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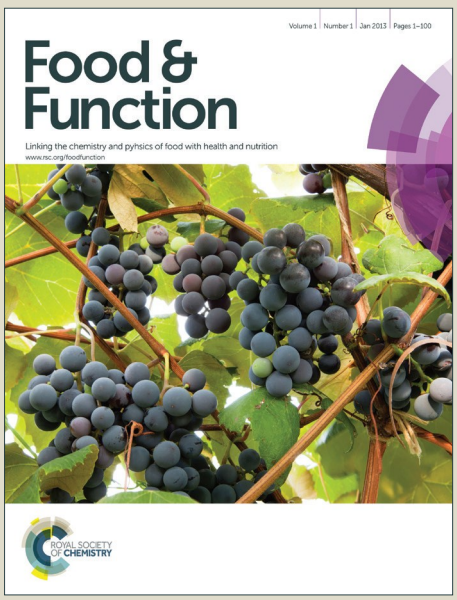

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Consumption of onion juice modulates oxidative stress and attenuates the risk of bone disorders in middle-aged and post-menopausal healthy subjects

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Abstract

Osteoporosis is a chronic inflammatory condition that is characterized by the loss of bone mineral density (BMD). The current study was undertaken to evaluate the impact of onion juice intake on the bone mineral density (BMD) and bone loss in corroboration

with antioxidant effect in human (*in vivo*) as well as inhibitory effects on the differentiation of osteoclast in the cell line (*in vitro*). For *in vitro* studies, the RAW 264.7 (osteoclast progenitor) cells were used to examine the anti-osteoclastogenic effect of onion. In the case of *in vivo* studies, twenty-four subjects were divided into two groups and advised to intake 100 mL of onion juice or placebo for 8 weeks. Anthropometric measurements and blood samples were collected at the initial, 2^{nd}, 6^{th}, 8^{th} and 10^{th} week. The result of *in vitro* studies indicated that onion extract would effectively inhibit the osteoclastogenesis and its differentiation. Significant changes in the levels of alkaline phosphatase (ALP), free radicals, total antioxidant capacity (TEAC) and various antioxidants were observed in onion administrated subjects. BMD of three postmenopausal women was also displayed mildly improved on supplementation with onion juice. Onion juice consumption showed a positive modulatory effect on the bone loss and BMD by improving antioxidant activity and thus, can be recommended for treating various bone-related disorders, especially osteoporosis.

Keywords: Osteoporosis, Onion, Antioxidation, Macrophage, Osteoclast

1. Introduction

Osteoporosis (OP) has become a worldwide health problem for the elderly population, particularly in postmenopausal women. The prevalence rate of OP in postmenopausal women is far higher than men due to loss of estrogen.[1] Approximately 1 in 2 women and 1 in 5 men older than 50 years will eventually experience osteoporotic fractures. OP is estimated to affect over 200 million people worldwide and 75 million people in Europe

and Asia.[2] OP ensues from imbalanced bone resorption (osteoclast) and bone formation (osteoblast) causing a net bone loss that may be due to hormonal imbalance, diseases, or medications.[3] Osteoclasts are belong to the lineage of monocyte/macrophage family, that begin from mononuclear form (stem cell) and end up with multinuclear form. Macrophage colony-stimulating factor (M-CSF) is a hematopoietic growth factor, involved in the proliferation, differentiation, and survival of monocytes/macrophages, and bone marrow progenitor cells.[4] Recent studies has indicated that these cells express the receptor for activation of NF-κB ligand (RANKL), which is essential for osteoclastogenesis.[5,6] The RAW 264.7 cell line (progenitors of the macrophage/osteoclast) was widely used as a model to assess the differentiation of osteoclast especially after stimulation with RANKL and M-CSF.[7]

Tremendous strides have been made in treating OP with medication classes such as bisphosphonates, synthetic estrogens, calcitonin, and parathyroid hormone derivatives.[8] Also, prolonged treatment with large doses of bisphosphonates could induce osteonecrosis of the jaw bone and atypical fractures.[9] Hence, scientist drew their attention on natural remedies with no or few adverse effects. Onion (*Allium cepa* L.) is a bulbous vegetable and is a widely used food ingredient globally, ascribed to its strong flavor and various beneficial properties.[10] Onions are classified based on their color into yellow, red and white and depending on their taste as sweet and non-sweet.[11] Onion is one of the richest sources of flavonoids (quercetin and its conjugates) and organo-sulfur compounds (S-methyl-L-cysteine sulphoxide, diallyl sulfide, alkyl sulfoxides, di-propyl trisulfide) that contribute for stronger antioxidant activities.[12] Traditionally, onion is also prescribed

for various ailments such as fever, dropsy, chronic bronchitis, colic, and scurvy. Pharmacologically, onion is recognized as an anti-asthmatic, antihypertensive, antihyperglycemic, anti-inflammatory and antioxidative agent.[13,14] Some preclinical studies proved that onion can inhibit bone resorption and differentiation of osteoclast and thereby maintaining normal bone mineral density.[15-17] However, till date no clinical studies are conducted to explore the efficacy of onion juice on risk factors of bone disorders (osteoporosis).

2. Material and Methods

2.1 Chemicals

Gallic acid, quercetin, Folin–Ciocalteu phenol reagent, sodium hydroxide (NaOH), aluminum chloride ($AlCl_3$), acetic acid and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, USA). Recombinant human RANKL and M-CSF from R&D Systems, (Minneapolis, MN, USA), RPMI 1640 and trypsin (GIBCO, NY, USA). Acid Phosphatase Kit (387-A; Sigma- Aldrich, MO, USA), iPTH intact RIA kit (Allegro; Japan Mediphysics Co, Tokyo, Japan), Randox kit (Antrim, UK). All other chemicals and solvents used in the present study were of analytical grade and highest purity.

2.2 Yellow onion juice

Yellow onion juice (commercially available) was provided by the Chou Chin Industrial Limited and its composition with placebo was summarized in table 1.

2.3 Freeze dried onion juice solid

Take one kilogram of onion juice extract and dried by a rotary evaporator (Eyela, Tokyo, Japan) under vacuum condition and frozen with nitrogen at -20˚C by the freeze drying system (FD4, Heto, Denmark) to yield 10.29 % of freeze dried onion juice (FDOJ).

2.4 Preparation of ethanolic extract of onion

Fresh yellow onion (*Allium cepa* Linn.), used for the present study were procured from local markets in Pingtung County, Taiwan. They were free of any pre-harvest chemical treatment. Onions were washed in water and chopped into uniform size (0.5 X 0.5 cm). The chopped onion was soaked in 10.5 % ethanol at 3:5 (W/V) and mixed well by blender and filtered through gauze layers and dried by a rotary evaporator (Eyela, Tokyo, Japan) under vacuum condition (removing moisture) and frozen with nitrogen at -20˚C by the freeze drying system (FD4, Heto, Denmark) to yield 5.77 % of freeze dried ethanolic extract of onion (EEO).

2.5 *In vitro* studies

2.5.1 Determination of Total phenolics and flavonoids contents

The total phenolic contents were determined by the method of Julkunen-Titto.[18] Testing sample (50 µL) with 1 mL of Folin–Ciocalteu phenol reagent were added and vigorously shaken, followed by 5 mL of 20% sodium carbonate was pipetted, and the mixture were made up to 10 mL, and they were shaken thoroughly again. After 20 min, the absorbance

of the mixture was read at 735 nm using a spectrophotometer (U-2100, Hitachi, Tokyo, Japan). Total phenolics contents were expressed as milligrams of gallic acid equivalents per liter or dry weight.

The total flavonoid contents were determined by the method described by Wang & Hwang.[19] 20 mL of methanol, and 1 mL of 5% $AlCl_3$ (w/v) were added to a testing sample (2 mL) and mixed vigorously. Then the absorbance was read at 425 nm without background measurements and using quercetin as a standard. Total flavonoid contents were expressed as milligrams of quercetin equivalents per liter.

2.5.2 Analysis of Hydrogen peroxide scavenging ability

The hydrogen peroxide scavenging ability was determined according to the method of Ruch *et al*.[20] A solution of hydrogen peroxide was prepared in phosphate buffer (pH 7.4). A sample of various concentrations (0.1-2 mg/mL) were mixed with distilled water and followed by hydrogen peroxide solution. The absorbance of hydrogen peroxide at 230 nm were determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of extract and standard ascorbic acid:

% scavenged $[H_2O_2]=[(A_0-A_1)/A0]X100$

where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample of extract and standards.

2.5.3 Cell culture studies

RAW 264.7 cells (a macrophage/osteoclast progenitor cell line obtained from the American Type Culture Collection) were grown in RPMI 1640 (GIBCO, NY, USA) supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in 5% CO_2. The media were changed twice a week. When cells reached confluence, they were detached with trypsin (0.25%; Gibco, NY, USA) and plated in 96-well cell culture plates.

2.5.4 Assay of cell viability

Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded on a 96-well plate with 100 µL (5×10^4/mL) of cells in each well and left overnight. The cells were treated with increasing concentrations of ethanolic extract of onion (EEO) and freeze dried onion juice (0.1, 0.2 and 0.4 mg/mL of DMSO) at 37˚C for 24 h in serum free medium. The medium was replaced with fresh medium (PBS), containing 0.5 mg/mL MTT, and cultured at 37˚C for 4 h. Then the culture medium was removed, and cells were dissolved in 100 µL of isopropanol and shaken for 10 min to dissolve the formazan crystals. The optical density (OD) at 570 and 630 nm was measured using a microplate (ELISA) reader after 5 min to check the purple-blue MTT formazan precipitate. The net absorbance ($OD_{570}-OD_{630}$) indicated the enzymatic activity of mitochondria and implicated the cell viability to evaluate cell toxicity of the drug. The viability (%) was plotted against the concentration of the extract.

Cell viability (%) = (A_{570}nm-A_{630}nm) sample/(A_{570}nm-A_{630}nm) control X 100%

2.5.5 Osteoclast differentiation from RAW264.7 cells (TRAP staining)

For the differentiation of osteoclasts, RAW264.7 cells (2×10^4, in a 24-well plate) were cultured in the presence of RANKL (50 ng/mL) and M-CSF (20 ng/mL) for 5 days. The culture medium was replaced every 5 days. Osteoclast formation was measured by quantifying cells that were positively stained for tartrate-resistant acid phosphatase (TRAP [Acid Phosphatase Kit 387-A; Sigma- Aldrich, St. Louis, MO, USA]) according to the manufacturer's instructions. Osteoclasts were determined to be TRAP-positive staining multinuclear (>3 nuclei) cells using light microscopy (Nikon Eclipse TE300, Tokyo, Japan). The total number of TRAP-positive cells and the number of nuclei per TRAP-positive cell in each well were counted.[7] Taking the mean of control values, we derived an individual control value as a ratio of the mean X 100, and data are expressed as percent change in the mean value of control for TRAP-positive cells.[21]

2.6 Removal of polyphenolic compounds by PVPP

The EEO and FDOJ (40 mL) were routinely treated at 20 ± 1°C with PVPP at different concentrations (0.1, 0.5, 1, 2, 3, 4 and 8 g/h) were stirred and filtration through Whatman No. 1 filter paper and centrifuged at 1200 g, to check the levels of total phenolic (quercetin) and non-phenolic contents and were confirmed by functional group analysis (sulfur content). The separated polyphenol compounds (quercetin and non-phenolic) were analyzed by MTT and TRAP strain method (as discussed earlier) to check the cell viability and the osteoclast differentiation ability of TRAP-positive cells.

2.7 *In vivo* Studies (Clinical Trial)

The current intervention was carried out in Chung Shan Medical University Hospital in accordance with the Declaration of Helsinki and subsequent revisions (ICH-GCP) and approved by the Human Research Governance Committee of the Institutional Review Board of Chung Shan Medical University Hospital, Taichung, Taiwan and registered (Approval No. CS10118). Healthy subjects were recruited by displaying posters at clinical laboratories of Chung Shan Medical University Hospital and public places. Written informed consent was obtained from all subjects before enrollment. Subjects involved in the present study were requested to avoid any medications/supplements as well as alcoholic beverages during the intervention. The exclusion criteria were a history of smoking, pregnant or lactating women, hepatic or renal dysfunction. Initially, 30 subjects were selected for the present intervention taking into consideration that minimum 30% of the subject may withdraw.

2.7.1 Experimental Study

Thirty healthy subjects (12 male and 18 female) aged between 40-80 were randomly selected for the present trial and segregated into experimental (onion juice, n=16) or placebo (control, n=14) group using a computer-generated random number lists obtained by an independent researcher (double-blind study). Each participant was prescribed to intake either 100 mL of onion juice or a placebo (either in lunch or dinner), based on Bordia[22] protocol. The sample bottle of both experimental and placebo groups were alike

in size, shape, odor and color (indistinguishable). The total duration of this experiment lasted for 11 weeks, which has been separated into three phases (fig 1). Phase I- one week (adaptation period) before intervention period, Phase II- eight weeks of intervention (experimental period) and Phase III- two weeks of stabilization period (follow-up). During the initial, 2^{nd}, 8^{th} and 10^{th} weeks (follow-up) fasting blood samples were withdrawn for biochemical assays, and anthropometric measurements were performed. From the subject's record, the average percentage intake of onion juice was 83.78% at the conclusion of the experiment. During the intervention, three female and two male from the experimental group and one male subject from the placebo group were excluded from the study (due to a personal problem and unwilling to continue due to the flavor of onion) and thus ended with 24 subjects (9 male and 15 female).

2.7.2 Blood collection

The fasting blood samples were collected in two tubes, one with EDTA coated for plasma and another tube without anticoagulant for serum preparation. Plasma was separated by centrifuging at 1500g (Supercentrifuge. 1K15, Sigma) and used for determining the various antioxidant indexes. Serum samples were used to determine the levels of minerals and parathyroid hormone (PTH). Separated blood samples after deducting the intermediate film, settling part was washed with isometric saline and centrifuged at 1500g to get erythrocytes and used for assaying antioxidant enzymes.

2.7.3 Biochemical analysis

The levels of calcium (Ca), phosphorus (P) and ALP were measured by using a commercial kit procured from the Randox kit (Antrim, UK)**.** Serum levels were measured by using a radioimmunoassay kit (intact PTH; Allegro; Mediphysics Co, Tokyo, Japan).

2.7.4 Bone mineral density

BMD was measured by Dual Energy X-ray Absorptiometry (DEXA) for post-menopausal women treated with onion and placebo. BMD was measured at the lumbar spine (L2-4) and hip region by DEXA using a LUNAR DPX densitometer (GE Lunar Corporation, Madison, WI, USA). The diagnosis of osteoporosis (OP) was based on the WHO criteria. They classified the results of BMD into four groups (normal BMD, T- is between standard deviation of +2.5 and -1.0; for osteopenia/low BMD, T-score is between -1.0 and -2.5; for osteoporosis, T-score is lower than -2.5 and for severe osteoporosis, T-score is lower than -2.5 scores with the presence of one or more fragility fractures.[23]

2.7.5 Various oxidative statuses in plasma

Trolox-Equivalent Antioxidant Capacity (TEAC) of plasma was determined by Arnao[24] and slightly modified by Miller method.[25] Briefly the absorbance against blank was determined at 734 nm as a function of extract concentration, and the scavenging percentage of $ABTS^{+}$ was calculated relative to Trolox (a water-soluble analogue of vitamin E adopted as an antioxidant standard) and expressed as mM in plasma.

GSH content in plasma was determined by the method of Halliwell and Gutteridge[26] with slight modification. To 250 µL of plasma add, 250 µL of 10% TCA and centrifuged (3000g at 4˚C). Adding 850 µL of phosphate buffer containing 0.1M sodium phosphate 5 mM EDTA, pH 8.0 to 100 µL of supernatant and combined with 50 µl of o-phthaldialdehyde reagent at room temperature. After 15 minutes, the absorbance was read at 350nm (excitation wavelength) and 420nm (emission wavelength). The GSH contents were expressed as µM in plasma.

2.7.6 Antioxidant enzymes in erythrocytes

Assay of glutathione peroxidase (GPx) and glutathione reductase (GR) were performed by using a commercial kit procured from the Randox kit (Antrim, UK)**.** Protein contents of RBCs were determined based on the Biuret reaction of the BCA kit from Thermo Fisher Scientific (Illinois, USA).

2.7.7 Determination of oxidative indexes in plasma

The total free radical contents superoxide anion were determined by the method of Zhou et al[27] and Lu et al[28] respectively, with some modification by adopting the chemiluminescence analysis (Ultrasensitive Chemiluminescence Analyzer, CLA-FS1, Tohoku, electronic industrial Co, Sendai, Japan). Amplifier luminol and lucigenin were used to detect the total free radicals and superoxide anion in the plasma. In brief, 100 µl of plasma were added to 100 µl of phosphate buffered saline and placed in the magnetic stirring reactor and analyzed by chemiluminescence analyzer, with the addition of 1mL

luminol and lucigenin respectively, for every 10 sec measure the photon emission (intensity) for 480 seconds. Calculate the total area under a curve minus the background intensity for each sample and CL were expressed as 10^3counts/10 sec.

2.8 Statistical analysis

The values were expressed as a mean ± standard deviation (S.D). The Paired t-test was used to compare the difference in the same group, and the Student's t-test was employed to compare the difference between the experimental and control groups (*in vivo*). *In vitro* test results achieved by One-way ANOVA. Both *in vitro* and *in vivo* results were analyzed by using software (SPSS version 17.0 for Windows; SPSS Inc., USA). All statistical results with a *p*-value less than 0.05 showed statistically significant. The results were the mean values of three replicates of the same sample.

3. Results

3.1 *In vitro* studies

3.1.1 The total phenolics and flavonoids contents in EEO and FDOJ

Table 2 showed the total phenolics and flavonoids contents in the ethanolic extract of onion and freeze dried onion juice. The total phenolics contents as estimated by Folin–Ciocalteu assay displayed that FDOJ group has the highest value of 0.28 ± 0.12 mg GAE/dry wt. in comparison with the EEO group that exhibits only 0.18 ± 0.03 mg

GAE/dry wt**.** In the case of total flavonoids contents, both EEO and FDO group showed similar results of 0.08 ± 0.01 and 0.06 ± 0.01 mg QE/dry wt.

3.1.2 Free radical scavenging activity in EEO and FDOJ

Antioxidants are chemicals that block the activity of other chemicals known as free radicals. The hydrogen peroxide (H_2O_2) radical scavenging ability of an ethanolic extract of onion and freeze dried onion juice were depicted in figure 2. EEO groups increased hydrogen peroxide radical scavenging activity, as the concentration increased and were near to standard (ascorbic acid). When concentrations reached to 0.20 mg/mL, EEO exhibited the maximum hydrogen peroxide radical scavenging capacity by 37.67%. However, the hydrogen peroxide radical scavenging capacity of FDOJ was only 5.68%.

3.1.3 Cell culture studies

Osteoclasts are belong to the lineage of monocyte/macrophage family that differentiate from mononuclear form (hematopoietic stem cells) to multinuclear form. During the present study RAW 264.7 a macrophage cell line (osteoclast progenitor cell), were used to evaluate the effects of EEO and FDOJ on TRAP-stained cells exposed with RANKL and M-CSF. Light microscopic appearance of RAW 264.7 cells, which were co-cultured in the presence of RANKL (50 ng/mL) and M-CSF (20 ng/mL) for five days to form a large multinucleated osteoclast. It was are characterized by phenotypic markers such as tartrate-resistant acid phosphatase (TRAP) as shown in figure 3. Mononuclear osteoclast was fused together to form multinucleated osteoclast, which were shown in dotted circle

(3A-100X) and a magnified form of multinucleated osteoclasts (3B-200X) as well as the effect of different concentration of EEO and FDOJ on the formation of multinuclear (fig. 4).

3.1.4 Cell viability (MTT Assay)

Effect of EEO and FDOJ on the viability of RAW 264.7 cells was displayed in figure 5. RAW 264.7 cell viability was not altered by various concentrations of EEO and FDOJ (0.1, 0.2 and 0.4 mg/mL). Slight improvement in cell viability was observed for EEO and FDOJ at concentrations 0.1 (128, 123%) and 0.2 mg/mL (108, 111%), in comparison with control cells (100%), but no significant difference were noted within or between the groups. Effect of PVPP treated onion extract on cell viability of RAW 264.7 cells was displayed in figure 6. RAW 264.7 cell viability was not altered by various concentrations of PVPP treated onion extract. Slight decrements in cell survival rate (cell viability) were observed for quercetin (Q), non-phenolic (NP) and quercetin + non-phenolic (Q+NP) group (91, 94.6 and 89.7%) respectively. In comparison with control cells (100%), no significant difference were noted within or between the groups.

3.1.5 Cell differentiation (TRAP stain)

Figure 7 illustrated the effect of EEO and FDOJ on the differentiation of RAW 264.7 cells (osteoclasts). Different concentrations 0.1 (68, 77%), 0.2 (46, 44%) and 0.4 (25, 19%) mg/mL of EEO and FDOJ showed significant difference ($p < 0.05$) over control

cells (100%). As the concentration increases, the differentiation rate of osteoclast (RAW 264.7) started to decline. Effect of phenolics in onion extract on the differentiation of RAW 264.7 cells were elaborated in figure 8. Q (quercetin; quercetin contents in 0.4 mg/mL of FDJO), NP (non-phenolics; PVPP treated in 0.4 mg/mL of FDJO) and Q+NP (30.79, 39.8 and 31.9%) concomitantly lowered ($p < 0.05$) the differentiation rate of osteoclasts (RAW 264.7) cells. The above results suggest that onion extract (EEO, FDOJ, Q, and Q+NP) directly inhibited RANKL-induced osteoclastogenesis.

3.2 *In vivo* Studies (Clinical Trial)

Thirty healthy subjects (12 male and 18 female) were randomly recruited and segregated into experimental (onion juice, n=16), and placebo (control, n=14) group. Each participant was requested to drink either 100 mL of onion juice or placebo. At the initial, 8^{th} and 10^{th} week body weight and BMI were noted. Table 3 showed the effect of onion juice on anthropometric parameters in healthy subjects. No significant changes were noted in body weight and BMI at initial and follow-up (10^{th} week) period, but on the 8^{th} week (during intervention) a concomitant decrease in body weight and BMI were noted. Moreover, onion juice consumed subjects did not show any abnormal symptoms or phenomena.

3.2.1 Biochemical analysis

The effect of onion juice on various biochemical parameters in healthy subjects were illustrated in table 4. The levels of PTH, bone minerals such as Ca, P depicted no marked alteration in onion consumed subjects, whereas the levels of ALP were substantially lower in onion treated groups especially in the 8th week than the initial and follow-up period.

3.2.2 Oxidative indexes and antioxidant status

The effect of onion juice on oxidative indexes and antioxidant status (enzymes) in healthy subjects were epitomized in table 5 and 6. The total free radicals and superoxide anion levels were significantly elevated at the initial stage, but the treatment for 8 weeks with onion juice concomitantly lowered the levels of total free radicals and superoxide anion (Table 5). The total antioxidant capacity and various enzymic antioxidants (GSH, Gpx, and GR) levels were also altered during the intervention. Intake of onion juice for 8 weeks revealed a pronounced elevation in the levels of total antioxidant capacity and glutathione content on compared to initial or baseline period. No significant alterations were observed in the case of antioxidant enzymes such as GPx and GR. In the placebo group, no significant alterations were noted in any of the above-discussed parameters.

3.2.3 DEXA analysis and oxidative indexes

A DEXA test is to measures the BMD and compares it to that of an established norm or standard to give a T-score. Table 7 elaborated the effect of onion juice consumption on

various oxidative indexes BMD (T-score) by DEXA in three postmenopausal women. The levels of BMD (T-score) of the lumbar, right and left hip were mildly changed from initial to 8th week. ALP was significantly decreased after drinking 8 weeks of onion juice. It also demonstrated the effect of onion juice consumption on oxidative indexes of three postmenopausal women. The levels of antioxidant enzymes (GSH and GR) were increased from the initial to 8th week. Marked declines in the levels of free radicals were observed at 8th week, compared to baseline (initial).

4. Discussion

The current study was designed to assess the effect of onion juice intake on the bone mineral density and bone loss in corroboration with antioxidant effect in human study (*in vivo*) as well as inhibitory effects on the differentiation of osteoclast in cell line model (*In vitro*). Preliminary phytochemical analysis clearly inferred that phenolic and flavonoids were rich in both extracts (EEO and FDOJ). Since the phenolic and flavonoids content were slightly higher in EEO than FDOJ, therefore EEO displayed better hydrogen peroxide scavenging ability than FDOJ. Numerous studies had demonstrated that phenolics and flavonoids molecules, probably acted as an electron (e^-) or proton (H^+) donor and could, terminate radical chain reactions by converting free radicals to more stable products.[29]

The MTT assay (viability) results apparently proved that EEO, FDOJ, and PVPP treated onion extract did not induce cytotoxicity even at higher concentrations, which also depicted that onion extract, could not trigger apoptosis. Our outcome were in

concordance with Tang *et al*.[30] who proved that different concentrations of onion powder did not alter the cell viability (survival rate) of RAW 264.7 macrophage cells, even at elevated concentrations. Tartrate-resistant acid phosphatase (TRAP or TRAPase), also called acid phosphatase 5, tartrate resistant (ACP5), is a glycosylated monomeric metalloprotein proenzyme expressed in mammals and efficiently participate in osteoclast-mediated bone turnover.[31] Osteoclast formation (osteoclastogenesis) and differentiation were measured by quantifying the positively stained cells for tartrate-resistant acid phosphatase. During the initial phase of osteoclast proliferation in RANKL and M-CSF alone treated cells trigger the ROS formation with the production of cytokines. Those ROS not only directly promote osteoclastogenesis but also induce apoptosis.

Both EEO and FDOJ (0.4 mg/mL) showed the perfect inhibition on cell differentiation rate than other concentrations (0.1 and 0.2 mg/mL). Q and Q+NP showed similarly and better inhibition on RAW 264.7 cells differentiation than NP group. The results obviously revealed that phenolic and non-phenolics in the FDOJ could directly inhibit RANKL-induced osteoclastogenesis and quercetin act as an important contributor. Quercetin effectively slows down the bone resorption by inhibiting the differentiation of osteoclast progenitor cells into mononuclear osteoclasts and by the disruptive effect on actin rings in mature osteoclasts.[21,32] Tang and his coworkers[30] had suggested that the inhibitory effect of onion powder on osteoclast differentiation into mature osteoclasts may be responsible for the regulation of phosphorylation of ERK and p38 as well as inhibiting NF-κB activation in RANKL-induced osteoclastogenesis. Several reports suggested that oxidative stress is an important contributor for various bone disorders.[33] Based on *in vitro*

results (anti-osteoclastogenic effect) with the several reference data, we framed a clinical trial to investigate the impact of onion juice on various risk factors related to bone disorders, especially OP.

Onion juice consumption for 8 weeks significantly reduced the body weight and BMI levels to a certain extent due to its anti-obesity and estrogenic effect of onion.[34] In the case of various biochemical parameters such as PTH, calcium and phosphorus did not exhibit any marked change between an onion and a placebo group, it might be due to short intervention period. ALP activities are directly correlated with the degree of bone disorders, especially demineralization and can be considered as markers of bone loss.[7] ALP levels were substantially lowered in onion juice drinking subjects as compared with the placebo-treated subjects. Serum ALP activities were significantly decreased when treating with antler extract of safflower combined with estrogen in osteoporosis-induced rats.[35] Our data were in corroborated with Block *et al*.[36] who also showed that administration of cinacalcet can effectively lower the ALP level in elderly patients.

Oxidative stress is characterized by an increased levels of reactive oxygen species (ROS) that disrupts the intracellular reduction–oxidation (redox) balance. Epidemiological evidence in pre-clinical and clinical studies indicates that aging process is directly associated with elevated ROS generation (oxidative stress) and osteoclastogenesis.[37,38] Treatment with onion juice for 8 weeks concomitantly lowered the levels of total free radicals and superoxide anion by increasing endogenous antioxidants attributed to antioxidant activities.[12,39]

Human defense mechanism is directly regulated by an antioxidant system that compressed of endogenous (superoxide dismutase, glutathione, glutathione peroxidases. glutathione reductase) and exogenous (vitamin A, C, E and various photo components), antioxidants which play a pivotal role in scavenging free radicals and thereby avoiding oxidative stress. Drinking 100 mL of onion juice every day for 8 weeks posted a concomitant escalation in the levels of total antioxidant capacity and glutathione content. No significant changes were observed in case of antioxidant enzymes such as GPx and GR in all subjects, but in three postmenopausal women the levels of antioxidant enzymes (GSH and GR) were increased as well as significant declines in the levels of free radicals were noted at 8th week when compared to the initial stage. Similarly, Sánchez-Rodríguez,[40] also indicated that supplementation with ascorbic acid and alpha-tocopherol positively lower the bone mineral density loss in an elderly person, due to antioxidant activity. Flavonoids in onion also involve indirect activation of nuclear transcription factors (e.g., Nrf2) which regulates the expression of genes encoding for antioxidant enzymes, especially glutathione.[41]

Estrogen plays a significant role in maintaining the balance between osteoclasts and osteoblasts and thus maintains the bone mineralization and remodeling (turnover) process.[42] Several studies show that BDM and T-score value of postmenopausal women are significantly altered, when compared to menopausal and healthy men.[1] The levels of BMD and T-score of the lumbar, right and left hip were mildly improved after 8 weeks of supplementation with onion juice in postmenopausal women. The presence of rutin,

myricetin, quercetin, several phytoestrogens (coumestrol, zearalenol, isoflavones, humulone), and vitamins (K and C) in onion might contribute to suppressing the osteoclast formation and differentiation.[43,44] Thus, trigger the synthesis of osteoblast and thereby normalizing the bone mineralization and remodeling process. [45]

Limitation of the present study, since it is a pilot study, we checked the beneficial effects of onion juice on various risk factors for bone disorders in healthy elderly subjects (instead of osteoporotic) for a short duration (10 weeks) and hence we did not encounter a significant change. Usually for bone re-modulation (turnover), it takes a quite longer period and thus in the future a long-term clinical trial with a large number of osteoporotic subjects will give an exact picture of the beneficial effects of onion juice. Extension of this work was recently initiated, to overcome the above-discussed limitation to check the beneficial effects of onion juice.

5. Conclusion

In vitro studies indicated that onion extract would effectively inhibit the osteoclast differentiation and formation (osteoclastogenesis), due to the presence of flavonoids (quercetin, rutin, myricetin) and phytoestrogens. Significant alterations in the levels of alkaline phosphatase (ALP), free radicals, total antioxidant capacity (TEAC) and various antioxidants were observed in onion administrated subjects. BMD of three postmenopausal women also displayed mildly improved on supplementation with onion juice. Onion juice consumption showed a positive effect on the bone loss and BDM by

improving the antioxidant activity. Therefore, we probably recommended onion for treating various bone-related disorders especially osteoporosis.

Author Contributions

YYL, YCS, KV and CKW conceived and designed the study protocol. HFC, YCS, and HHL helped in conducting the clinical trial. CKW and KV collected data and wrote the manuscript. All authors read and approved the final manuscript.

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Figure Legends

Fig. 1 Experimental design of the clinical trial. Phase I- one week (Adaptation period) before intervention period, Phase II- eight weeks of Intervention (Experimental period) and Phase III- two weeks of monitoring period (Follow-ups).

Fig. 2 The scavenging efficiency on hydrogen peroxide in ethanolic extract of onion and freeze dried onion juice (solid). Values were expressed as means ± SD. Data bearing different letters were significantly different (p<0.05).

Fig. 3 Light microscopic appearance of RAW 264.7 derived mononuclear osteoclasts. RAW 264.7 cells were co-cultured with RANKL (50 ng/ml) and M-CSF (20 ng/ml) and stained for tartrate-resistant acid phosphatase (TRAP). Mononuclear osteoclast was fused together to form multinucleated osteoclast, which are shown in a dotted circle (A-100X) and a magnified form of multinucleated osteoclasts (B-200X).

Fig. 4 Effect of ethanolic extract of onion and freeze dried onion juice on the formation of TRAP-positive cells using light microscope. RAW 264.7 cells alone (A), RANKL (50 ng/ml) + M-CSF (20 ng/ml) alone (B), RANKL + M-CSF + 0.1 mg/ml of EEO (C), RANKL + M-CSF + 0.1 mg/ml of FDOJ (D), RANKL + M-CSF (20 ng/ml) + 0.2 mg/ml

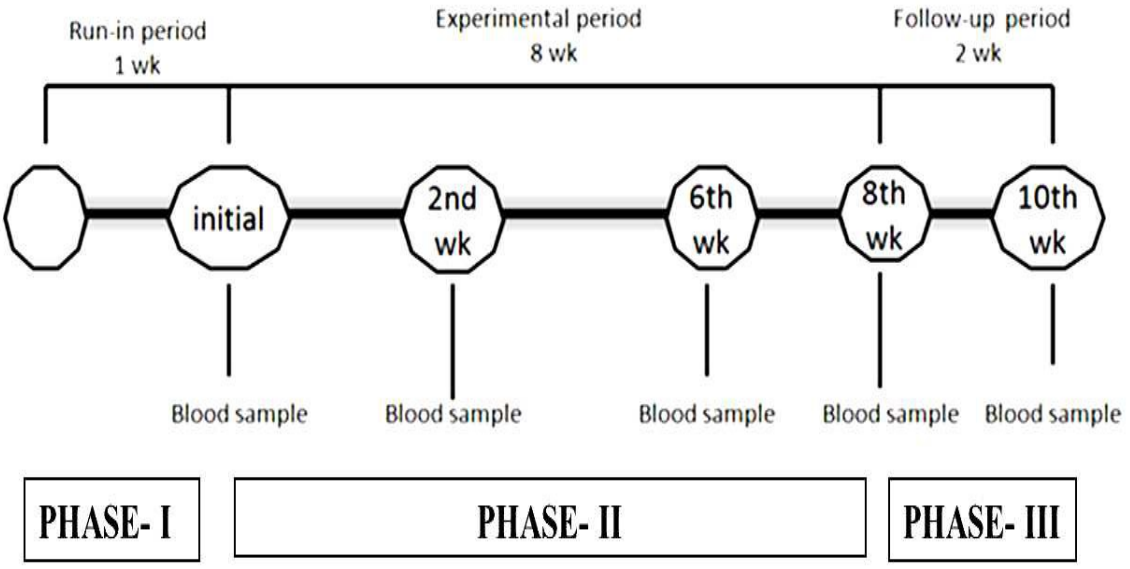
of EEO (E), RANKL + M-CSF + 0.2 mg/ml of FDOJ (F), RANKL ++ M-CSF + 0.4 mg/ml of EEO (G), RANKL ++ M-CSF + 0.4 mg/ml of FDOJ (H). (100X)

Fig. 5 Effect of ethanolic extract of onion and freeze dried onion juice on the cell viability. Values were expressed as means ± SD. Data bearing different letters were significantly different (p<0.05).

Fig. 6 Effect of phenolic and non-phenolics compounds on cell viability. Concentration of quercetin: 31.44 μM. Values were expressed as means ± SD. Data bearing different letters were significantly different (p<0.05).

Fig. 7 Effect of different concentrations of ethanolic extract of onion and freeze dried onion juice on the formation of TRAP-positively stained cells during differentiation. Values were expressed as means ± SD. Data bearing different letters were significantly different (p<0.05).

Fig. 8 Effect of phenolic compounds and phenolics-free compounds on the formation of TRAP-positively stained cells during differentiation. Concentration of quercetin: 31.44 μM. Values were expressed as means ± SD. Data bearing different letters were significantly different (p<0.05).



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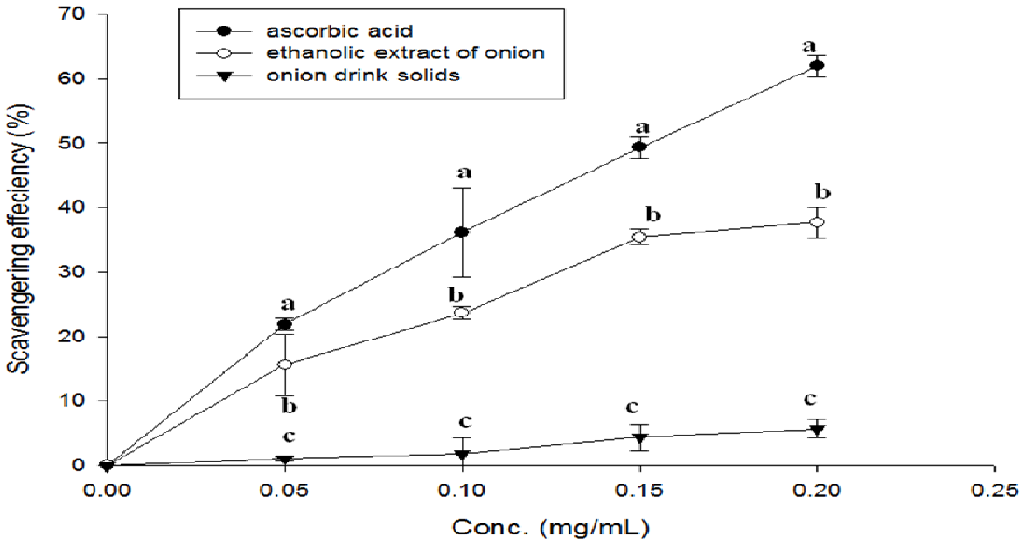

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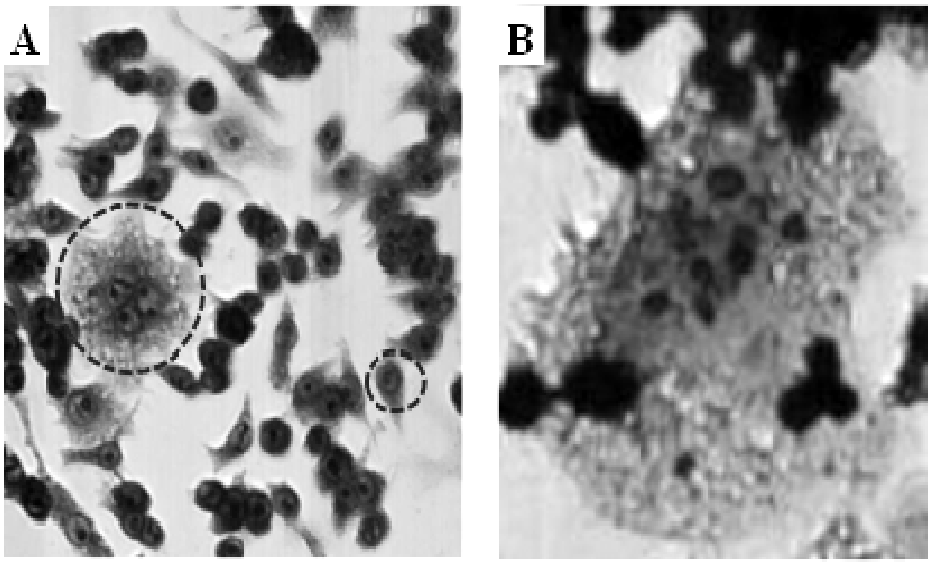

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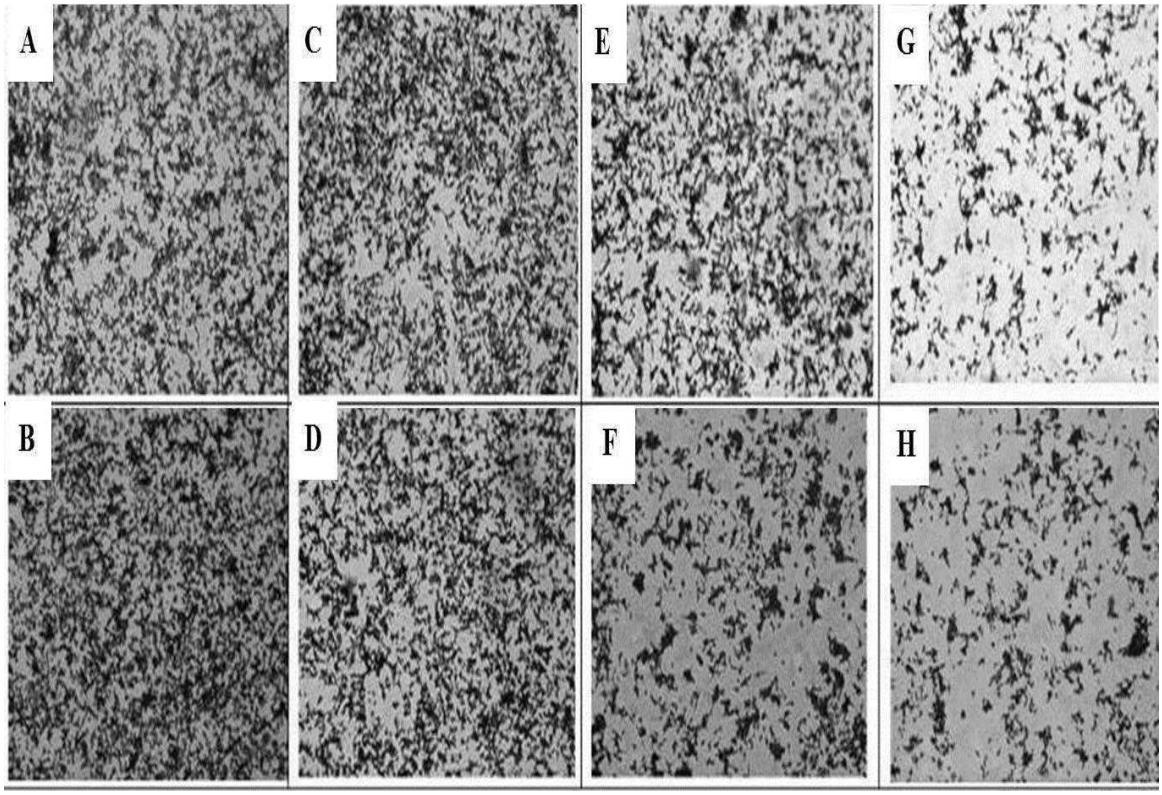

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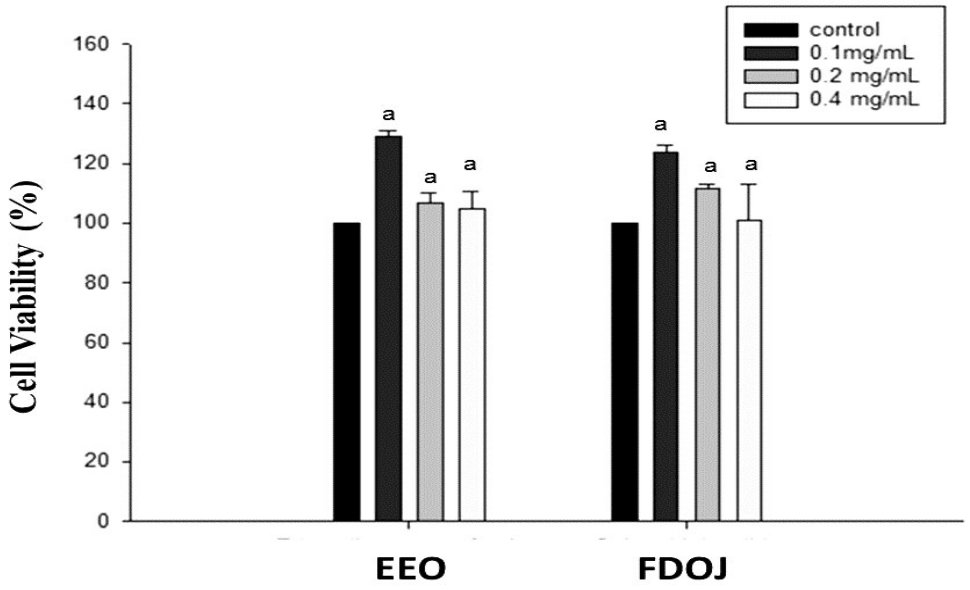

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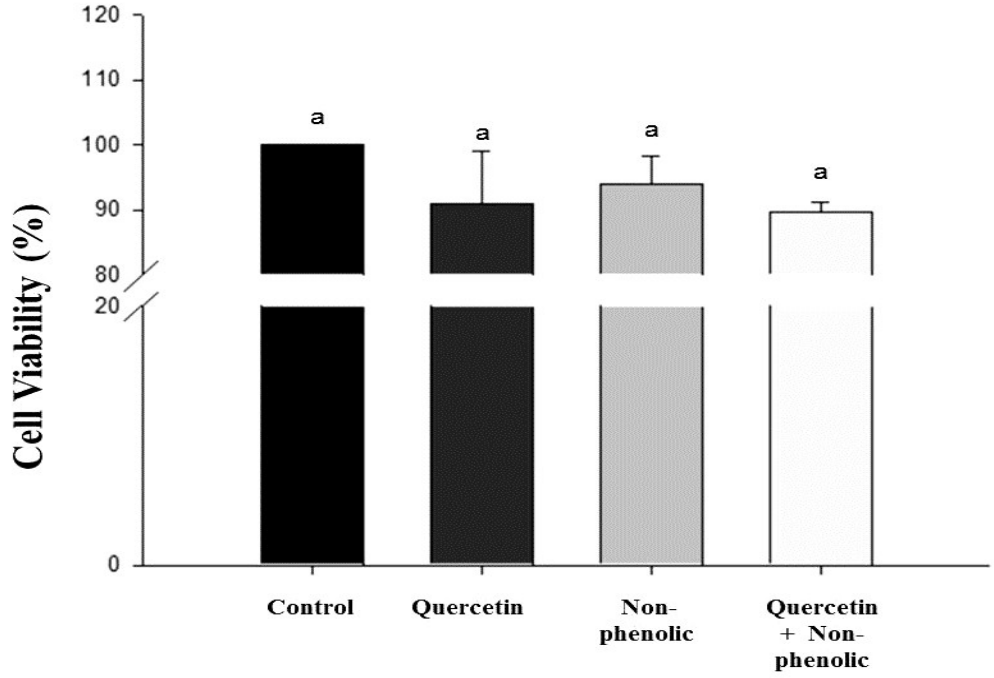

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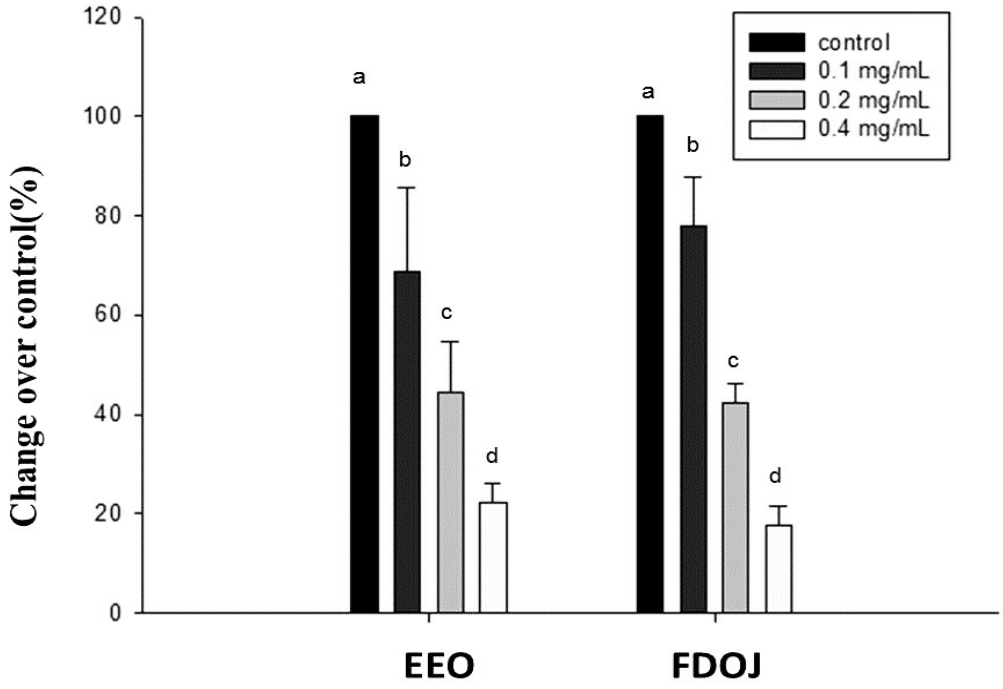

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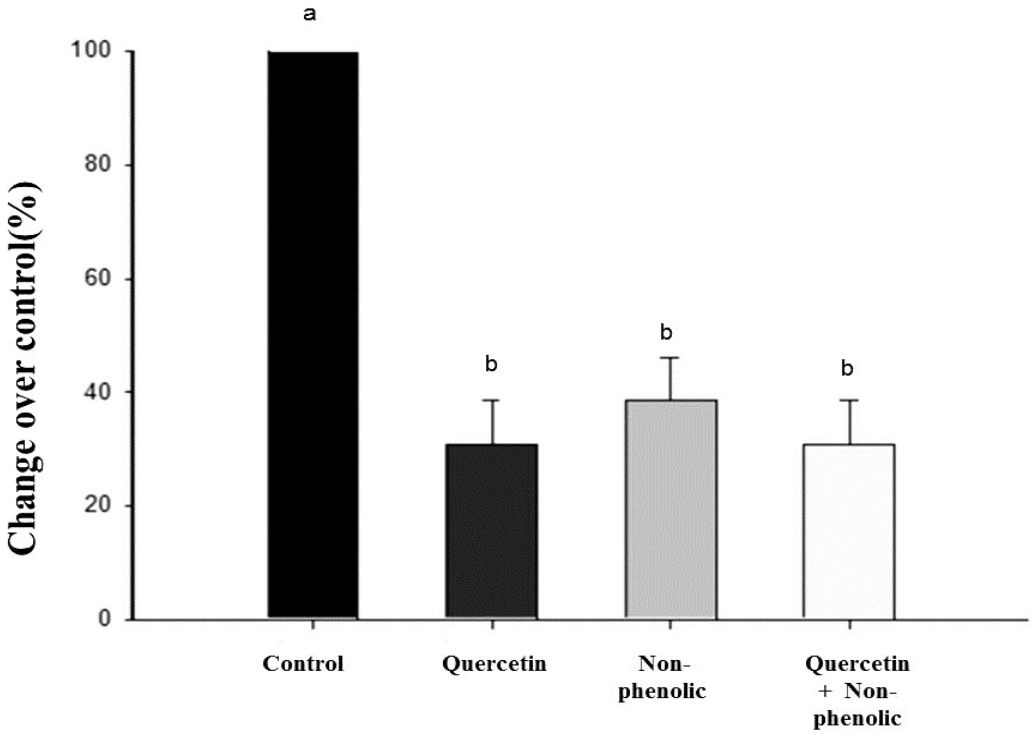

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Table 1. Composition of onion juice (Exp group) and placebo (Control group)

	Onion Juice (Exp)	Placebo (Control)
Onion Juice (%)	50	-
Onion washing water[1] (%)	-	20
Sugar (%)	9.72	10
Citric acid (%)	0.28	0.28
Caramel (%)	-	0.04
Water (%)	40	69.68

[1] Onion washed water: providing onion's flavor (Yellow onion)

Table 2. The total phenolics and total flavonoids contents in ethanolic extract of onion and freeze dried onion juice

	Ethanolic extract of onion	Dried onion juice
Total phenolics	0.18±0.03 [b]	0.28±0.12[a]
Total flavonoids	0.08±0.01 [a]	0.06±0.01 [b]

mg gallic acid equivalent /g dry wt.

mg quercetin equivalent /g dry wt.

Values were expressed as means ± SD. Data within the same column of each group sharing different superscript letters were significantly different (p<0.05).

Table 3. The effect of onion juice on anthropometric parameters in healthy subjects

	Group	Weight (Kg)	BMI[1](Kg/m^2)
Initial	Exp	68.60 ± 9.14[a]	24.73 ± 1.62[a]
	Placebo	65.30 ± 15.97[a]	25.68 ± 3.47[a]
8th week	Exp	68.10 ± 9.14[b]	24.24 ± 1.59[b]
	Placebo	65.43 ± 15.53[a]	25.74 ± 3.26[a]
Follow Up[2]	Exp	68.50 ± 9.04[a]	24.61 ± 1.50[a]
	Placebo	68.78 ± 15.55[a]	25.87 ± 4.23[a]

[1] BMI：body mass index

[2]F/U：follow up period, two weeks after the end of the experimental period.

Values were expressed as means ± SD. Data within the same column of each group sharing different superscript letters were significantly different (p<0.05).

Table 4. The effect of onion juice on various biochemical parameters in healthy subjects

	Group	PTH (pg/mL)	Ca (mg/dL)	P (mg/dL)	ALP (IU/L)
Initial	Exp	42.3 ± 8.3[a]	9.50 ± 0.1[a]	3.40 ± 0.4[a]	64.50 ± 10.5[a]
	Placebo	45.0 ± 14.8[a]	9.80 ± 0.4[a]	3.50 ± 0.6[a]	57.60 ± 10[a]
8th	Exp	39.3 ± 13.9[a]	9.50 ± 0.3[a]	3.50 ± 0.3[a]	56.60 ± 9.1[c]
week	Placebo	49.10 ± 13.9[a]	9.70 ± 0.3[a]	3.60 ± 0.6[a]	58.10 ± 8.5[a]
Follow	Exp	42.6 ± 12.9[a]	9.50 ± 0.2[a]	3.50 ± 0.3[a]	59.40 ± 10.9[b]
Up[1]	Placebo	41.70 ± 12.1[a]	9.60 ± 0.4[a]	3.70 ± 0.6[a]	58.10 ± 5.0[a]

[1]F/U：follow up period, two weeks after the end of the experimental period.

Values were expressed as means ± SD. Data within the same column of each group sharing different superscript letters were significantly different (p<0.05).

Table 5. The effect of onion juice on oxidative indexes in healthy subjects

Group	Exp	Placebo
Total free radical[1]		
Initial	11.20±8.72[a]	13.20±8.72[a]
8[th] wk	7.46±2.58[b]	15.18±4.39[a]
[2]F/U	10.90±3.64[a]	14.60±2.19[a]
Total superoxide anion[1]		
Initial	5.39±3.83[a]	7.72±3.70[a]
8[th] wk	3.99±1.34[b]	7.74±1.73[a]
[2]F/U	5.67±1.79[a]	7.67±3.86[a]

[1] Chemiluminescence level (10^3 counts / 10 sec)

[2]F/U：follow up period, two weeks after the end of the experimental period.

Values were expressed as means ± SD. Data within the same column of each group sharing different superscript letters were significantly different (p<0.05).

Table 6. The effect of onion juice on antioxidant status in healthy subjects

	Group	TEAC (mM)	[1]GSH (μM)	GPx (IU/gHb)	GR (IU/gHb)
Initial	**Exp**	0.73 ± 0.02^{a}	3.04 ± 0.46^{a}	52.87 ± 8.41^{a}	14.75 ± 4.38^{a}
	Placebo	0.74 ± 0.02^{a}	3.74 ± 1.40^{a}	63.48 ± 9.00^{a}	15.57 ± 1.96^{a}
8th	**Exp**	0.85 ± 0.01^{b}	4.30 ± 1.30^{b}	54.10 ± 7.55^{a}	15.32 ± 3.59^{a}
Week	**Placebo**	0.75 ± 0.02^{a}	3.53 ± 1.13^{a}	63.34 ± 4.60^{a}	15.45 ± 4.00^{a}
Follow	**Exp**	0.84 ± 0.02^{b}	4.68 ± 1.66^{b}	52.60 ± 7.33^{a}	14.69 ± 1.57^{a}
Up[2]	**Placebo**	0.76 ± 0.02^{a}	3.94 ± 0.85^{a}	62.74 ± 6.49^{a}	15.33 ± 3.24^{a}

[1]GSH：glutathione

[2]F/U：follow up period, two weeks after the end of the experimental period.

Values were expressed as means ± SD. Data within the same column of each group sharing different superscript letters were significantly different (p<0.05).

Table 7. The effect of onion juice on various oxidative indexes and Bone mineral density (T-score) by DEXA on three postmenopausal women

	Case 1		Case 2		Case 3	
Age (year)	**70**		**60**		**60**	
	Initial	8th wk	Initial	8th wk	Initial	8th wk
Weight (kg)	57.4	58.4	60.4	59.3	62.7	63.2
BMI (kg/m^2)	24.2	24.6	23.9	23.5	25.4	25.6

Lumbar						
BMD (g/cm^2)	1.184	1.173	1.111	1.097	1.013	1.009
T-score	-0.1	-0.2	-0.7	-0.8	-1.2	-1.7
Left hip						
BMD (g/cm^2)	0.841	0.894	0.984	0.997	1.085	1.074
T-score	-1.3	-1.7	-0.1	0	0.7	0.6
Right hip						
BMD (g/cm^2)	0.776	0.776	1.048	1.054	1.076	1.074
T-score	-1.9	-1.9	0.4	0.3	0.6	0.6
ALP (IU/L)	59.40	56.12	58.95	55.23	57.12	53.23
GSH[1] (μM)	3.1	2.93	2.38	3.22	3.43	4.45
GR (IU/gHb)	12.7	16.4	9.23	9.13	12.11	14.98
Total Free Radicals[2]	9.58	4.85	10.34	8.13	18.24	17.25

[1]GSH：glutathione

[2] Chemiluminescence level (10^3 counts / 10 sec)