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Full Length Research Paper

Protective effect of *Citrus sinensis* and *Citrus aurantifolia* against osteoporosis and their phytochemical constituents

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The efficacy of *Citrus aurantifolia* L. *cv*. Swingle and *Citrus sinensis* L. *cv*. Liucheng against osteoporosis was evaluated in an ovariectomized rat model. Administration of *Citrus* extracts increased trabecular bone mineral content and bone mineral density of tibia, improved the levels of phosphorus and calcium. The results demonstrated that *Citrus* extracts reduced bone loss in ovariectomized rats. These findings have prompted the authors to investigate the phytochemical constituents of those plants, collected in Saudi Arabia, for the first time. Eighteen compounds were obtained from both plants and classified into, eight coumarins (1 - 8), eight flavonoids (9 - 16) and two sterols analogues (17 and 18). Leaves and peels of *C. aurantifolia* afforded eleven components, while those of *C. sinensis* afforded fourteen compounds. Structures of the isolated compounds were deduced by UV, NMR and MS spectra, and comparison with related structures, in which isobergapten (6), marmesin (7), myricetin (11), 4',5,7-trihydroxy-3,6-dimethoxy flavone (12), and quercetin-3-*O*-robinobioside (14), were reported to first time from *Citrus* species, Coumarins, flavonoids and sterols from *C. aurantifolia* and *C. sinensis* could be responsible for their antiosteoporotic activity and the action mechanism of these constituents needs to be further studied. Therefore, *Citrus* extracts have the potential to develop a clinically useful antiosteoporotic agent.

Key words: *Citrus sinensis*, *Citrus aurantifolia*, osteoporosis, bone mineral density, coumarins, flavonoids, sterols.

INTRODUCTION

Osteoporosis, characterized by a loss of bone mass, is a major health problem, especially for elderly women. After the onset of menopause, a reduction in the circulating level of estrogen results in bone loss and increases the incidence of osteoporosis (Branca, 2003; Wong and Rabie, 2006). Of all the natural alternatives currently under investigation, phytoestrogens appear to offer the most potential for the prevention of bone loss. The use of dietary phytoestrogens 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 (Branca, 2003; Morrow et al., 2009). Dietary influences the bone mass and bone metabolism. There are evidence of a positive link between fruits and vegetables consumption and bone health (Wong and Rabie, 2006; Woo et al., 2010). Many plants and plant-derived compounds employed in prevention of the osteoporosis. The genus *Citrus* (Rutaceae) are rich sources of many interesting constituents; such as volatile oils, steroids, limonoids, flavonoids, coumarins and pectins of different bioactivities and nutritional values (Tripoli et al., 2007; Khan et al., 2010). *Citrus* juice of orange and grapefruit

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positively affects serum antioxidant status and bone strength in orchidectomized rats (Deyhim et al., 2008). The powdered pericarp parts of *Citrus aurantium* were reported to have anti-osteoporotic activity in ovariectomized rat (Mandadi et al., 2009). Recently, feeding of orange pulp was reported to improve bone quality in a rat model of male osteoporosis (Morrow et al., 2009). Different bioactive compounds isolated from *Citrus* were reported to improve bone quality in orchidectomized rats especially flavonoids and limonoids (Horcajada-Molteni et al., 2008; Mandadi et al., 2009). Here in, we investigated the potential protective effects of *Citrus aurantifolia* and *Citrus sinensis* collected from Saudi Arabia on ovariectomy-induced bone loss in adult female rats and determine their bioactive phytochemical constituents. The biological and phytochemical investigations of the two plants were carried out for the first time.

MATERIALS AND METHODS

Animals

All the experimental animal work was carried out according to the standard regulations and the ethical committee of medical research at National Research Center (NRC) of Egypt. Adult female Sprague-Dawley rats, weighing 130 - 150 g were obtained from the animal house of the institute (NRC, Dokki, Egypt).

Plants

The leaves and mature fruits (selected for their uniformity, size, color and shape) of the two plants were collected in September 2008; *C. sinensis* L. *cv*. Liucheng, from Nigran, South of Saudi Arabia and *C. aurantifolia* L. *cv*. Swingle from Asfan, Jeddah, West of Saudi Arabia. Identity was confirmed by Dr. Farag Abd-Allah Elghamdy, Botany Department, King Abdul-Aziz University, Jeddah, Saudi Arabia.

Materials used for chromatographic analysis

Whatman No. 1 sheets (Whatman Ltd., Maidstone, England), precoated silica gel 60 F_{254} plates and silica gel (Si) 60, microcrystalline cellulose (Merck, Darmstadt, Germany), and Sephadex LH-20 (Pharmacia, Uppsala, Sweden) were used for paper chromatographic (PC), thin layer chromatographic (TLC) and column chromatographic (CC) analysis, respectively.

Solvent systems

S_1: C_6H_6/EtOAc (9.5:0.5); S_2: C_6H_{14}/EtOAc (3:1); S_3: $CHCl_3$/MeOH (9:1); S_4: *n*-BuOH/HOAc/H_2O (4:1:5, top layer); S_5: 15% aqueous HOAc.

Spray reagents

I. 10% KOH, II. Iodine/potassium iodide (I_2/KI), III. Naturstoff (NA/PE): (a) 1% Diphenyl boryloxyethanolamine in ethanol, (b) 5% polyethylene glycol 400 in methanol. IV. 1% Ethanolic $AlCl_3$, and V. *p*-Anisaldehyde-sulphuric acid.

Instruments

DEXA (Norland X 46, Version 3.9.6/2.3.1) instrument equipped with dedicated software for small animal measurements was used for analysis of BMD and area of femoral bones. Koffler's heating stage microscope. GC/MS Finnigan mat SSQ 7000, Digital DEC EL eV 70. Electron spray ionization mass spectrometry (ESI-MS) measurements were run on a double focusing Mat 95 sector field mass spectrometer (Finnigan, Bremen, Germany). UV analyses for pure samples were recorded, separately, in methanol solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 (P/N 240 – 58000). The NMR spectra were recorded at 300 (^{1}H) and 75 (^{13}C) MHz, on a Varian Mercury 300 NMR spectrometer and δ-values are reported as ppm relative to TMS in the convenient solvent.

Biological evaluation

Preparation of extracts

Dried powdered samples (100 g) were percolated in ethanol (3 × 500 ml) and then filtered. The residues obtained after removal of solvents were dissolved in water in appropriate concentrations for biological activity. All doses were expressed in terms of dried extracts (mg/kg body weight). The yields were 3.13, 3.69, 2.90, 3.37% for the leaves and peels of *C. aurantifolia* and of *C. sinensis*, respectively.

Design of bioactivity study

The animals were ovariectomized surgically and were kept in wire-bottomed cages at room temperature (25 ± 1°C) under 12 h dark/light cycle for three months. The animals were fed standard diet consisted of casein 10%, salts mixture 4%, vitamin mixture 1%, corn oil 10%, cellulose 5% and completed to 100 g with corn starch (A.O.A.C., 1995). Then, after three months following surgery, the animals were classified into six groups; each was composed of 8 rats. The first group was untreated control. The second and third groups were treated orally with lemon leaves and peels extract respectively, 5 mg/kg b.wt (Trivedi et al., 2008). The fourth and fifth groups were treated orally with orange leaves and peels extract respectively (5 mg/kg b.wt.). The sixth group was treated orally with conjugated estrogen (50 mg/kg b.wt.) daily for three months (Burdette et al., 2002). Additional gonad intact control group was included in this study.

At the end of the experimental period, all animals were fated for 12 h and the blood samples were collected from retro orbital plexus (Schermer, 1967) under diethyl ether anaesthia. The blood samples were left to clot and the sera were separated by cooling centrifugation (4°C) at 3000 rpm. for 10 min. Then the right femoral bone of each animal was carefully removed cleaned and stored in formalin buffer 10% for analysis of bone mineral density (BMD) and area by dual energy X-ray absorptiometry (DEXA).

Biological methods

Serum calcium (Ca) concentration was assayed by colorimetric method (Tietz, 1995) using kit purchased from Cromatest Linear Chemicals Co. (Spain). Serum phosphorous (P) concentration was measured by kinetic method (Daly and Ertingshausen, 1972) and serum alkaline phosphatase (ALP) activity was estimated by enzymatic–kinetic method (Scherwin, 2003) using kit purchased

from Elitech Co. (France). Serum parathyroid hormone (PTH) and osteocalcin were estimated by immunoenzymtric assay (Bouillon et al., 1990; Power and Fottrell, 1991) using kit purchased from Biosource Europe S.A. (Belgium). Serum receptor activator nuclear factor kappa B ligand (RANKL) was determined using R and D ELISA kits (USA) as described by Crotti et al. (2002). BMD was calculated by the bone mineral content (BMC) of the measurement area. The scanned area of the femur was equally divided into three regions (proximal, mid shaft and distal femur) to assess the regional differences.

Statistical analysis

The obtained data are presented as means ± standard error (SE). The difference between two groups was calculated using independent unpaired Student t-test (Woolson, 1987) with a present probability level of $P < 0.05$ that was considered to be statically significant.

Isolation of compounds

General procedure

The air dried leaves and peels of *C. aurantifolia* and of *C. sinensis* were grounded in a mixer and exhaustively extracted with 80% methanol at room temperature. The methanolic extract of each fraction was concentrated under vacuum at 40 °C and defatted with light petroleum (60-80°C) to afford a residue. This residue was chromatographed on Si CC using gradient elution with petroleum ether-benzene. β-Sitosterol 17 was isolated from the leaves of the two *Citrus* species and stigmasterol 18 was obtained only from leaves of *C. sinensis.* The methanolic fractions were chromatographed on Si CC using gradient elution with C_6H_6-EtOAc and $CHCl_3$-MeOH. The fractions eluted by C_6H_6-EtOAc were further done separated by repeated chromatographed on Si CC and more purified by flash chromatographs. Six coumarins 1-3, 5, 7, 8 were isolated from leaves and four 1, 2, 5, 8 from peels of *C. sinensis*. Furthermore, four coumarins 1-3, 6 were obtained from leaves and four 1, 3-5 from peels of *C. aurantifolia*. The eight coumarins were characterized mainly by spectroscopic methods; UV, MS, ^1H and ^{13}C NMR as well as comparison of the melting points with authentic samples or those in the literatures. On the other hand, the fractions eluted by $CHCl_3$-MeOH were chromatographed on different columns *viz*., Si CC using methylene chloride/methanol, cellulose using 40% methanol/water and Sephadex LH-20 using *n*-butanol/*iso*-propanol/water (4:1:5, top layer), methanol or methanol/water mixture (9:1) as different eluents to yield eight flavonoids 9-16. The purity of compounds 1-8 were being checked by TLC using solvent systems S_1 for compounds 1, 3, 6 and 17, 18 while, S_2 and S_3 for compounds 2, 4, 5, 7, and 8. Spray reagents I and II were used for compounds 1-8 and V for 17 and 18. While the purity of the isolated compounds 9-16 is being checked by PC using solvent systems S_3 - S_5 and spray reagents III and IV. The chromatographic properties of compounds 13-16 (color under UV light, change with ammonia vapor and responses towards spray reagents, III and IV) can suggest their flavonoid glycosides characters. From peels and leaves of *C. sinensis* five flavonoids 10, 12-15 and three 10, 15, 16, respectively were isolated. In addition to four flavonoids 9, 10, 12, 15 and three 11, 15, 16 were obtained from leaves and peels of *C. aurantifolia*, respectively. The identification of the isolated flavonoids were done on the basis of chromatographic properties, UV spectral data (using shift reagents), MS, ^1H and ^{13}C NMR spectroscopy as well as comparison with the available references samples.

Compound (6): 46 mg, white needles, m.p. 219-221°C, R_f 0.67 (S_1), 0.58 (S_2). UV: λ_{max} (MeOH): 248, 308 nm. EIMS m/z (100%): 216 [M^+, 100], 201 [M^+-CH_3, 63], 188 [M^+-CO, 23], 173 [201-2CO, 94], [145 201-3CO, 34], 89 [201-4CO, 27]. ^1H NMR (DMSO-d_6 300 MHz): δ ppm 8.13 (1 H, d , *J*= 9.9 Hz, H-4), 7.59 (1 H, d , *J*= 2.4 Hz, H-2'), 7.11(1 H, s , H-6), 7.01 (1 H, d , *J*= 2.4 Hz, H-3'), 6.25 (1 H, d , *J*= 9.9 Hz, H-3), 4.26 (3H, s, OCH_3-5).

Compound (12): 427 mg, yellowish white crystals, m.p. 210-212 °C, R_f 0.48 (S_1), 0.90 (S_2). UV: λ_{max} (MeOH): 268, 342; (+NaOMe): 272, 328, 396; (+$AlCl_3$): 277, 309 sh, 363, 416 sh; (+$AlCl_3$+HCl): 279, 303 sh, 353, 404 sh; (+NaOAc): 272, 301 sh, 353; (+ NaOAc + H_3BO_3): 272, 342 nm. EIMS m/z (100%): 330 [M^+, 100], 329 [M-H, 42], 316 [M^+-CH_3/+H, 21], 315 [M^+-CH_3, 76], 312 [M^+ -H_2O, 46], 298 [316-H_2O, 16], 288 [316-CO, 10], 287 [315-CO, 53], 269 [315-H_2O-CO, 39]. ^1H NMR (DMSO-d_6 300 MHz): ppm 12.73 (1H, s, OH-5), 7.91 (2 H, d, *J*= 8.4 Hz, H-2'/ 6'), 6.93 (2 H, d, *J*= 8.7 Hz, H-3'/ 5'), 6.52 (1 H, s, H-8), 3.77 (3 H, s, OCH_3-6), 3.76 (3 H, s, OCH_3-3). ^{13}C NMR (DMSO-d_6, 125.7 MHz): δ ppm 178.1 (C-4), 160.1 (C-4'), 155.6 (C-2), 157.2 (C-7), 152.3 (C-5), 151.5 (C-9), 137.2 (C-3), 131.1 (C-6), 130.0 (C-2'/6'), 120.6 (C-1'), 115.5 (C-3'/5'), 104.5 (C-10), 93.9 (C-8), 59.9 (OCH_3-6), 59.6 (OCH_3-3).

Compound (13): 54 mg, dark yellow crystals, m.p. 232-234 °C, R_f 0.37 (S_1), 0.57 (S_2). UV: λ_{max} (MeOH): 256, 298 sh, 356; (+NaOMe): 271, 326, 412; + $AlCl_3$ 269, 306 sh, 427; (+$AlCl_3$+HCl): 273, 300, 356 sh, 400; (+NaOAc): 263, 365; (+ NaOAc + H_3BO_3): 261, 380 nm. negative ESI-MS m/z (100%): 464 [M^-, 25], 463 [(M-H)$^-$, 100] at low volt 300.9 [(Q-H) $^-$, 48], 299.9 [(Q-2H) $^-$, 100]. ^1H NMR (DMSO-d_6 300 MHz): ppm 12.60 (1 H, s, OH-5), 7.65 (1 H, dd , *J*= 8.4, 2.4 Hz, H-6'), 7.54 (1 H, d, J=2.4 Hz, H-2'), 6.81 (1 H, d, *J*= 8.4 Hz, H-5'), 6.41 (1 H, d, *J*= 2.1 Hz, H-8), 6.21 (1 H, d, *J*= 2.1 Hz, H-6), 5.35 (1 H, d, *J*= 7.5 Hz, H-1''), 3.70-3.20 (m, Sugar Protons).

Compound (14): 10 mg, yellow amorphous, R_f 0.52 (S_1), 0.40 (S_2). UV: λ_{max} (MeOH): 250 sh, 269, 340; (+NaOMe): 273, 296 sh, 376; (+$AlCl_3$): 272, 303 sh, 427; (+$AlCl_3$+HCl): 272, 303 sh, 351; (+NaOAcm): 266, 299 sh, 400; (+ NaOAc + H_3BO_3): 266, 399 sh, 400 nm. CI-MS m/z (100%): 611 [M^++H, 23], 465 [M^+-deoxyrhamnose+2H, 14.5], 303 [M^+-deoxyrobinose+2H, 100]. ^1H NMR (DMSO-d_6, 300 MHz): δ ppm 7.30 (2 H, m, H-2'/6') ; 6.53 (1 H, d, *J*= 8.4 Hz, H-5'), 6.02 (1H, d, *J*= 1.8 Hz, H-8), 5.88 (1 H, d, *J*= 1.8 Hz, H-6), 4.60 (1 H, d, *J*=7.5 Hz, H-1''), 4.24 (1 H, brs, H-1''), 3.80- 3.04 (m, sugar protons), 0.77 (3 H, d, *J*= 6 Hz, CH_3-6''').

Compound (15): 25 mg, yellow amorphous, R_f 0.49 (S_1), 0.53 (S_2). UV: λ_{max} (MeOH): 258, 299 sh, 360; (+NaOMe): 270, 327, 411; (+$AlCl_3$): 273, 305 sh, 361, 431; (+$AlCl_3$+HCl): 265, 302 sh, 361 400; (+NaOAc): 265, 298 sh, 373; (+ NaOAc + H_3BO_3): 265, 398 sh, 389 nm. negative ESI-MS m/z (100%): 609 [(M-H) $^-$, 100], 463 [(M-deoxyrhamnose) $^-$, 10], 301 [(quercetin-H) $^-$, 6]. ^1H NMR (DMSO-d_6 300 MHz): δ ppm 7.53 (2 H, m, H-2'/6') ; 6.83 (1 H, d, *J*= 9.0 Hz, H-5'), 6.38 (1H, d, *J*= 2.1 Hz, H-8), 6.19 (1 H, d, *J*= 2.1 Hz, H-6), 5.33 (1 H, d, *J*=7.0 Hz, H-1''), 4.39 (1 H, brs, H-1'''),3.70 (1H, brd, *J*= 10.2 Hz, H-6a'') 3.80- 3.06 (m, sugar protons), 0.99 (3 H, d, *J*= 6 Hz, CH_3-6''').

RESULTS AND DISCUSSION

The ovariectomized rats have been proposed as a good model of postmenopausal osteoporosis in women and have been validated as a clinically relevant model of this condition. Currently there exist two well-established small animal models of local osteoporosis: the rat ovariectomy (OVX) and the immobilization (IM)-induced bone loss models. The OVX rat is an excellent preclinical animal model that correctly emulates the important clinical feature of the estrogen depleted human skeleton and the response of therapeutic agents (Jee and Yao, 2001; Li et

al., 2009).

Oral administration of either leaves or peels of *C. aurantifolia* and *C. sinensis* extracts in OVX rats produced significant increase in serum calcium level when compared to that in untreated OVX rats (Table 1). Flavanones, flavones, and flavonols are the flavonoids present in *Citrus*, these types of compounds have been show to be powerful oestrogen–like activity (Tripoli et al., 2007). It has been reported that intake of the *Citrus* flavonoids including rutin, quercetin and kaempferol might be useful in preventing symptoms arising from estrogen deficiency and could be have a positive effect on bone remodeling as well as inhibitory action on ovariectomy–induced osteopenia in rats (Kim et al*.,* 2006; Woo et al., 2010). Bioflavonoids are a class of flavonoids that includes rutin, hesperidin, quercetin, eriodictyl and citron, and they are essential for the absorption of vitamin C. Normally found in highly nutritious foods, such as *Citrus* and they improved bone quality (Morrow et al., 2009).

Quercetin and kaempferol were reported to inhibit proliferation and increase osteogenic differentiation in human adipose stromal cells (Kim et al., 2006). The possible role of flavonone and flavonol isolated from *Citrus* extracts, in this study, is that these compounds act on bone cells through estrogen receptors (ER_s) (Wattel et al., 2003; Prouillet et al., 2004). ERs have been found in osteoblasts, bone forming cells, and bone marrow stromal cells. The binding affinity of these flavonoids for ER is high (Prouillet et al., 2004; Morrow et al., 2009). In particular, quercetin, rutin and hesperidin could modulate nuclear ER_s proteins, by increasing ER_β and decreasing ER_α, this acting as selective ER modulators (Prouillet et al., 2004). Thus, by activation of ER_β, these compound showed estrogenic potency and could decrease calcium excretion and restore serum calcium level in OVX rats towards normal value (Horcajada-Molteni et al., 2008). The coumarin compounds isolated in this study as xanthotoxin, isopimpinellin, bergapten and imperatorin were reported to inhibit the formation and differentiation of multinucleated osteoclasts, decreased the activity of Tartrate-resistant acid phosphatase (TRAP) and bone resorption *in vitro,* which led to the prevention of experimental osteoporosis in ovariectomized rats (Qin et al., 2003).

As shown in Table 1, treatment of OVX rats with the investigated extracts of *C. aurantifolia* and *C. sinensis* resulted in insignificant reduction in total alkaline phosphatase (TAP) activity as compared to untreated OVX rats. This finding could be attributed to the bioactive compounds like *Citrus* flavonoids present in these extracts which exerted an inhibitory action on bone turnover induced by estrogen deficiency and this suggested that these compounds appear to prevent bone loss by its anti-resorptive activity (Wattel et al., 2003; Trivedi et al., 2008; Woo et al., 2010). They exhibited significantly higher bone mineral density (BMD) in the trabecular regions (femur neck, proximal tibia and vertebrae). Also, Woo et al. (2004) demonstrated that quercetin could suppress bone resorption by inhibiting the differentiation and activation of osteoclasts. While, Kim et al. (2006) reported that quercetin inhibits proliferation and increases osteogenic differentiation in human adipose stromal cells. *Citrus* flavonoids have antioxidant action and have influence on neutrophil apoptosis and oxidative metabolism (Przyjemska and Ignatowicz, 2008). Moreover the antioxidant activity of *Citrus* bioactive compounds may be contributed in the anti-resorptive effect of these compounds and improve bone quality (Ghasemi et al., 2009; Mandadi et al., 2009). Their ability to scavenge highly reactive species might be responsible for the inhibition of osteoclastic superoxide availability and consequent reduction of bone resorption. Therefore, these active compounds induced in the tested extracts may act in a synergistic pathway to inhibit bone turnover marker "alkaline phosphatase" *via* their anti resorpative activity and improve bone quality.

Treatment of OVX rats with either one of the tested extracts resulted in significant decrease in serum osteocalcin level as compared to untreated OVX rats (Table 1). This finding could be interpreted *via* the positive effect of bioactive compounds like flavonoids on reducing bone remodeling sites (Horcajada-Molteni et al., 2008). This effect of compounds may be attributed to their antioxidant properties which participated in reducing osteoclastic bone resorption *via* lowering reactive oxygen species (ROS) involved in increasing osteoclastic activity (Mandadi et al., 2009). Many *Citrus* flavonoids had a reducing effect on osteoclastic bone resorption *in vitro* via the direct targeting on the mature osteoclasts by a mechanism involving, at least in part, the estrogen receptor (Wattel et al., 2003). Moreover, kaempferol has been shown to inhibit bone turnover as we mentioned before and this property of kaempferol may also contribute in reducing bone turnover marker "osteocalcin" level in the present study.

Significant reduction in serum RANKL level were resulted in either one of *Citrus* extracts treated groups as compared to that in untreated OVX rats (Table 1). The bioactive flavonoid, in particular kaemferol and quercetin have been shown to inhibit the activation of two key transcription factors; $NF\text{-}K_B$ and activator protein-1 (AP-1) involved in differentiation, survival and activation of osteoclasts (Jimi et al., 1998). They strongly attenuated the RANKL-induced formation of multinucleated osteoclasts and considered as potential agents for clinical application following the insults of bone loss (Pang et al., 2006). Recent study demonstrated the role of rutin in inhibiting RANKL-induced osteoclast formation *in vitro* by decreasing ROS production (Kyung et al., 2008).

Table 2 showed that treatment with either one of the tested extract led to significant increase in BMD of each of proximal and distal areas of rat femur. Treatment with lemon leaves or peels extracts in OVX rats led to

Table 1. Effects of *Citrus* extracts on different parameters in ovariectomized (OVX) rats (n = 6).

Groups	Ca mg/dl	P mg/dl	Alkaline phosphatase IU/l	Osteocalcin ng/ml	RANKL pg/ml	PTH pg/ml
Gonad intact	9.0 ± 0.2	5.8 ± 0.2	105.1 ± 6.4	7.8 ± 0.1	62.0 ± 2.4	34.4 ± 0.6
OVX control	7.0 ± 0.1a**	7.0 ± 0.1a**	122.3 ± 4.2a*	9.6 ± 0.2a**	88.9 ± 3.5 a**	41.8 ± 1.1a**
Conjugated estrogen	8.5 ± 0.2 b**	5.5 ± 0.2b**	111.9 ± 3.8bNS	8.8 ± 0.08b**	70.3 ± 3.0b**	35.3 ± 0.9b**
C. aurantifolia leaves	8.6 ± 0.1b**	6.1 ± 0.3b*	113.1 ± 4.9bNS	8.5 ± 0.07b**	72.4 ± 2.9b**	35.8 ± 1.2b**
C. aurantifolia peels	8.8 ± 0.1 b**	5.9 ± 0.2b**	112.4 ± 3.5bNS	8.4 ± 0.1b**	69.8 ± 2.6b**	35.0 ± 1.5b**
C. sinensis leaves	8.1 ± 0.2b**	6.1 ± 0.1b**	115.8 ± 2.8bNS	8.7 ± 0.1b**	77.2 ± 2.4b*	36.5 ± 1.0b**
C. sinensis peels	8.5 ± 0.3b**	6.3 ± 0.1b**	114.1 ± 2.6bNS	8.6 ± 0.2b**	75.9 ± 3.2b*	36.2 ± 1.3b**

All values are expressed as mean ± S.E.; a: Difference changes in relation to gonad intact control group; b: Difference changes in relation to OVX group; *: Significant change at P < 0.05; **: Significant change at P < 0.01; NS: insignificant P > 0.05.

Table 2. Effects of *Citrus* extracts on bone mineral density (BMD) in ovariectomized (OVX) rats (n = 6).

Groups	Proximal (mg/cm^2)	Mid (mg/cm^2)	Distal (mg/cm^2)	Total (mg/cm^2)
Gonad intact	105.2 ± 2.0	112.3 ± 0.38	120.7 ± 2.0	116.6 ± 1.3
OVX control	101.2 ± 0.38^{aNS}	105.5 ± 0.5^{a**}	109.1 ± 0.37^{a**}	109.3 ± 1.2^{a**}
Conjugated estrogen	111.5 ± 1.0^{b**}	106.8 ± 1.5^{bNS}	118.3 ± 1.8^{b**}	112.1 ± 0.8^{bNS}
C. aurantifolia leaves extract	111.8 ± 1.3^{b**}	109.9 ± 0.6^{b**}	115.2 ± 1.3^{b**}	113.1 ± 1.1^{b*}
C. aurantifolia peels extract	113.6 ± 0.25^{b**}	111.0 ± 0.8^{b**}	120.2 ± 0.9^{b**}	115.3 ± 1.0^{b**}
C. sinensis leaves extract	105 ± 0.9^{b**}	106.1± 0.3^{bNS}	113.2 ± 0.4^{b**}	110.2 ± 0.9^{bNS}
C. sinensis peels extract	110.5 ± 0.3^{b**}	107.3 ± 0.9^{bNS}	119.3 ± 1.2^{b**}	112.3 ± 1.0^{bNS}

All values are expressed as mean ± S.E. a: Difference changes in relation to gonad intact control group. b: Difference changes in relation to OVX group. *: Significant change at P < 0.05. **: Significant change at P< 0.01. NS: insignificant P > 0.05.

significant increase in BMD of each of mid and total areas of rat femur, while insignificant increase in BMD of mid and total areas of rat femur was detected in OVX rats treated with orange leaves or peels extracts as compared to untreated OVX rats. These findings might be explained due to the presence of the detected active ingredients in these extracts. These active compounds included rutin, quercetin and kaempferol. Rutin has been found to inhibit ovariectomy-induced trabecular bone loss in rats, both by slowing bone resorption and increasing osteoblastic activity. Through this action of rutin, it could increase femoral strength and total femoral density due to the increase in trabecular bone density (Horcajada-Molteni et al., 2008). Rutin also could regulate bone metabolism and increase bone mass in rats (Woo et al., 2004). Quercetin has been found to increase calcium content in the diaphyseal tissues of rat femoral culture system *in vitro*. This means that quercetin had unique effects on bone calcification (Kim et al., 2006).

Promotion of osteoblasts mineralization and inhibition of resorption by osteoclasts have been occurred simultaneously due to the presence of kaempferol. Ovariectomized rats treated with kaempferol showed significant increase in BMD of trabecular bones, including the femur neck and tibia proximal with a concomitant increase in bone strength suggesting that kaempferol treatment improves bone quality (Trivedi et al., 2008).

The results of the present study clearly indicate that anti-osteoporosis activity is proportionate to the content of both flavonoids and coumarins of *C. aurantifolia* and *C. sinensis*.

Phytochemical constituents

The methanol extracts of the *Citrus* species were fractionated by column chromatography to obtain two fractions. The first fraction resulted from C_6H_6-EtOAc afforded eight coumarins: psoralene (1), xanthotoxin (2), bergapten (3), isopimpinellin (4), imperatorin (5), isobergapten (6_a), marmesin (7), umbellifrone (8). Psoralene (1) has isolated before from the fruits of *C. limon* L *var*. Eureka (Chouchi and Barth, 1994).

Compounds 2-5 and 8 have been reported from the leaves of *C. aurantifolia* (Stanley and Jurd, 1971; Haggag et al., 1999). Compounds 6_a and 7 have not been showed previously to be constituents of *Citrus* species. From the second fraction ($CHCl_3$-MeOH) eight flavonoids were isolated. Compound 10 and 15 have been reported from fruits of grapefruit and lemon (Robards et al., 1997; Belajova and Suhaj, 2004). Compounds 16, 17, and 18 have been isolated from *C. sinensis* and *C. aurantium* (Haggag et al., 1999). Compounds 11, 12 and 14 have not been isolated from *Citrus* species.

Table 3. ^{13}C NMR spectral data of compounds 3, 6_a and 6_b.

No. of C	Compound 3	Compound 6_a	Compound 6_b
C-2	161.0	160.7	162.2
C-3	112.6	112.5	114.5
C-4	139.2	139.1	144.5
C-5	149.6	154.1	123.9
C-6	112.7	93.7	108.8
C-7	158.4	158.3	157.3
C-8	93.9	109.3	116.9
C-9	152.7	148.8	148.5
C-10	106.5	106.3	113.5
C-2'	144.8	144.7	145.9
C-3'	105.0	105.0	104.0
OCH_3-5	60.1	60.0	-

Compound (6_a): UV spectral data of 6 showed absorption maxima at 248, 308 nm which characteristic of an angular type of coumarins (Murray et al., 1982). Its ^1H NMR spectrum displayed an AB system (J = 9.9 Hz) at d 6.25 and d 8.13 ppm characteristic of a coumarin substituted at C-3 and C-4, respectively. Another two doublets signals at δ 7.59 and 7.01 ppm were assigned to the protons at C-2' and C-3', respectively. Moreover, the signal of the methoxyl group appeared at s 4.26 ppm. The proposed structure of isobergapten was confirmed by ^{13}C NMR spectrum which supported the angular type furanocoumarin (Table 3). The presence of OMe group at the 5 position resulted in the appreciable downfield of C-5 and upfield of C-6 compared with angelicin 6_b (Table 3) (Macias et al., 1990). Furthermore, the observed upfield shift of C-6 and downfield of C-8 of isobergapten when compared with bergapten (Table 3) (Duddeck and Kaiser, 1982).

Compound (12): On the basis of chromatographic properties and UV spectral data (Markham, 1982), compound 12 was expected to be kaempferol like structure. On addition of NaOMe band I was shifted to longer λ_{max} (+54 nm) with increasing in the intensity indicating to free OH-4'. A bathochromic shift was observed for band I and II by addition of $AlCl_3$/HCl indicated the presence of free OH-5 fraction along with OR-6 grouping (Mears and Mabry, 1972). The presence of an oxygen function at C-6 is also corroborated by suppression of band II for a free OH-7 (Mabry et al. 1970). ^1H NMR spectrum of compound 12 showed in its aromatic region two spin coupling system AX, each of two doublets. The first one consists of two *ortho* doublets at δ 7.91 and 6.93 ppm assigned to H-2'/6' and H-3'/5', respectively of 4'-hydroxy B ring (Mahmoud et al., 2001). In addition a singlet at δ 6.52 ppm was assigned to H-8 indicated 5, 6, 7-trisubstituted ring A. The 3-substituted kaempferol moiety was confirmed from its characteristic resonances in the ^{13}C NMR spectrum (Table 4) and it was interpretated specially signals at 130.0 and 115.5 ppm of C-2'/6' and C-3'/5' intrinsic for 1, 4-disubstituted B ring of kaempferol aglycone. The methoxylation of C-3 and C-6 were simply deduced from the characteristic downfield δ values at 137.2 and 131.1 ppm, respectively. Therefore, compound 12 may be 4',5,7, trihydroxy-3,6 dimethoxy flavone (Horie et al, 1998).

On the basis of chromatographic properties and UV spectral data, compounds 13-15 were expected to be quercetin-3-glycoside (Mabry et al., 1970). Acid hydrolysis of the three compounds afforded quercetin as an aglycone and D-galactose for 13, L-rhamnose and D-galactose for 14 and L-rhamnose and D-glucose for 15 as glycoside moieties (Markham, 1982).

^1H NMR of the compounds 13-15 showed in the aromatic region, the two characteristic spin coupling systems ABX (d H-2', dd H-6', d H-5') of 1, 3, 4-trisubstituted ring B and AM (d H-8, d H-6) for A ring. ^{13}C NMR spectra of the three compounds (Table 4) showed the typical 15 ^{13}C resonances for a quercetin moiety, among them C-4' and C-3' at 148.1-149.1 ppm and 144.2-145.4 ppm, respectively, which can be considered the key signals of 3', 4'-disubstituted B-ring in all quercetin glycosides. Glycosidation at OH-3 was proved by the relative upfield shift of C-3 at 133.4-134.3 ppm (Δ~ 3-4 ppm) and downfield shift of C-2 (Δ~ + 10 ppm) relative to those of free aglycone (Harborne and Mabry, 1982).

Stereostructure of a 3-*O*-galactoside moiety (compound 13) was established as β-$^4C_1$-pyranose depending on δ and *J*-values in both ^1H (5.35 ppm, *J*=6.9 Hz) and ^{13}C (101.8 ppm) NMR spectra (Harborne and Mabry, 1982; Agrawal, 1989). The large differences between δ values of C-3'' and C-5'' (~2 ppm) was diagnostic to a galctoside moiety (Agrawal, 1989) as compared with that of the glucoside (~< 1 ppm). Hence, compound 13 was identified as quercetin -3-*O*-β-D-$^4C_1$-galactoside or hyperin (Harborne and Baxter, 1999). The presence of robinoside moiety for compound 14 was evidenced from the two

Table 4. ^{13}C NMR spectral data of compounds 13 - 15.

No. of C	Compound 12	Compound 13	Compound 14	Compound 15
C-2	155.6	156.3	156.6	157.3
C-3	137.2	133.4	134.3	134.1
C-4	178.1	177.4	177.6	178.1
C-5	152.3	161.1	161.3	161.9
C-6	131.1	98.7	99.0	99.4
C-7	157.2	164.4	164.3	164.8
C-8	93.9	93.5	93.7	94.3
C-9	151.5	156.1	157.5	157.1
C-10	104.5	103.8	104.1	104.7
C-1'	120.6	121.0	121.3	121.9
C-2'	130.0	115.1	115.1	116.0
C-3'	115.5	144.8	144.2	145.4
C-4'	160.1	148.4	148.1	149.1
C-5'	115.5	115.9	116.6	117.0
C-6'	130.0	121.9	122.1	122.3
C-1''	-	101.8	99.0	101.4
C-2''	-	71.2	70.3	74.8
C-3''	-	73.1	73.9	77.3
C-4''	-	67.9	69.5	70.8
C-5''	-	75.8	75.4	76.7
C-6''	-	60.1	66.9	67.7
C-1'''	-	-	100.6	102.0
C-2'''	-	-	70.9	71.1
C-3'''	-	-	70.3	71.4
C-4'''	-	-	72.7	72.7
C-5'''	-	-	69.5	68.7
C-6'''		-	17.4	18.4
OCH_3-3	59.6	-	-	-
OCH_3-6	59.9	-	-	-

resonances at 4.60 ppm (J = 7.5 Hz) and 4.24 ppm broad singlet assigned to the two anomeric proton signals of β-galactosyl and α-rhamnosyl moiety, respectively. Anomeric carbons of the sugar moieties appeared at 99.0 and 100.6 ppm indicating *O*-linked sugar (Table 4). The downfield shift of C-6'' (66.9 ppm) of galactose which appeared ~ 6.0 ppm downfield showed 6''→1''' linkage of D-galactose and L-rhamnose which established the structure as quercetin-3-*O*-α-L-rhamnopyranose-1'''→6''-β-D-galactopyranoside (quercetin-3-*O*-robinobioside) as compared with published data of structurally related compounds (Agrawal, 1989).

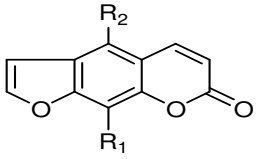
^1H NMR of compound 15 showed a β–anomeric proton signal of inner glycoside moiety H-1'' at 5.33 ppm (J=7.0 Hz) with a characteristic anomeric doublet signal and CH_3-6'' as a doublet at 0.99 ppm (J= 6.0 Hz) of a terminal α-rhamnosyl moiety. The δ value of H-1''' at brs 4.39 ppm together with downfield of H-6a'' at 3.70 ppm was a confirmative evidence for (1'''→6'' rhamnosyl glucoside linkage. Therefore, compound 15 was identified as quercetin-3-*O*-α-L-rhamnopyranosyl-1'''→6''-β-D-glucopyranoside or rutin (Harborne and Baxter, 1999). ^1H and ^{13}C resonances were achieved through a comparison with reported data in the literatures (Markham et al., 1978; Harborne and Mabry, 1982; Agrawal, 1989).

Relationship between the obtained compounds

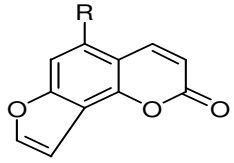
Distribution of the isolated compounds (1-18) in Leaves and peels of both *Citrus* species was remarked in Table 5 (Figure 1). Despite of the similarity of both plants in their constituted compounds, certain isolated compounds were specifically restricted to their own plants. Accordingly, isobergapten (6_a) and kaempferol (9) were specific to leaves of *C. aurantifolia*, while quercetin-3-*O*-robinobioside (14) and stigmasterol (17) were definite to leaves of *C. sinensis*. Isopimpinellin (4) and myricetin (11) were detected only in both parts of *C. aurantifolia* while, xanthotoxin (2), umbellifrone (8) and quercetin (10)

Table 5. Relationship between the obtained compounds from leaves and peels of *C. aurantifolia* and *C. sinensis*.

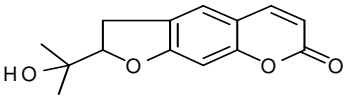
Obtained compounds	Leaves		Peels	
	C. aurantifolia	***C. sinensis***	***C. aurantifolia***	***C. sinensis***
Psoralene (1)	+	+	+	+
Xanthotoxin (2)	-	+	-	+
Bergapten (3)	+	+	+	-
Isopimpinellin (4)	+	-	+	-
Imperatorin (5)	+	+	+	+
Isobergapten (6)	+	-	-	-
Marmesin (7)	-	+	-	-
Umbellifrone (8)	-	+	-	+
Kaempferol (9)	+	-	-	-
Quercetin (10)	-	+	-	+
Myricetin (11)	+	-	+	-
4',5,7- Trihydroxy-3,6- dimethoxy flavone (12)	+	+	-	-
Hyperin (13)	-	+	-	-
Quercetin-3-*O*-robinobioside (14)	-	+	-	-
Rutin (15)	+	+	+	+
Hesperidin (16)	-	-	+	+
Stigmasterol (17)	-	+	-	-
β-Sitosterol (18)	+	+	-	-



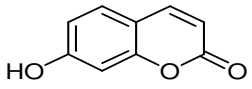
		R_1	R_2
1	Psoralene	H	H
2	Xanthotoxin	OCH_3	H
3	Bergapten	H	OCH_3
4	Isopimpinellin	OCH_3	OCH_3
5	Imperatorin		H



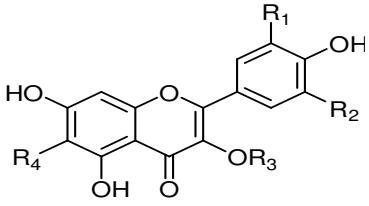
(**6a**) Isobergapten R= OCH_3
(**6b**) Angelicin R=H



Marmesin (**7**)

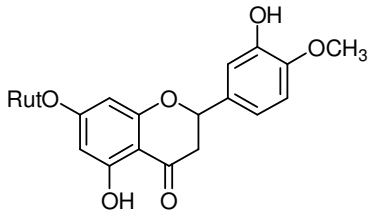


Umbelliferone (**8**)



		R_1	R_2	R_3	R_4
9	Kaempferol	H	H	H	H
10	Quercetin	OH	H	H	H
11	Myricetin	OH	OH	H	H
12	4',5,7- Trihydroxy-3,6- dimethoxy flavone	H	H	CH_3	OCH_3
13	Hyperin	OH	H	Gal	H
14	Quercetin-3-robinobioside	OH	H	Rob	H
15	Rutin	OH	H	Rut	H

Gal= galactose; Rob=robinobioside (2-*O*-α-rhamnosyl-D-galactose); Rut =rutinose (6-*O*-α-rhamnosyl-D-glucose)



Hesperidin (**16**)

Figure 1. Structures of isolated compounds 1 - 16.

were detected in both parts of *C. sinensis*. Psoralene (1), imperatorin (5) and rutin (15) are major constituents in leaves and peels of *Citrus* species. While 4',5,7 trihydroxy-3,6-dimethoxy flavone (12) and β-sitosterol (18) were distinguished in the leaves of both *Citrus* species. Hesperidin (16) was in their peels and was isolated as minor compound from peels of *C. sinensis* although these peels were considered as a commercial source for the compound in another study (Kanaze et al., 2009).

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