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Full Length Research paper

Biochemical markers of bone metabolism after a 3-mo *Zizyphus spina-christi* supplementation on postmenopausal women with osteoporosis

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The use of dietary phyto-oestrogens as a possible option for the prevention of osteoporosis has raised considerable interest because of the increased concern about the risks associated with the use of hormone-replacement therapy. However, the evidence in support of a bone-sparing effect in post-menopausal women is still not sufficiently convincing. Most studies have been performed on soyabean isoflavones (genistein and daidzein). The purpose of this study is to evaluate *Zizyphus spina-christi* extract and compare to rutin in postmenopausal women with osteoporosis. This study was done on 46 patients suffering from osteoporosis. Patients were classified into three groups: Gp 1: patients were given orange juice in addition to calcium (1000 mg daily) and vitamin D (0.25 µg daily), Gp 2: patients were given orange juice fortified with rutin, a synthetic isoflavone (180 mg/d), in addition to a daily dose of Ca and vitamin D_3 as in group 1, Gp 3: patients were given orange juice fortified with *Z. spina-christi* extract (50 mg/d), in addition to a daily dose of Ca and vitamin D_3 as in group 1. These regimens continued for a period of 3 months and followed up weekly. The serum levels of calcium, osteocalcin (OC), urinary deoxypyridinoline (U-Dpd) and urinary creatinine were estimated for all patients before and after the intervention. There were a significant increase ($p< 0.05$) in the level of serum OC and a significant decrease ($p< 0.01$) of U-Dpd of postmenopausal women in groups (2, 3) after three months follow-up. There was a highly significant positive correlation ($p< 0.05$) between serum OC and U-Dpd in postmenopausal women before intervention trials. The Ca/Cr ratio ($p< 0.01$) was significantly decreased in postmenopausal women after given rutin or ZSC extract, but the decrease in case of ZSC extract was higher relative to rutin. No significant change in the level of serum calcium was noticed as for the three routes of intervention. It was concluded that *Z. spina-christi* extract is a novel functional food ingredients which suggests that this product could potentially be used for the prevention and treatment of osteoporosis that may occur among women after menopause. Also, it is a more potent alternative with less oestrogen-related side effects for hormone replacement therapy in postmeno-pausal osteoporosis,

Key words: Rutin, *Zizyphus spina-christi,* bone markers, vitamin D, calcium, osteoporosis and postmenopausal women.

INTRODUCTION

Osteoporosis as well as fractures that result from this condition, has become a significant social concern with the increasing age of the population, calling for prompt action toward establishment of appropriate prevention and therapeutic strategies. Such intervention should be readily undertaken for women and especially for postmenopausal women, whose bone mass rapidly decreases.

Estrogen replacement therapy has long been used to prevent osteoporosis in women and treatment with estrogen inhibits bone loss, bone turnover and increase bone mineral density (T'sjoen, 2009). However, estrogen treatment is associated with a higher risk of uterine and breast tumors. Besides, the discovery of new phytochemical is able to substitute estrogens without having their negative

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side effects offer a good alternate.

Research in nutrition has led to awareness that the human diet contains, in addition to essential macro- and micronutrients, a complex array of naturally occurring bioactive molecules called phytochemicals, which may confer health benefits (Arjmandi et al., 2000; Weaver et al., 2005; McCarty, 2006; Poulsen and Kruger, 2008 and Coxam, 2008). Soy food and isoflavones have received considerable attention for their potential role in preventing osteopaenia induced by ovariectomy in rats (Watkins et al., 2005) or by menopause in women (Register et al., 2003; Chen et al., 2003). They have estrogenic properties and compete with endogenous estradiol for binding to the estradiol receptor.

Very recently, attention also has been focused on the possible role of other polyphenols. Matheson et al. (2009) had shown that several vegetables can inhibit bone resorption in the rats. The strongest effect was observed with onions, while the same dose of soy (gram for gram of actual raw food) was ineffective. Onions extracts are rich in flavonol glycosides (Zielińska et al., 2008; Justesen et al., 2003); the main flavonol is quercetin (200-600 mg/ kg onions). However, quercetin is mainly found as a glycoside (rutin [quercetin-3-o-rutinose]) that is very abundant in fruits and vegetables (Nöthlings et al., 2008; Somerset and Johannot, 2008). Rutin is one of the polyphenolic compounds found in ZSC, and may be the richest among other flavonoids. Based on this, it is assumed that a similar effect to onion can be extracted *Zizyphus spina-christi*.

The aim of the present study is to evaluate the therapeutic effect of rutin as a standard polyphenol to compare the effect of *Z. spina-christi* extract, regarding their benefit on bone turnover in postmenopausal women.

SUBJECTS AND METHODS

Preparation of the extract

The fruit and seeds of *Z. Spina Christi* was cleaned, dried in an oven of 60°C till complete dryness, then ground to fine powder. Five hundred grams of the dried powder was subjected to successive extractions in soxhelt apparatus using methanol. The solvent was completely removed by distillation under reduced pressure in a rotary evaporator at a temperature not exceeding 60˚C and dried over anhydrous calcium chloride to constant weight in vacuum desiccators. The residue that remained was 41.6% of the dried powder. The residue was added to orange juice (50 mg of residue/ 200 ml juice) and used in the study.

Subjects

Fifty four women in the postmenopausal stage (Age: 54.6 - 70 years old) were recruited. Of these, 46 completed the test period. Two women interrupted the intervention of product and other one due to influenza; the remaining five dropped out for familiar problems which made the follow up impossible. Those patients were selected among those attending the orthopedic out-patient Clinic in El-Haram hospital, Ministry of Health, Giza, Egypt. They were at least 10 years post natural menopause; the mean time after menopause was 14.7 ± 3.37 years and they were free from any critical illness or medical problems. Bone mineral density (BMD) of those patients was measured using dual-energy X-ray Absorpiometery (DPX-L; lunar, Madisom, WE). They were subjected to full clinical investigation.

Design of study

The postmenopausal women were randomly classified into three groups:

Group 1: 13 patients were given orange juice (citrus flavor-noids are limited bioavailability) (Nielsen et al., 2006), in addition to a daily dose of calcium carbonate (Wyeth-Ayrst- Canada INC. Montreal, Canada) equivalent to 1000 mg Ca, 0.025 mg vitamin D_3 (LEO pharmaceutical products- Ballerup Denmark).

Group 2: 18 patients were given orange juice fortified with rutin; a synthetic isoflavone (180 mg/d) in addition to a daily dose of Ca and vitamin D_3 as in group 1.

Group 3: 15 patients were given orange juice fortified with *Z. spina-christi* extract (50 mg/d) in addition to a daily dose of Ca and vitamin D_3 as in group 1. These regimens continued for 3 months and followed up weekly.

Fasting blood samples were taken from each patient in the morning at 10 am before starting the therapeutic regimen and at the end of the 3 month interval. Blood samples were left to clot, and then centrifuged at 3500 rpm for 10 min. Serum was separated and frozen at -20°C until analyzed. The second morning urine samples were collected and frozen at -20°C until analyzed.

Serum calcium and osteocalcin; urinary calcium and deoxypyridinoline (U-Dpd) were determined.

Analytical methods

The individual flavonoids of *Z. spina-christi* in the extract were performed by HPLC method according to Ben-Hammouda et al. (1995), using a reverse-phase column and eluting with acetonetrile/water, acetic acid system. These flavonoids were detected at 335 nm. Serum calcium was estimated colorimetrically by the method given by Weatherburn et al. (1982) as cited in Linear Chemicals. Serum OC was measured by a competitive enzyme linked immuno sorbent assay (ELISA) method according to the manufacture's instructions (BioSource host ELISA kit, BioSource Europe A.S., Rue de l'Indestrie, 8, B-1400 Nivelles, Betgium). U-Dpd was estimated by a competitive enzyme linked immuno sor-bent assay (ELISA) method according to the manufacture's instructions (Pyrilinks_D, Metra Biosystem, Polo AILO, USA) and the results were corrected for urinary creatinine concentration. Urinary createnine was quantified kinetically according to the method of Labbee et al. (1996) as cited in Biosystem.

Statistical analysis

SPSS for windows version 7.0 computer program was used for statistical analysis. All numeric data were expressed as mean ± SE. Data were analyzed using the student t- test to compare means before and after treatment. Pearson's correlation coefficient was used to determine the relationship between different values. For all tests a probability < 0.05 was considered as significant.

RESULTS AND DISCUSSION

Age, time after menopause and T-score of postmenopau-

Table 1. Age, time of menopause and T-score of BMD values of women with postmenopausal osteoporosis.

Groups	Age (ys)	Time of menopause (ys)	T-score
Gp1	61.8 ± 1.98 (54.3- 68.2)	13.6 ± 3.91	-2.5 ± 3.50
Gp2	64.6 ± 2.37 (52.1- 9.3)	14.9 ± 2.89	-3.1 ± 5.21
Gp3	69.2 ± 1.84(51.0-69 6)	15.7 ± 3.31	-2.4 ± 4.11
Mean ± S.E.	56.9 ± 2.02 (51-69.6)	14.7 ± 3.37	-2.67 ± 4.26

Values expressed as means ± SE., * means a significant p<0 .05 & ** means a significant p<0 .01, Groups: (1) Ca + Vit-D3, (2) Ca + Vit-D3+ rutin, (3) Ca + Vit-D3 +, *Zizyphus spina-christi* extract.

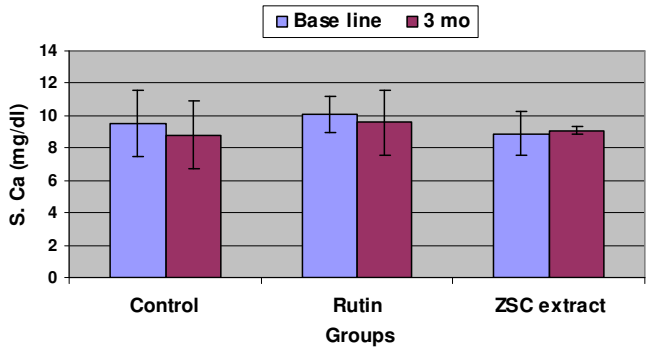

Figure 1. Serum total calcium of postmenopausal women before and after fortification, 3 month intervention. Values expressed as means ± SE. * means a significant p<0 .05 & ** means a significant p<0 .01, Groups: (1) Ca + Vit-D3, (2) Ca + Vit-D3+ rutin, (3) Ca + Vit-D3 + *Zizyphus spina-christi* extract.

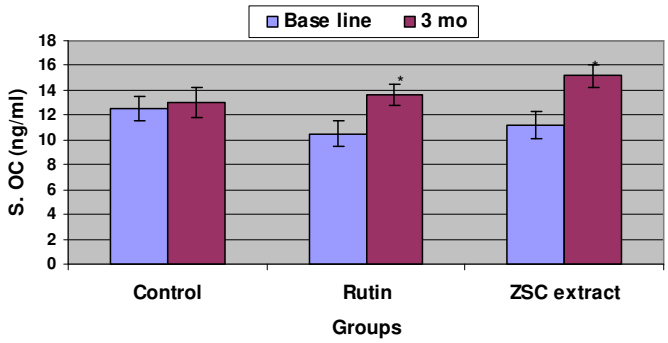

Figure 2. Serum osteocalcin of postmenopausal women before and after fortification, 3 month intervention. Values expressed as means ± SE,. * means a significant p<0 .05 & ** means a significant p<0 .01, Groups: (1) Ca + Vit-D3, (2) Ca + Vit-D3+ rutin, (3) Ca + Vit-D3 + *Zizyphus spina-christi* extract.

sal women are shown in Table 1. As shown from this table most of the cases included in the study are old women that have passed the menopause time long age and having T-score indicating established osteoporosis. As shown in Figure 1, no significant change in serum calcium level occurred due to any of the therapeutic regimen followed by patients in different groups; however, serum osteocalcin was significantly increased in case of groups 2, 3 (Figure 2). This is an indication to improved bone formation most probably caused either by rutin or the phytochemicals present in *Zizyphus* extract.

Phytoesterogens have many biological properties either estrogenic or anti-estrogenic which are useful in prevention of several chronic diseases. It has been suggested that flavonoids could have a beneficial effect in prevention or reduction of bone loss in osteoporosis (Wattel et al., 2001). The increased level of osteocalcin in patients who received rutin or *Zizyphus* extract confirms the biological activity of these compounds with decreasing to bone resorption.

The bone-sparing effects of rutin or its derivatives might result from an increased osteoblastic activity, as indicated by increase of plasma OC level in postmenopausal women after ingestion of rutin or *Zizyphus* extract (Figure 2). It seems that the flavonoids are able to enhance bone formation and might partly explain why onion extract, naturally rich in glycosides of querctin (Nöthlings et al., 2008; Somerset and Johannot, 2008) have a beneficial effect on bone density in pre-menopausal and postmenopausal women (Matheson et al., 2009) and how some vegetables protect postmenopausal women against bone loss (Rassi et al., 2005). Genistein is among several photochemical compounds present in *Zizyphus* extract. It is the most abundant, with a concentration of 24.2 ug/ 50 mg extract. Genistein stimulated protein synthesis in osteoblast cell lines *in vitro* and exerts an anabolic effect on bone in some animal studies (Albertazzi, 2002).

The level of U-Dpd showed marked decrease due to therapeutic ingestion of orange juice fortified with rutin or *Zizyphus* extract (Figure 3). The percentage decrease of U-Dpd level was 46.4% in case of rutin and 57.1% in case of *Zizyphus* extract. There was a highly significant positive correlation between serum osteocalcin and urinary deoxypyridinoline of postmenopausal women in all groups (r= 0.515 and P< 0.01) before supplementation. Although the dose of rutin (180 mg/day) was higher as quantity relative to the dose from *Zizyphus* extract (50 mg/day), yet the effect of the latter on U-Dpd was more pronounced. The effect of either rutin or *Zizyphus* extract on U-Dpd is another indication that these compounds decrease bone resorption. Phytochemical screening of *Zizphus* fruits by HPLC revealed the presence of flavo-

Table 2. The concentrations of individual flavonoids in *Zizyphus Spina Christi* extract (µg/50mg) by HPLC.

Individual flavonoids	Concentrations(µg/50mg)
Genistin	24.200
Dadzain	0.230
Dadzin	0.071
Rutin	0.600
Kaempherol	0.148
Phenol	5.700
Salicylic	2.340
Fuerulic	0.770
Euganol	0.015
Pinostrobin	0.148
Total	35.232

The individual flavonoids of *Zizyphus spina-christi* in the extract were performed by HPLC using a reverse-phase column and eluting with acetonitrile/water, acetic acid system. These flavonoids were detected at 335 nm.

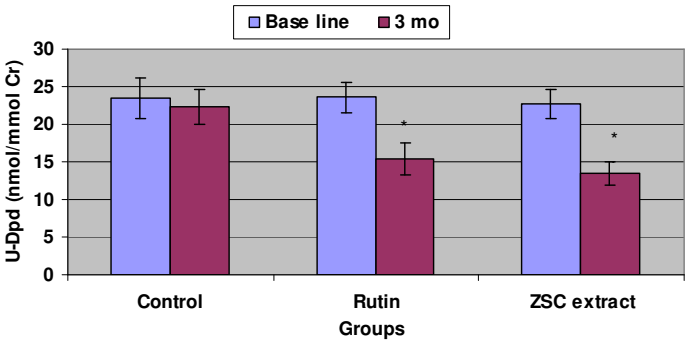

Figure 3. Urinary deoxypyrindinoline of postmenopausal women before and after fortification, 3 month intervention. Values expressed as means ± SE. * means a significant p<0 .05 & ** means a significant p<0 .01, Groups: (1) Ca + Vit-D3, (2) Ca + Vit-D3+ rutin, (3) Ca + Vit-D3 +, *Zizyphus spina-christi* extract.

noids (such as genistin, rutin, phenol, dadzain, dadzin, kaempherol, fuerulic, pinostrobin and euganol). The total amount of flavonoids in 50 mg extract was 35.232 µg (Table 2). It seems that the mixture of flavonoids present in *Zizyphus*, although quantitatively is less, yet is more potent as inhibitor bone resorbing factor. It was reported that genistin acts as osteoclast inhibitor by losing their bone degradation potency in *in vitro* studies. The inhibition of osteoclast-like cell could also occur via an adenosine monophosphate signaling pathway (Williams et al., 1998; Fukushima et al., 2005). Daidzein is one-fourth as estrogenic as genistein. Purified daidzein was more effective than genistein at 10 g/g body weight in preventing ovariectomy-induced bone loss from cancellous bone after 3 months in 12-months-old rats (Picherit et al., 2000). A combination of genistin (159 mg), daidzin (156 mg), and glycitin (33 mg) did not prevent trabecular bone

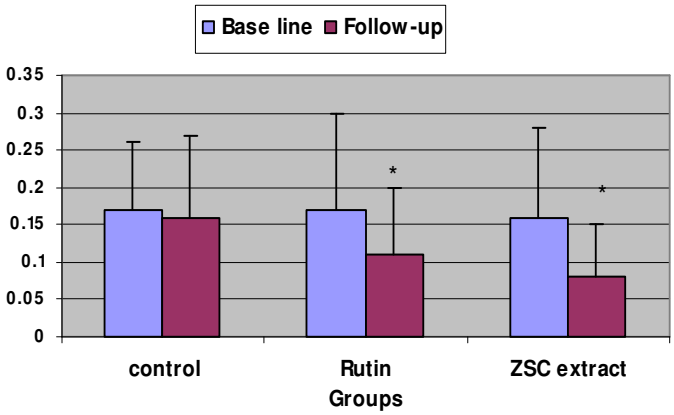

Figure 4. Urinary calcium/ creatinine ratio of postmeno-pausal women before and after fortification, 3 month intervention. Values expressed as means ± SE, * means a significant p<0 .05 & ** means a significant p<0 .01, Groups: (1) Ca + Vit-D3, (2) Ca + Vit-D3+ rutin, (3) Ca + Vit-D3 + *Zizyphus spina-christi* extract.

loss in 7-months-old rats for 12 wk at 0, 20, 40, or 80 mg/kg body weight/d (Picherit et al., 2001).

Most of the phyto-compounds detected in *Zizyphus* sample have marked antioxidant capacity through their ability to donate H atoms/ electrons from their hydroxyl group to free radicals (Lamien-Meda et al., 2008). Since the activities of superoxide dismutase are low in the mineralized cartilage zone (Beecher et al., 2007), the presence of these phytochemicals would influence oxidative stress and consequently decrease bone resorption.

Another mechanism would be that rutin or quercetin present in *Zizyphus* extract exerts its role through an estradiol receptor (ER), as phytoestrogens (Yang et al., 2006). Ranking of the estrogenic potency of phytoestrogens for ER shows that the potency of rutin, which equals ipriflavone, formentin and chrysin is significant and triggers many of the biological responses induced by these compounds (Windahl et al., 2000).

The urinary Ca/Cr ratio was significantly decreased in postmenopausal women after ingestion of rutin or Zizyphus extract; the decrease in the case of *Zizyphus* extract was higher relative to rutin (Figure 4). This shows that the ingestion of flavonids resulted in a decrease of calcium excretion in postmenopausal women. It seems that the decrease in calcium excretion was consistent with the maintenance of bone mass as was reported by Adolphi et al. (2009). It was reported that the bone density did not correlate with excretion of calcium in the urine and that the calcium in the urine accounted only for a small amount of the calcium from the bones. It suggested that rutin or *Zizyphus* extract may affect the calcium balance through mechanisms in the bowel. This would agree with studies of the effect of estrogen on calcium balance via a combination of increased intestinal absorption and decreased renal excretion (Zhang et al., 2006). Thus, phytoestrogen would affect calcium balance in the same way.

Conclusion

Our results show that rutin or *Zizyphus* extract can neutralize estrogen deficiency-induced bone loss, both by decreasing bone resorption and by increasing osteoblastic activity. In this case it can be used to fortify diets of postmenopausal women to help in prevention of osteoporosis. Although the quantity of rutin given to those women was higher ~ 2.5 times than that present in *Zizyphus* extract, the effect of the extract was more pronounced. These findings suggested that polyphenolic compounds present in *Zizyphus* may be attractive candidate as a cheep therapeutic agent against osteoporosis. Also, it may be useful in preventing bone loss and it is a more potent alternative with less estrogen-related side effects for hormone replacement therapy in postmenopausal osteoporosis.

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