resulted in an improvement in pathological changes in the form of increased number of osteocytes and increased thickness of the shaft cortical width compared with the group of rats only administered valproate.

According to Yildiz *et al.* [50], the bone density of the rats treated with caffeic acid was increased. Also, Tang *et al.* [51] reported that caffeic acid exerted the most powerful inhibitory effects on osteoclastogenesis. Caffeic acid suppressed the expression of NFATc1, a key transcription factor for the induction of osteoclastogenesis. Elwakkad *et al.* [45] reported that Fish liver oil and propolis are protective natural products against the effect of the AED valproate in rats.

In the present study, the treatment of rats with valproate, only 400 mg/kg/day, for 6 months led to an increase in the protein content. The results of the present work were in agreement with those of Gould *et al.* [52], who observed that in-vivo treatment of rats with lithium or valproate for 9 days, at therapeutically relevant concentrations, resulted in a significant increase in the soluble fraction of β-catenin protein in the frontal cortex (representing largely cytoplasm).

Brauser [53] found that valproate reduced the formation of two key proteins important for bone strength, decreasing the production of collagen by 60% and levels of osteonectin by 28% in patients with spinal muscular atrophy. Defects in these proteins are reflected as instability of the repetitive DNA sequences (microsatellites) [54,55].

Bone morphogenetic proteins (BMPs) are considered to be key regulators of embryonic skeletogenesis [56], endochondral ossification [57], bone remodeling [58,59], fracture repair [60], and bone regeneration [61].

The balance between bone formation and bone resorption at sites of bone remodeling is controlled by paracrine and autocrine growth factors including BMPs [59].

BMPs play well-established roles in pattern formation, organogenesis, and skeletal morphogenesis during vertebrate development. At the cellular level, BMPs regulate cell proliferation, differentiation, and apoptosis in embryonic and postnatal chondrocytes, osteoblasts, and osteoclasts [62].

In the present study, treatment of rats with valproate in combination with fish liver oil or propolis led to an increase in the protein content in comparison with rats treated with valproate only. According to Watkin's *et al.* [48], ω-3 fatty acids modulate cyclooxygenase-2 protein expression, reduce prostaglandin E2, increase osteoblastic bone formation markers, and decrease the activation of RANKL on T cells.

Conclusion

Treatment with antiepileptic drugs (AEDs) is generally chronic or even lifelong; it may be associated with significant metabolic effects such as decreased bone mass. Treatment with the dietary natural products, fish liver oil, and propolis along with the AED valproate might be of great help to epileptic patients who depend on AED for a long time as they improve histopathological changes and morphometric parameters in bone associated with AED-induced osteoporosis.

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Conflicts of interest

None declared.

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