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The protective role of almond and thyme in carbonated beverage-induced osteoporosis in male albino rats

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***Abstract*---**Osteoporosis is a common health problem distinguished by low bone mass and microarchitectural deterioration of bone tissue, thus increase bone fragility and fracture. Nutrition possesses a critical role in reducing osteoporosis risks among its impact on fragility factors, particularly on bone mass development and maintenance. The current study was carried out to assess and compare the protective effect of almond and thyme against osteoporosis induced by excessive intake of carbonated beverage (Coca-Cola) in rats. Forty-eight adult male Sprague–Dawley rats were divided into six groups: G1, control negative fed balanced diet; G2, control positive fed balanced diet and received Coca-Cola orally by gavage (20 ml/day) (CB); G3, almond powder (5g/100g diet) (A); G4, thyme powder (5g/100g diet) (T) and G5, (CB) +(A); 6, (CB)+(T)). Almond and thyme supplementation significantly reduced the development of osteoporosis as well as inflammation and oxidative stress. Our findings show that almond and thyme can effectively reduce soft drink-induced bone loss and could be used as dietary supplements to prevent bone resorption and osteoporosis.

***Keywords*---**Osteoporosis, Coca-Cola, Almond, Thyme.

Introduction

Osteoporosis is a skeletal disease characterized by reduced bone mass and mineral density, as well as bone degradation, that increases the bone fracture and skeleton fragility risks, causing serious complications [1]. Calcium is an essential mineral bone density development and maintenance. A lack of sufficient levels of calcium causes fractures and osteoporosis [2]. Cola beverages consumption, which contain phosphoric acid and often caffeine, could increase the fragility of bones in children

and adolescents through interactions with bone mineral content thus increased the risk of bone fractures [3]. Excessive soft drink consumption may decrease consumption of healthy drinks like milk, resulting trace elements loss like calcium and magnesium, thus elevating the risk of osteoporosis and fracture [4]. Nutrition plays an important role in bone formation. Choosing a well-balanced, of high-nutritional contents enhance skeletal health significantly. Appropriate nutrition is crucial for bone health, which aids in treatment and avoidance of bone diseases [5]. Various medicinal herbs and nuts, on the other hand, inhibit osteoporosis and heal bone resorption [6, 7].

Almond (*Prunus amygdalus dulcis*)) is the most popular nuts and widely used as snack foods and as ingredients in a variety of processed foods [8]. Almond consumption may lower the possibility of chronic diseases and assist in preventing colon cancer [9]. Besides, almond contains proteins, certain minerals (calcium, magnesium, zinc, manganese, copper), vitamins E and D, phytochemicals including phenolic compounds, phytosterols, flavonoids, phenolic acids, and about 50% oil. The highest concentrations of phytochemicals are found in the dark brown almond skins. Almond oil is also extracted for medicinal purposes. Almond oil contains a high concentration of monounsaturated fatty acids (MUFA), particularly oleic fatty acids [10], that may reduce cellular apoptosis, oxidative stress, mitochondrial dysfunction, and inflammation in hepatocytes [11]. Moreover, Almond oil was used as a medicine substitute and indicated promise in pharmacological and biomedical and research [12].

Thyme (*Thymus vulgaris L.*) is a medicinal herb rich in various volatile components and essential oils. It is commonly used all over the world and is believed to have a possible protective influence against bone loss [13]. The essential oils are nontoxic and biodegradable compounds with antimicrobial activity that cause no side effects or intestinal problems when consumed [14]. The main constituents of thyme essential oil are thymol, carvacrol, -terpinene, and -caryophyllene [15]. It has been established that thymus vulgaris' biochemical effects are mainly due to the existence of phenolic compounds, particularly thymol and carvacrol [16].

Materials and Methods

This study was performed on adult male albino rats (Sprague-Dawley) initially weighing 103±7g. Rats were obtained from the King Fahd Medical Research Center's Animal House Colony in Jeddah. Rats were housed in stainless steel cages within an air-conditioned animal house at 24°C. Rats were fed adequate standard diet and allowed water ad libitum and kept under normal laboratory conditions through the whole experimental period.

Carbonated beverage doses and routs of administration

Coca-Cola beverage was purchased from local supermarket and was orally administered to rats at consecutive doses throughout the day (20 ml/day) for 42 days.

Almond and Thyme

Fresh dry herb of thyme (*Tymus vulgaris L.*) and almond seeds (*Prunus amygdalus dulcis*) were obtained from the local markets. Dried samples were ground into a fine powder.

Experimental Design

Forty-eight male rats were divided into six groups (8 rats/group) as follows:
Group 1 (C): Rats fed standard balanced diet (Negative control).
Group 2 (**CB**): Rats fed balanced diet and received Coca-Cola (20 ml/day) by oral gavage (Positive control).
Group 3 (**A**): Rats fed standard balanced diet supplemented with almond powder (5% w/w).
Group 4 (**T**): Rats fed standard balanced diet supplemented with thyme powder (5% w/w).
Group 5 (**CB+A**): Rats fed balanced diet supplemented with almond powder (5% w/w) and received Coca-Cola (20 ml/day) by oral gavage.
Group 6 (**CB+T**): Rats fed balanced diet supplemented with thyme powder (5% w/w) and received Coca-Cola (20 ml/day) by oral gavage.

Analytical Procedures

I-Serum Analysis:
At the end of the experimental period (6 weeks), rats were fasted for 12 hrs., then anesthetized with ether, blood samples were taken from the hepatic portal vein and transferred into centrifuge tubes. To obtain serum for the biochemical analysis, tubes were centrifuged at 10000 x g for 15 minutes at 25°C. Serum samples were collected and stored in dry clean plastic tubes at -20°C until used for various biochemical analyses. Serum levels of calcium, inorganic phosphorus, vitamin D_3, C-terminal telopeptide (CTX), parathyroid hormone (PTH), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), malondialdehyde (MDA), protein carbonyl (PC), 4-Hydroxynonenal (4-HNE) levels, and alkaline phosphatase (ALP) activity were determined in according to analytical methods.

II- Bone Ash Analysis:
The percentage of femur bone ash magnesium, zinc, and copper concentrations were determined by atomic absorption spectrophotometry.

Statistical Analysis

Data were statistically analyzed by SPSS version 20.0 statistical packages. Data were represented as the means ± SE; statistical differences between groups were performed using t-test. Differences were considered significant when $p<0.01$.

Results

Supplementation with almond and thyme significantly ($p\leq0.01$) increased serum calcium and vitamin D3 levels in both the CB+A and CB+T groups when compared to the positive control group, as shown in Table 1. On the other hand, phosphorus

levels in the same treated groups were significantly (p≤0.01) decreased compared to positive control.

Table 1
Effect of different experimental diets on serum calcium, phosphorus, and vitamin D_3 levels

Groups	Calcium (mmol / L)	Phosphorus (mmol / L)	Vitamin D_3 (pg/ml)
Negative Control (C)	3.10^{a} ± 0.05	1.44^{a} ± 0.01	70.02^{a} ± 1.03a
Positive Control (CB)	1.79^{b} ±0.03	2.25^{b} ±0.0**2**	56.04^{b} ± 1.05d
Almond (A)	3.50^{a} ± 0.05	1.48^{a} ± 0.02	70.10^{a} ± 1.00a
Thyme (T)	3.11^{a} ± 0.04	1.40^{a} ± 0.01	72.50^{a} ± 1.50
CB + A	2.80^{c} ± 0.04	1.66^{c} ± 0.04	61.03^{b} ± 1.02b
CB + T	1.92^{b} ± 0.02	1.90^{d} ± 0.03	65.57^{b} ± 0.85bc

Values are expressed as means ± SE. (n= 8). Means with different letters in each column are significantly different (p≤0.01).

Results in Table 2 showed that levels of bone biomarkers (PTH, CTX, and ALP) were significantly higher in the control positive group compared to all experimental groups. The bone resorption marker CTX levels, PTH, and ALP activity were significantly (p≤0.01) enhanced by administering either almond or thyme in both G5 and G6 groups respectively as compared to the positive control. In the same context, results showed that the almond supplemented group (CB+A) had the most significant improvement in bone biomarker levels, followed by the thyme supplemented group (CB+T) when compared to the control positive group (CB).

Table 2
Effect of different experimental diets on serum levels of bone biomarkers

Groups	PTH (pg/mL)	CTX (pg/mL)	ALP (IU/L)
Negative Control (C)	48.10 ^{a} ±9.25	340.00 ^{a} ± 10.00	162.23^{a}±2.5
Positive Control (CB)	75.25 ^{b} ±8.50	499.80 ^{b} ± 11.26	200.05^{b}±6.0
Almond (A)	47.10 ^{a} ± 6.50	339.05 ^{a} ± 9.40	160.23^{a}±2.1
Thyme (T)	49.25 ^{a} ± 4.50	344.00 ^{a} ± 7.50	164.23^{c}±2.4
CB + A	50.15 ^{a} ± 9.80	365.70 ^{c} ± 7.55	159.23^{a}±3.0
CB+ T	52.15 ^{a} ± 7.05	381.60 ^{c} ± 8.00	166.23^{c}±2.6

Values are expressed as means ± SE. (n= 8). Means with different letters in each column are significantly different (p≤0.01).

Table 3 shows the effects of various treatments on serum inflammatory biomarkers in all experimental groups. Coca-Cola administration elicited a potent inflammatory response, evidenced by significant (p ≤ 0.01) increase in serum IL-6, TNF-, and CRP levels when compared to the control group. Almond and thyme supplementation, on the other hand, significantly (p≤0.01) enhanced inflammatory biomarkers levels. Similarly, thyme supplemented group (CB+T) showed the greatest improvement in

inflammatory biomarker levels, followed by the almond supplemented group (CB+A) when compared to the control positive group (CB).

Table 3
Effect of different treatments on serum levels of inflammatory markers in all studied groups

	IL-6 (pg/ml)	TNF-α (pg/ml)	CRP (mg/dl)
Negative Control (C)	6.50[a] ± 0.55	29.80 [a] ± 1.30	0.95 [a] ± 0.05
Positive Control (CB)	21.05 [b] ±2.50	55.25 [b] ± 5.95	2.15 [b] ± 0.20
Almond (A)	7.25[a] ± 0.85	32.50 [a] ± 2.40	0.90 [a] ± 0.09
Thyme (T)	5.98[a] ± 0.45	29.75 [a] ± 2.30	0.85 [a] ± 0.088
CB+ A	15.55[c] ± 1.5	46.43 [c] ± 6.55	1.15 [c] ± 0.09
CB+ T	8.95[d] ± 0.95	38.50 [d] ± 3.96	0.97 [a] ± 0.07

Values are expressed as means ± SE. (n= 8). Means with different letters in each column are significantly different (p≤0.01).

Coca-Cola induced oxidative stress denoted as a significant (p ≤ 0.01) increase in serum protein oxidation biomarker (PC), marker of oxidation of unsaturated fatty acids (4-HNE), and malondialdehyde (MDA) levels (lipid peroxidation marker), as compared to control group. On the other hand, supplementing either almond (G5) or thyme (G6) significantly (p ≤ 0.01) improved the level of oxidative stress biomarkers.

Table 4
Effect of different experimental diets on serum levels of oxidative stress biomarkers

Groups	MDA (µmol/l)	PC (µmol/ mg)	4-HNE (ng\ml)
Negative Control (C)	1.56 [a] ± 0.10	9.29 [a] ±.0.33	201.30 [a] ±6.71
Positive Control (CB)	2.33 [b] ± 0.11	14.50 [b] ±0.49	221.50 [b] ±6.60
Almond (A)	1.50 [a] ± 0.10	8.99 [a] ±.0.21	200.90 [a] ±5.81
Thyme (T)	1.52 [a] ± 0.10	9.00 [a] ±.0.32	201.80 [a] ±6.20
CB+ A	2.04 [c] ± 0.10	12.02 [c] ±.0.52	207.50 [c] ±4.22
CB+ T	1.79 [d] ± 0.11	11.52 [c] ±.0.49	205.30 [c]±3.53

Values are expressed as means ± SE. (n= 8). Means with different letters in each column are significantly different (p≤0.01).

Results in Table 5 indicated that there were no significant differences in the mean values of bone ash copper concentration. On the other hand, Coca-Cola induced significant reduction (p ≤ 0.01) in bone ash magnesium and zinc. While supplementation with almond (G3 and G5) significantly improved (p ≤ 0.01) the level of bone minerals content (Mg and Zn) when compared with positive control and thyme groups.

Table 5

The effect of different experimental diets on bone ash calcium, phosphorus, and magnesium

	Magnesium (g/100g ash)	Zinc (g/100 g ash)	Copper (g/100g ash)
Negative Control (C)	1.050 [a] ±0.009	0.255 [a] ±0.002	0.040±0.005
Positive Control (CB)	0.500 [b]±0.001	0.130 [b] ±0.001	0.038±0.002
Almond (A)	1.120 [c] ±0.010	0.375 [c] ±0.004	0.045±0.008
Thyme (T)	1.055[a] ±0.005	0.245 [a] ±0.001	0.040±0.005
CB+ A	1.140[c] ±0.00**7**	0.285 [a] ±0.004	0.048±0.003
CB+ T	0.650[b] ±0.001	0.135 [b] ±0.001	0.038±0.002

Values are expressed as means ± SE. (n= 8). Means with different letters in each column are significantly different (p≤0.01).

Discussion

Osteoporosis is a common bone disorder; its incidence is likely raised markedly in the coming years. Treating osteoporosis still a major challenge, it is therefore regarded as common health problem [17]. This study confirmed that consuming large amounts of carbonated beverages during adolescent development reduces bone mineral content and elevate the risk of future fractures [18]. Phosphorus is an essential bone component which is necessary for proper skeletal mineralization. Most of the body's phosphorus is stored in the bones [19]. A high phosphoric acid content in soft drinks was proposed as one of the mechanisms that links soft drinks and fracture. Extreme consumption of phosphoric acid changes calcium/phosphorus ratio and disturbs body's acid-base balance, resulting in decreased bone density and even osteoporosis and fractures. [20]. The results of the current study showed a significant decrease in serum calcium and vitamin D levels in control positive group which coincided with increased serum phosphorus and different parameters of bone biomarkers levels (PTH, CTX, ALP), indicating calcium transfer from the blood to the bone Calcium deficiency induced the thyroid to secrete calcitonin and deliver more calcium to the site of bone loss. Numerous mechanisms explain our findings on the direct correlation between soft drink consumption and bone loss. Soft drinks contribute to phosphorus intake in the diet. Increase dietary phosphorus and reduced calcium may encourage parathyroid hormone causing bone resorption [21]. Increased phosphorus consumption reduces renal activation of 25-hydroxyvitamin D and influences calcium homeostasis [22]. Meanwhile, particular ingredients in soft drinks can also affect bones such as sugar and sodium that increase calcium loss [23]. Furthermore, increased intake of caffeinated beverages enhances the risk of fractures [24, 25]. One more potential mechanism for the indirect effect of soft drinks on bone loss and fractures is the mediating effect of obesity. It was shown in many studies that the consumption of soft drinks increases the possibility of obesity [26]. Studies found that fats affect bone regulation and participates in bone active hormones metabolism [27]. Increased muscle fat content causes more falls, which can increase fracture risk in specific areas [28].

This study demonstrated that supplementing almond significantly increased serum calcium and vitamin D3 levels and reduced levels of serum phosphorus and bone

biomarkers in all experimental groups. Almonds provide a nutrient-dense source of calcium, phosphorus, copper, iron, and zinc as well as protein and dietary fiber [29]. The presence of phosphorus in almond powder appears to hasten the healing process of bone. Almond also considered as a rich source of potassium and low sodium thus promoters of healthy balance of electrolytes as well as good calcium/phosphorus ratio [30]. The vitamins, minerals, and phosphorus content of almond are found to be linked with bone development and strengthening [31].

The results of the current study suggested that thyme supplementation in the Coca-Cola diet improves serum calcium levels, which may lead to the prevention or reduction of bone loss and the restoration of serum calcium levels to normal levels. Our data showed a significant increase in vitamin D3 and decrease in PTH levels after treatment with thyme compared to positive control rats. These suggestions are consistent with the findings of Banu et al. [32], who observed that thyme and red clovers, influence bone metabolism, and improve calcium absorption from the gastrointestinal tract, and have an which maintains calcium homeostasis.

This study results represented the positive effect of almond and thyme on bone minerals (Zn, Mg). Magnesium and Zinc both are necessary in bone metabolism. Supplementation with almond (G3 and G5) significantly improved ($p \leq 0.01$) the level of bone minerals content (Mg and Zn) when compared with positive control and thyme groups. In addition to improving bone formation, our data show that almond and thyme supplementation reduces bone resorption, as indicated by a reduced serum level of CTX. It was noted that different nutritional, molecular and immunological factors affect the relation between inflammation and osteoporosis [33]. In the present study, An increased levels of malondialdehyde was noted in positive control rats as compared to normal control one. These elevated levels of malondialdehyde in cases of bone resorption, indicated free radical production and oxidative stress due to calcium deficiency [34]. Inflammation is typically associated with some inflammatory biomarkers recorded in case of rheumatoid arthritis, osteoporosis, and osteomyelitis. In these conditions, pro-osteoclastic cytokines such as tumor necrosis factor- (TNF-α) and interleukin-6 (IL-6) are elevated [35]. The production of liver C-reactive protein was regulated by IL-1, IL-6, and TNF-α and regarded as a sensitive inflammation biomarker [36]. CRP levels and bone mineral density have been linked in inflammatory diseases as well as in healthy people, implying a link between subclinical systemic inflammation and bone loss [37]. The study findings showed that almond and thyme possess a powerful anti-inflammatory and anti-oxidative stress effect. According to Boruga et al. [38], thymol was considered the most abundant component in thymus vulgaris essential oil, followed by c-terpinene and p-cymene. Thymol is a monoterpene that is one of the most important dietary constituents in thyme species and has been used in traditional medicine; it has anti-inflammatory, free-radical scavenging, analgesic, antimicrobial, and antispasmodic properties [39]. Because of their potent antioxidant activity, carvacrol and thymol combination was able to reduce oxidative stress [40]. Moreover, A study by Sapkota et al. [41] examined the effect of thymol in osteoclastogenesis and bone loss in mice and discovered that thymol suppressed osteoclast formation, blocked bone resorption, and protected against proinflammatory cytokines; representing the crucial role of thymol as a therapeutic agent preventing bone diseases. They proposed that the monoterpenes borneol,

thymol, and camphor inhibit bone resorption by direct action on bone cells and influencing calciotropic hormones or by stimulating intestinal calcium absorption.

Almond anti-inflammatory properties may be attributed primarily to its high MUFA content, which has been linked to lower levels of CRP. Furthermore, other almond components, such as magnesium and phytochemicals, may help to reduce the levels of inflammatory mediators. [42].

The results of our study revealed that almond powder supplementation improved the serum levels of protein oxidation biomarker (PC) and marker of oxidation of unsaturated fatty acids (4-HNE) as compared to control positive group. The unique combination of MUFAs, phytosterols, and antioxidants, as well as almonds' high nutrient density in terms of vitamin E, folate, calcium, magnesium, potassium, and low sodium, may all contribute to the health benefits observed in epidemiological studies and human trials [43]. Whole almond seed, brown skin, shell, and green shell cover (hull) extracts have powerful free radical-scavenging properties [44]. The presence of flavonoids and other phenolic compounds in nuts may be responsible for these activities [45].

Conclusion

Our findings clearly demonstrated that excessive Coc-Cola consumption alters the calcium/phosphorus ratio, causing reduced bone density osteoporosis and fractures. Almond and thyme supplementation significantly decreased bone loss, enhanced calcium and vitamin D3 levels in serum, improved bone minerals (Mg, Zn), and reduced inflammation and oxidative stress. However, almond has a stronger anti-bone resorption and osteoporosis effect than thyme.

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