Effect of Ethanol Extract of *Capparis Cartilaginea* on Osteoporosis-Induced Rats

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ABSTRACT

Capparis cartilaginea is widespread throughout different regions of Saudi Arabia. The local population commonly uses the fruit, roots and leaves of this species for the treatment of back pain, arthritis and rheumatism. This research aimed to investigate the effect of *C. cartilaginea* on bone mineral density (BMD), bone turnover markers (BTMs), procollagen type I propeptides (PINPs) and carboxy-terminal type I collagen cross-linking telopeptide (CTX) in osteoporosis-induced rats. *C. cartilaginea* fruit was harvested, freeze-dried, ground and extracted using ethanol. Fifty-eight Wister rats (30 female and 28 male) were used in the experiment. Osteoporosis was induced by the ovariectomy procedure in females and introducing a high dose of vitamin A in males. The daily dose of the fruit administered to the rats was 500 mg/kg over the period of one month. PINPs, CTX and BMD in rat sera were determined by ELISA kits. The results showed a significant increase in the BMD of the treated osteoporotic groups versus the non-treated osteoporotic groups of female (P=0.008) and male rats (P=0.0001). The levels of CTX in the treated osteoporotic groups were significantly lower than those in the non-treated groups (female and male: P=0.0001, P =0.0001, respectively). The PINP levels increased significantly in the treated osteoporotic females versus the non-treated osteoporotic females (P=0.009). In conclusion, *C. cartilaginea* was found to have potential to reduce and treat osteoporosis. Further research is needed to identify the dose and the period needed for bone to benefit from the fruit as an anti-osteoporotic agent.

Key Words: *Capparis Cartilaginea*, Osteoporosis, Bone Turnover Markers, Bone Mineral Density.

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INTRODUCTION

Osteoporosis is a skeletal bone disease that is characterized by a decrease in bone mass and microarchitectural structures of bone tissue that results in the risk of fracture [1]. Fractures cause major morbidity to the patient, such as severe pain, disabilities, decreased mobility, impaired respiratory function, and in the worst case, death [2]. An epidemiological analysis in the Kingdom of Saudi Arabia showed that healthy Saudi women (34%) and men (30.7%) between the ages of 50-79 years are osteoporotic [3].

The common type of osteoporosis is postmenopausal osteoporosis, which occurs as a consequence of oestrogen deficiency. Oestrogen deficiency enhances bone turnover and imbalance in bone formation and resorption. The diagnosis of osteoporosis can be performed by bone density measurements of the lumbar spine and proximal femur [4]. Ovariectomy in rats has been commonly used as a model for postmenopausal osteoporosis in humans [5]. Scientists have used rats to provide accurate data that are applied to the adult human skeleton. In rats after ovariectomy, the female skeleton became sensitive, and the loss of sex hormones occured [6] in addition to oestrogen deficiency [7]. Additionally, most of the characteristics of human postmenopausal osteoporosis have been exhibited by ovariectomized rats [8]. In an ovariectomy, the choice of the operative method is very important, particularly when a few dozen animals need to be operated on in a short time [9].

Another mechanism that induces osteoporosis is the consumption of a high dose of vitamin A, which can lead to bone resorption, and can inhibit bone formation [10]. Furthermore, long-term intake of a diet high in vitamin A can increase bone loss, decrease bone mineral density, and

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increase the osteoporotic fracture rick [11]. Rodents and many other species have been established and used as animal models in osteoporosis research [12].

Bone mineral density (BMD) testing has been approved as a standard test for screening osteoporosis [13]. The method available to assess BMD is dual-energy X-ray absorptiometry (DEXA). This method is used due to its flexibility, excellent precision, high reproducibility, and low radiation exposure. Additionally, DEXA is used for assessing the fracture risk and for monitoring the patients' response to osteoporotic treatment [14].

Bone turnover markers (BTMs) indicated osteoblast activity during the development in different phases, and can be measured in serum or plasma. In osteoporosis, the most sensitive marker of bone formation was procollagen type I propeptides (PINPs) [15]. Examples of bone resorption markers include hydroxyproline (HYP), hydroxypyridinium crosslinks of collagen, pyridinoline, deoxypyridinoline and carboxy-terminal type I collagen cross-linking telopeptide (CTX-I) [15].

In postmenopausal women, hormone replacement therapy (HRT) has proven successful in inhibiting bone loss, and decreasing the occurrence of skeletal fractures [16]. However, long-term HRT raises the risk of many types of cancers and other diseases, and it is not favoured by many women [17].

As the population ages, there would be a high incidence of hip fractures, and the expenses associated with treatments would increase dramatically, except for the effective prophylactic actions [18].

According to the World Health Organization (WHO), 4000 million people depend on herbal medicine, and 25% of the medical drugs have been based on plant-derived chemicals [19]. In developed countries, the use of medicinal plants for chronic diseases is favoured because there is a concern about the side effects of chemical drugs compared to the treatment with the medicine of natural origin, which appears to have a more gentle effect in disease management [20]. Quran and Hadith have referred to numerous wild plants that are still used in traditional medicine [21]. The flora of Saudi Arabia has been expected to contain more than 1200 medicinal species out of a total of 2250 [22]. *Capparis cartilaginea* is a plant that grows in stony and rocky areas and compact silty soils in depressions and on roadsides [23]. The whole herb (fruit, root and leaves) of *C. cartilaginea* is used for bruises, childbirth, paralysis, swelling [24], arthritis and rheumatism [25]. *C. cartilaginea* fruit contains vitamins, carbohydrates, protein [24], glucosinolates and flavonoids. The four flavonoids that have been isolated from *C. cartilaginea* are kaempferol-3-o-rutinoside, quercetin-3-O-rutinoside, quercetin-7-O-rutinoside and quercetin-3-glucoside-7-O-rhamnoside. Additionally, isothiocynates have been isolated and identified, such as butyl isothiocyanate,6- methylsulphonylhexyl isothiocyanate, 7-methylsulphonylheptyl isothiocyanate and 5-benzylsulphonyl-4-pentenyl isothiocyanate [26]. *C. cartilaginea* extracts contain alkaloids, carbohydrates, protein, coumarin, phytosterols, bitter principles, phenols and tannins [27] and rutin [28]. To the knowledge of the authors of this study, no studies have evaluated whether *C. cartilaginea* has anti-osteoporotic activity in rats. The present study investigated the influence of *C. cartilaginea* fruit extract in preventing and treating osteoporosis in a rat model induced by vitamin A (males) and ovariectomy (females).

MATERIALS AND METHODS

Plant material

Fresh fruit of *C. cartilaginea* was collected from Umluj Mountains in Tabuk Province, Northwest Saudi Arabia. All the collected fruit was freeze-dried at -64°C under 5 m Torr pressure, and ground using a Waring blender (Sigma, USA). Four hundred and sixty-seven grams of fresh fruit yielded 100 g of dried powder. The ethanol extraction method was performed as stated in the previously published research [29]. The *C. cartilaginea* fruit extract was administered daily; it was freshly prepared each day by dissolving the extract in distilled water (2 ml), and was administered orally using an oral gavage needle (Cadence Science, USA). The total duration of the study was two months.

Acute oral toxicity

Acute toxicity tests of the *C. cartilaginea* ethanolic extract were performed according to the Organisation for Economic Cooperation and Development (OECD – 420,2008) to select a proper dose for the oral gavage. The groups of animals were dosed using fixed doses of 5, 50, 300, 2000 and 5000 mg/kg. The doses used did not indicate any sign of toxicity; consequently, a dose lower than 5000 mg/kg was considered safe for the treatment.

Animals

The present study was approved by the Research Ethics Committee, Unit of Biomedical Ethics, King Abdulaziz University, Jeddah, Saudi Arabia. Three-month-old female (n=30) and male (n=28) Wister rats weighing 200 to 260 g were obtained from the Animal House Unit at King Fahad Medical Research Centre (KFMRC), Jeddah, Saudi Arabia. The rats were accommodated under standard laboratory conditions (22±1ºC and 60% humidity) for two weeks prior to the experiment. They were exposed to 12/12 h light/dark cycles, and were fed a slandered pelleted diet (Grain Soils and Flour Mills Organization Jeddah, Saudi Arabia) with free access to water. The animals received care according to the institutional guidelines for the care and use of laboratory animals in KFMRC.

Osteoporosis induction was performed by ovariectomy in females [30]. The induction of osteoporosis in males was performed by administrating a high dose of vitamin A [31].

Experimental design

After 2 weeks of acclimatization, the female rats were randomly divided into 3 groups: Group I: control rats (healthy animals), including non-ovariectomized untreated rats (n=8). Group II: osteoporotic rats, including those that were ovariectomized (n=8). Group III: treated osteoporotic rats, including those that were ovariectomized and that received the *C. cartilaginea* fruit extract (500 mg/kg) daily for one month (n=14).

After 2 weeks of acclimatization, the male rats were randomly divided into 3 groups: Group (I): control rats (n=8), including those that were not treated and did not receive vitamin A. Group II: Osteoporotic rats (n=6), including those whose osteoporosis was induced by the administration of a high dose of vitamin A.

Group III: Treated osteoporotic rats (n=14), including those whose osteoporosis was induced by the administration of a high dose of vitamin A once a day for 21 days. Subsequently, these rats were treated with the *C. cartilaginea* fruit extract (500 mg/kg) daily for 30 days.

Ovariectomy

After 2 weeks of acclimatization, osteoporosis induction was performed by ovariectomy in females. The rats were anesthetized with ether. A 2-cm skin incision along with the dorsal midline, and through the abdominal musculature was made. The ovarian fat pad was gently grasped using forceps. The ovaries were exposed and removed. Then, cautery was used to control any bleeding. The muscle was stitched with 3-0 absorbable sutures, and stainless steel wound clips were used to close the skin incision. The wound clips were removed after 7 days of surgery. The surgery took 10 minutes to completely remove the ovary of one animal. The instruments were sterilized with Betadine between each ovariectomy procedure [32]. At the end of the trial, all rats were sacrificed.

Vitamin A

Osteoporosis was induced by the administration of a high dose (daily dose 80 mg/kg) of vitamin A (Kahira Pharm. & Chem. Ind. Co. Cairo, Egypt).Vitamin A was mixed with 1% carboxymethyl cellulose [31], and each rat was administered this mixture orally once a day for 21 days.

Measurement of BMD by DEXA

DEXA scans were performed under anaesthesia (chloral hydrate; 300 mg/kg). An injection was administered intraperitoneally in the lower left abdominal quadrant [30]. DEXA scanned the female rats twice, three months after the ovariectomy procedure and after one month of treatment with the *C. cartilaginea* fruit extract. With respect to male rats, DEXA scans were performed after 21 days of vitamin A administration and after one month of the treatment with the *C. cartilaginea* fruit extract.

Measurement of Rat s-CTX

This assay employed the competitive inhibition enzyme immunoassay technique in which s-CTX was measured using a commercial s-CTX ELISA kit (CUSABIO Biotech CO. Ltd, China). The analysis was carried out according to the manufacturer's instructions.

Measurement of Rat s-PINP

In the measurement of s-PINP in rats, the competitive inhibition enzyme immunoassay technique was performed using a commercial s-PINP ELISA kit (CUSABIO Biotech CO. Ltd, China). The analysis was carried out according to the manufacturer's instructions.

Blood collection

Blood was collected twice from the female rats; three months after the ovariectomy procedure and after one month of treatment with the *C. cartilaginea* fruit extract (500 mg/kg). With respect to the male rats, blood was collected after 21 days of vitamin A administration, and after one month of the treatment with the *C. cartilaginea* fruit extract (500 mg/kg). Blood was collected from the eyes via the intraorbital sinus [33] using a capillary tube (75 mm; Hirschmann Laborgerate, Germany). Serum was prepared by centrifugation of the blood for 15 min at 3000 rpm at 4°C. The blood was preserved at -80°C until further analysis.

Statistical analysis

The Statistical Package for Social Science (SPSS) version 20 for Windows was applied to analyse the data. The data were expressed as the means +/- standard deviation (SD). The comparison of the variables between groups was performed using a paired Student's *t*-test for the parametric parameters at the beginning and end of the experiments, and one-way ANOVA was used for the comparison between groups as appropriate. Statistical significance was considered at P-value < 0.05.

RESULTS

In female rats at the beginning of the experiment, BMD was significantly lower in the non-treated and treated osteoporotic groups than in the control (P =0.001 and P =0.017, respectively). At the end of the experiment, BMD was significantly lower in the non-treated and treated osteoporotic groups than in the control (P =0.0001 and P =0.016, respectively), and was significantly higher in the treated than in the non-treated osteoporotic female groups (P =0.008). In the control and treated osteoporotic groups, BMD was significantly higher at the end than at the beginning of the experiment (P =0.030 and P= 0.002, respectively) (Table 1).

Table 1: Comparison of the measured parameters in female rats

Variable	Control group (n= 8)	Non-treated osteoporotic group (n= 8)	Treated osteoporotic group (n= 14)
BMD (gram/cm^2)			
Beginning of experiment	0.153±0.004	0.145±0.004	0.148±0.004
Significance		^{1}P =0.001	^{1}P =0.017, ^{2}P =0.076
End of experiment	0.156±0.003	0.146±0.005	0.151±0.006
Significance	P =0.030	P =0.676, ^{1}P =0.0001	P =0.002, ^{1}P =0.016, ^{2}P =0.008
CTX (U/L)			
Beginning of experiment	98.02±15.68	142.75±19.81	136.51±17.88
Significance		^{1}P =0.0001	^{1}P =0.0001, ^{2}P =0.438
End of experiment	108.87±9.00	219.05±27.24	165.64±24.15
Significance	P =0.205	P =0.0001, ^{1}P =0.0001	P =0.004, ^{1}P =0.0001, ^{2}P =0.0001
PINP (U/L)			
Beginning of experiment	4811.35±864.62	4231.27±339.52	4201.11±221.69
Significance		^{1}P =0.027	^{1}P =0.010, ^{2}P =0.903
End of experiment	5224.69±605.13	4117.93±565.93	4775.38±458.70
Significance	P =0.163	P =0.479, ^{1}P =0.0001	P =0.0001, ^{1}P =0.066, ^{2}P =0.009

Data are expressed as the mean +/- standard deviation. P: significant at the beginning versus the end of the experiment based on a paired Student's t-test, ^{1}P: significant versus the control, ^{2}P: significant versus the non-treated osteoporotic group based on one-way ANOVA (LSD).

At the beginning of the experiment, CTX in female rats was significantly higher in the non-treated and treated osteoporotic groups than in the control (*P* =0.0001 and *P* =0.0001, respectively). At the end of the experiment, CTX was significantly higher in the non-treated and treated osteoporotic groups than in the control (*P* =0.0001 and *P* =0.0001, respectively). CTX was significantly lower in the treated osteoporotic group than in the non-treated osteoporotic group (*P*=0.0001), and was significantly higher in these groups at the end of the experiment compared with the beginning (*P*= 0.0001 and *P*=0.004, respectively) (Table 1).

PINP in female rats at the beginning of the experiment was significantly lower in the non-treated and treated osteoporotic groups than in the control (*P* =0.027 and *P* =0.010, respectively). At the end of the experiment, PINP was significantly lower in the non-treated osteoporotic group than in the control (*P* =0.0001). At the end of the experiment, PINP was significantly higher in the treated than in the non-treated osteoporotic group (*P* =0.009) (Table 1).

Table 2 shows that in male rats at the beginning of the experiment, BMD was significantly lower in the non-treated and treated osteoporotic groups than in the control (*P* =0.0001 and *P* =0.0001; respectively). In addition, higher BMD values were measured in the treated than in the non-treated osteoporotic group (*P* =0.0001).

Table 2: Comparison of the measured parameters in male rats

Variable	Control group (n= 8)	Non-treated osteoporotic group (n= 6)	Treated osteoporotic group (n= 14)
BMD (gram/cm^2)			
Beginning of experiment	0.152±0.004	0.127±0.004	0.129±0.006
Significance		^{1}P =0.0001	^{1}P =0.0001, ^{2}P =0.404
End of experiment	0.153±0.006	0.120±0.008	0.150±0.005
Significance	P=0.638	P=0.175, ^{1}P =0.0001	P=0.0001, ^{1}P =0.178, ^{2}P =0.0001
CTX (U/L)			
Beginning of experiment	104.04±12.80	158.46±8.47	148.64±11.80
Significance		^{1}P =0.0001	^{1}P =0. 0001, ^{2}P =0.092
End of experiment	101.78±6.81	156.50±36.58	108.10±22.71
Significance	P=0.754	P=0.911, ^{1}P =0.0001	P=0.0001, ^{1}P =0.572, ^{2}P =0.0001
PINP (U/L)			
Beginning of experiment	5129.87±117.22	4233.56±477.36	4339.54±776.33
Significance		^{1}P =0.015	^{1}P =0.010, ^{2}P =0.727
End of experiment	5385.61±351.38	4389.30±238.98	4470.53±768.79
Significance	P=0.112	P=0.357, ^{1}P =0.007	P=0.663, ^{1}P =0.003, ^{2}P =0.785

Data are expressed as the mean +/- standard deviation. P: significant at the beginning versus the end of the experiment based on a paired Student's t-test, ^{1}P: significant versus the control, ^{2}P: significant versus the non-treated osteoporotic group based on one-way ANOVA (LSD).

In the treated osteoporotic group, BMD at the end of the experiment was significantly higher than that at the beginning (P =0.0001), and BMD was significantly higher in the treated than in the non-treated osteoporotic group (P=0.0001)

In male rats at the beginning of the experiment, CTX was significantly higher in the non-treated and treated osteoporotic groups than in the control (P =0.0001 and P =0.0001, respectively). At the end of the experiment, CTX was significantly higher in the non-treated osteoporotic group than in the control (P =0.0001). CTX was lower in the treated than in the non-treated osteoporotic group (P =0.0001). CTX in the treated osteoporotic group was lower at the end than at the beginning of the experiment.

In male rats at the beginning of the experiment, PINP was significantly lower in the non-treated and treated osteoporotic groups than in the control (P =0.015 and P =0.010; respectively). At the end of the experiment, PINP was significantly lower in the non-treated osteoporotic group and treated osteoporotic group than in the control (P =0.007 and P =0.003; respectively). In addition,the values measured in the nontreated osteoporotic group were lower (not significant) than those in the treated osteoporotic group (Table 2).

DISCUSSION

Osteoporosis and osteopenia were reported to be 27.2% and 29.8%; respectively, in Saudi women (20-80 years of age) [34]. The occurrence of osteoporosis in Saudi Arabian males was higher than that in the western world, and in the last decade, this occurrence has amplified [35]. Medicines based on the phytochemicals currently have been widely used for the treatment of different diseases [36]. The Capparis family is a herbal group from which the oriental medicine has been prepared that provided cures for many diseases. For example, its extracts as well as the fruit, roots and leaves of the plants [24]. have been used to treat patients with diabetes mellitus, rheumatism and rheumatoid arthritis [25]. The influence of *C. cartilaginea* extracts on bone turnover and bone mineral density in osteoporosis has not been sufficiently examined. Because *C. cartilaginea* is a traditional medication for arthritis and other types of back pain, it has been believed that it could also have an effect on osteoporosis.

According to this expectation, an animal model was developed, and the effect of fruit extracts of *C. cartilaginea* on osteoporotic-induced rodents was observed.

In this study, *C. cartilaginea* had a significant positive effect on BMD in female and male rats. Fruit extracts of *C. cartilaginea* contain glucosinolates, flavonoids and isothiocynates [26]. One of these flavonoids is quercetin, which is a typical flavonol that has been widely used as a phytochemical assisting in bone metabolism [37, 38].

Quercetin, administered to ovariectomized mice to study the effect on bone loss, resulted in a significant increase in bone mineral density [39].

Similarly, the scientists have proposed a positive association between flavonoid intake and bone mineral density in perimenopausal women. Additionally, flavonoids have been shown to prevent osteoporosis in animal research [40-42]. Zhang et al. [43] and Ma et al. [44] found that the flavonoid extract of the *Epimedium sagittatum* prevents osteoporosis that was induced by ovariectomy, and therefore inferred that the extract contains anti-osteoporotic constituents [44]. In the same way, Welch et al [45]. reported that the total flavonoid intake has a positive relationship with BMD. Anthocyanins, which are a subclass of flavonoids and flavanones, have a role in bone health [45]. Orsolic et al. [46] reported that quercetin has the ability to prevent the development of osteoporosis in rats that were administered retinoic acid to induce osteoporosis [46]. Quercetin has been reported to have a unique effect on BMD in male rats with retinol-induced osteoporosis [47].

CTX and PINP were used as the markers of bone turnover in experimental rats in the conducted research. PINP is a very selective marker of bone turnover that has been used for monitoring bone anabolism [48]. CTX is the most sensitive marker of bone resorption after the surgical menopause [49]. The fruit extract of *C. cartilaginea* enhanced the PINP levels in the treated osteoporotic female group compared to the non-treated osteoporotic group. However, the fruit extract decreased the CTX levels in the treated osteoporotic group compared to the non-treated osteoporotic group but did not affect the CTX level within the same group before and after the treatment. This could be due to the severe osteoporosis induced by ovariectomy in the female group. The results of this study were in agreement with those presented by Kim et al. [50], who demonstrated that luteolin (flavonoid) inhibited osteoclast bone resorption activity. These authors also suggested that when ovariectomized mice were orally administered different concentrations of luteolin, there was a significant increase in the BMD and bone mineral content of trabecular and cortical bones in the femur compared to the ovariectomized controls. The serum CTX value in ovariectomized mice was significantly higher than that in the control group. Additionally, CTX levels were higher in the ovariectomized group than in the ovariectomized group treated with luteolin. Luteolin also prevented decreases in bone strength indexes induced by ovariectomy surgery, and inhibited bone loss resulting from the decreased bone turnover [50].

The results from the present study were in agreement with the findings reported by Gunn et al. [51] These authors observed a lower bone turnover in postmenopausal women who consumed the Scarborough Fair Diet rich in

flavonoids, including quercetin. Additionally, the results showed positive changes in both calcium conservation and turnover markers [51]. Consistent with these findings, quercetin was found to enhance bone strength, particularly by influencing bone formation, based on CTX analysis in cirrhotic rats. In addition, quercetin and isothiocynates obtained from pycnogenol (maritime pine plant extract) and dried plum respectively, decreased resorption markers, increased formation markers, prevented BMD loss and deterioration of the bone trabecular structure and generally inhibited bone turnover, indicating a positive effect of flavonoids on bone formation [52, 53]. Muraleva et al. [54] showed the same effect of quercetin in male senescence-accelerated OXYS rats as a model of osteoporosis [54]. According to Horcajada-Molteni et al. [55], quercetin in ovariectomized rats prevents trabecular bone loss by increasing osteoblast activity and decreasing bone resorption [55]. Other data also showed that quercetin influences osteoclast activity and the cell cycle by inhibiting the differentiation of progenitor cells into osteoclasts, and arresting mature osteoclast activity [56, 57]. The investigations conducted *in vitro* by Wattel et al. [58] showed that quercetin also reinforced the differentiation of stromal cells to osteoblasts as well, inhibited their differentiation into adipocytes.

Quercetin and rutin play a role as antioxidants. They have been reported to inhibit bone loss in ovariectomized rats. Oxidative stress plays an important and critical role in bone loss, which is associated with oestrogen deficiency [39, 55]. From the previous finding, it can be hypothesized that the increase in BMD and PINP and the decrease in CTX were due to the effect of flavonoid components in the fruit extract of *C. cartilaginea* that may increase osteoblasts, and decrease osteoclast activity, consequently decreasing bone resorption, enhancing bone formation, and acting as anti-osteoporotic agents.

In conclusion, this study was designed to induce osteoporosis in rats by ovariectomy in females and by high doses of vitamin A in males, and study the effect of the fruit extract of *C. cartilaginea* on BMD, PINP and CTX. The results showed that the fruit extract might have an effect on BMD, CTX and PINP in female and male rats. Future studies would be needed on a large sample size for a longer period of treatment time. To improve the accuracy, in this study, it was proposed that DEXA can be performed at the beginning of the experiment, after inducing osteoporosis and after subjecting the rats to the treatment. The mechanism involved in the entire process must also be studied further.

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