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Evidence for anti-inflammatory and antioxidative properties of dried plum polyphenols in macrophage RAW 264.7 cells

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This study presents the anti-inflammatory and antioxidative properties of dried plum (*Prunus domestica* L.) polyphenols in macrophage RAW 264.7 cells. We hypothesized that dried plum polyphenols have strong anti-inflammatory and antioxidant properties against lipopolysaccharide (LPS)-induced production of the pro-inflammatory markers, nitric oxide (NO) and cyclooxygenase-2 (COX-2), and the lipid peroxidation product, malondialdehyde, in activated macrophage RAW 264.7 cells. To test this hypothesis, macrophage RAW 264.7 cells were stimulated with either 1 μg/ml (for measurement of NO production) or 1 ng/ml (for measurement of COX-2 expression) of LPS to induce inflammation and were treated with different doses of dried plum polyphenols (0.0, 0.1,1,10,100 and 1000 μg/ml). Dried plum polyphenols at a dose of 1000 μg/ml was able to significantly ($P < 0.05$) reduce NO production by 43%. Additionally, LPS-induced expression of COX-2 was significantly ($P < 0.05$) reduced by 100 and 1000 μg/ml dried plum polyphenols. To investigate the antioxidant activity of dried plum polyphenols, macrophage RAW 264.7 cells were stimulated with 100 μg/ml of FeSO4 + 1mM/ml of H_2O_2 to induce lipid peroxidation. Dried plum polyphenols at a dose of 1000 μg/ml showed a 32% reduction in malondialdehyde production. These findings indicate that dried plum polyphenols are potent anti-inflammatory and antioxidative agents *in vitro*.

1. Introduction

The bone-protective properties of dried plums (*Prunus domestica* L.) have been demonstrated in ovariectomized,[1-6] orchidectomized,[7,8] and aging rodent models,[9] as well as in postmenopausal women.[10-12] These beneficial effects, in part, have been attributed to their polyphenolic content. Collectively, the polyphenols present in dried plums have one of the highest oxygen radical absorbance capacity (ORAC) values among the most frequently consumed fruits and vegetables.[13,14] Dried plum polyphenols have been shown to stimulate osteoblast activity through enhanced alkaline phosphatase activity, calcified nodule formation, and type I collagen cross-linking.[15] These alterations were reported to be mediated by the up-regulation of runt-related transcription factor (Runx2), osterix, and insulin-like growth factor-I (IGF-I). In addition to their ability to stimulate bone formation, dried plum polyphenols decrease bone resorption by osteoclasts through the down-regulation of the receptor activator of nuclear factor kappa-B ligand (RANKL) expression by osteoblasts thereby inhibiting osteoclastogenesis from osteoclast precursors, i.e. macrophages and monocytes.[8,16] Although the mechanism of action by which dried plum polyphenols exert their bone-protective properties has been evaluated using osteoblasts, there is only one known study that has investigated the bone-protective properties of dried plum polyphenols under normal, inflammatory, and oxidative stress conditions through their anti-inflammatory properties in osteoclasts.[17] The production of pro-inflammatory molecules can disrupt normal bone remodelling[15,18-20] and chronic inflammation has been associated with progressive bone loss and microarchitecture deterioration[21-23] through excessive production of pro-inflammatory cytokine mediators, e.g. tumor necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2),[18] interleukin-1β[24] and products of oxidative stress[25]. Many different types of polyphenolic compounds, such as flavonoids[26,27] and caffeic acid derivatives,[28] founds in fruits and vegetables are capable of scavenging reactive oxygen species[29,30] and down-regulating inflammatory mediators.[31] These studies suggest that plant-based foods, especially those

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rich in polyphenolic compounds with potent anti-inflammatory and antioxidative properties, may inhibit osteoclastogenesis and osteoclast activity.[32] Hence, the hypothesis of this study was that dried plum polyphenols would prevent the production of pro-inflammatory molecules and oxidative markers under inflammatory conditions *in vitro*. To test this hypothesis, the effects of dried plum polyphenols on nitric oxide (NO), COX-2 and malondialdehyde production under inflammatory and oxidative conditions in macrophage RAW 267.4 cells were evaluated.

2. Materials and Methods

2.1. Polyphenol Extraction

Dried plums were provided by the California Dried Plum Board. Dried plum polyphenols were extracted according to a method described by Kim and Lee[33] with some modification. Briefly, dried plums were chopped and freeze-dried to form a powder. Polyphenols were extracted from dried plum powder (10 g) by 100 ml of 80% ethanol (v/v) in an ultrasonic bath and sonicated for 20 minutes at room temperature, subdued light with continual nitrogen gas purging. The mixture was filtered through Whatman no.2 filter paper (8 µm, Florham Park, NJ, USA) by vacuum suction. The residue was re-extracted twice and all the filtrates were transferred to a 1000-ml round-bottom evaporating flask with additional 50 ml of 80% ethanol (v/v). Ethanol was evaporated in a rotary evaporator (Buchi, Flawil, Switzerland) under a vacuum at 62ºC until the volume of extract was reduced to 10-30 ml. After the ethanol was completely removed, the crude extract was freeze dried and stored at -20ºC for future use. Total polyphenols were detected by Folin Ciocalteu's reagent (Sigma, St Louis, MO, USA) using gallic acid (TCI America, Portland, OR, USA) as a standard, described by Kim *et al*.[33] The polyphenolic sample was compared to the gallic acid standard curve and content was expressed as mg gallic acid equivalents (GAE)/g of extract.

2.2 Quantification of Dried Plum Polyphenols

High performance liquid chromatography (HPLC) coupled with a photodiode array detector (DAD; Waters 996, Sydney, Australia) was used to quantify the major polyphenols present in total polyphenols extracted from dried plum. The HPLC system consisted of an autosampler (Waters 717 plus) and Waters 600 Solvent Delivery system and equipped with the Empower Software. The polyphenols were eluted using a reversed phase column, Alltech C18 (5 µm; 250 × 4.6 mm, Alltech, Sydney, Australia) with a mobile-phase gradient program at a flow rate of 1 mL•min–1. UV spectra of the different compounds were recorded with a diode array detector. Compounds were identified by comparison with authentic standards and UV/vis spectra. The results are reported as parts per million (ppm).

2.3 Cell Culture

Macrophage RAW 264.7 cells were purchased from ATCC (Manassas, VA, USA). Cells were maintained at 37°C, 5% CO_2 in Dulbecco Modified Eagle's Medium (DMEM; ATCC; Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; ATCC; Manassas, VA, USA), 100 U/ml penicillin and 100 µg/ml streptomycin. Medium was changed every 3 days.

2.4 Cell Viability Assessment

Cells were seeded in 96-well plates at a density of 2 × 104 cells per well. After 24 hours, cells were treated with grading doses of dried plum polyphenols (0.0, 0.1, 1, 10, 100, 1000 and 10, 000 µg/ml) and 1 µg LPS/ml medium for 24 hours. The number of viable cells was determined as a function of metabolic activity using the dye resazurin according to the manufacturers' directions (Promega, Madison, WI, USA). Briefly, 20 µl of CellTiter-Blue® reagent was added to each well, the plate was shaken gently for 10 seconds and then incubated at 37°C for 3 hours. Absorbance was recorded at 573 nm using a microplate reader (Bio Tek, Winooski, VT, USA). The results were plotted against concentrations of the test compound.

2.5 Measurement of NO

The marker used to assess inflammation in macrophages, NO, was assessed by measuring nitrite levels using the Griess Reagent system (Promega, Madison, WI, USA). Culture supernatants were collected from 96-well plates after 12-hours treatment with dried plum polyphenols (0.0, 0.1, 1, 10, 100, 1000 µg/ml) and 1 µg/ml LPS. Sodium nitrite dissolved in tissue culture media (0.1 µg/ml–100 µg/ml) was used to create the standard curve for this assay. Samples (50 µl) and standard media were combined with 50 µl of sulfanilamide, incubated for 10 minutes at room temperature. Then, 50 µl N-(1-naphthyl)-ethylenediamine dihydrochloride solution was added to all wells and incubated for another 10 minutes. Absorbance was measured at 550 nm. Nitrite levels were determined using the standard curve generated for each plate.

2.6 Measurement of COX-2

The COX-2 protein, an inducible pro-inflammatory molecule, was measured by the western blot method as described by our laboratory.[34] Cells were seeded in 6-well plates at a density of 5 × 105 cells per well. After 80% confluence, cells were treated with grading doses of dried plum polyphenols (0, 0.1, 1, 10, 100, 1000 µg/ml) for 1 hour, followed by 10 ng/ml LPS for 6 hours. Cells were then washed twice with ice cold phosphate-buffered saline (PBS) twice. Breaking solution (200 µl of 0.1% Triton X-100 PBS) was added to each well and the cells were collected. Cells were sonicated at 20% amplitude, 3 seconds pulse for 3-4 times and centrifuged at 3000 × g for 10 minutes at 4ºC. The protein content in cytosolic fraction was determined in each sample by the bicinchoninic acid assay (BCA; Fisher Scientific, Rockland, IL, USA) for the equal loading of protein in the gel. Twenty µg protein was loaded in 12% sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) and

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separated for 2.5 hours (100 V) in mini gel electrophoretic cells (Bio-Rad Laboratories, Hercules, CA, USA). The separated protein was transferred to a polyvinylidene diflouride membrane (Millipore, Bedford, MA, USA) using a semidry blotting apparatus for 30 minutes at 15 V. Membranes were blocked with TBS-Tween (50 mM Tris, 150 mM NaCl, 0.2% Tween-20, pH 7.5) containing 5% skim milk for 1 hour. After washing in TBS, blots were incubated with a 1:500 dilution of goat polyclonal COX-2 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody overnight. This was followed by 2-hour incubation with 1:1500 dilutions of secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer. Membranes were treated with equal volumes of horseradish peroxide buffer and horseradish peroxidase enhancer (Bio-Rad Laboratories, Hercules, CA, USA). The bands were developed and the intensity of bands was calculated using Versadoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 Measurement of Malondialdehyde

Malondialdehyde, a marker of lipid peroxidation, was measured by colorimetric microplate assay for lipid peroxidation (Oxford Biomedical Research, Rochester Hills, MI, USA). RAW 264.7 cells (80% confluence) were treated with 100 µmol/L $FeSO_4$, 1 mmol/L H_2O_2 and 1000 µg/ml of dried plum polyphenols for 4 hours. Cells were collected at a density of 1.5 × 107 cells per ml of PBS and protein of cytosolic fractions were obtained by centrifuging cell lysates at 3000 × g at 4°C for 10 minutes. The protein concentration was estimated by a BCA assay kit (Fisher Scientific, Rockland, IL, USA) according to the manufacturers' instructions.

2.8 Statistical Analysis

Data is presented as mean ± SE. Values were compared using analysis of variance (ANOVA). When ANOVA indicated statistical significance, Tukey's post hoc multiple comparisons test was used to determine which means were significantly different. GraphPad software version 3.0 (San Diego, CA, USA) was used for all statistical analyses. Significance was accepted at $P \leq 0.05$.

3. Results

3.1. Characterization of Dried Plum Polyphenols

The compounds characterized in dried plum extract are presented in Table 1. Total phenolic content of ethanol extraction of dried plum was 450.06 ± 16.55 mg gallic acid equivalent (GAE)/100 g of dried plum. The most abundant polyphenols present in the dried plum extract were neochlorogenic acid (3-o-caffeoy;-quinic acid), gallic acid and chlorogenic acid (5-o-caffeoyl-quinic acid).

Table 1. Quantification of polyphenols in dried plum extract

Compound	Concentration (ppm)
Gallic Acid	739.6
3-o-caffeoyl-quinic acid	1,253.1
4-o-caffeoyl-quinic acid	15.3
5-o-caffeoyl-quinic acid	621.9
P-coumaric acid	60.7
Rutin	7.5

3.2. Effects of Dried Plum Polyphenols on Viability of Macrophage RAW 264.7 Cells

Treatment with LPS (1µg/ml) did not negatively affect the viability of macrophage RAW 264.7 cells compared to the control group. Dried plum polyphenols in doses of 0.0, 1.0, 10, 100 and 1000 µg/ml did not affect the viability of macrophages under LPS-induced conditions. However, cytotoxicity was detected in cells treated with very high concentration of dried plum polyphenols (10,000 µg/ml; Fig. 1).

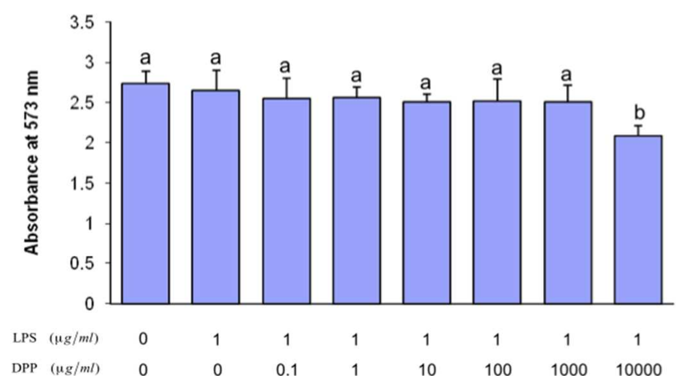

Figure 1. Effect of dried plum polyphenols on cell viability in lipopolysaccharide (LPS)-activated macrophage RAW 264.7 cells. Cells were cultured in the absence or presence of LPS (1 µg/ml) with 0.0, 0.1, 1.0, 10, 100, 1000, 10000 µg/ml of dried plum polyphenols for 24 hours. The cell viability was measured by resazurin method and was represented as mean ± standard error (SE) in the bars. Different letters indicate significant different values at $P \leq 0.05$ (n = 4).

3.3. Effects of Dried Plum Polyphenols on NO Production in LPS-induced Macrophage RAW 264.7 Cells

Cell culture supernatant NO levels increased significantly ($P < 0.05$) as a result of LPS treatment. Dried plum polyphenols at a dose of 1000 µg/ml reduced NO production by 43% in comparison with the LPS-treated control cells. The other doses of dried plum polyphenols had no significant inhibitory effects on increased NO production due to treatment with LPS (Fig. 2).

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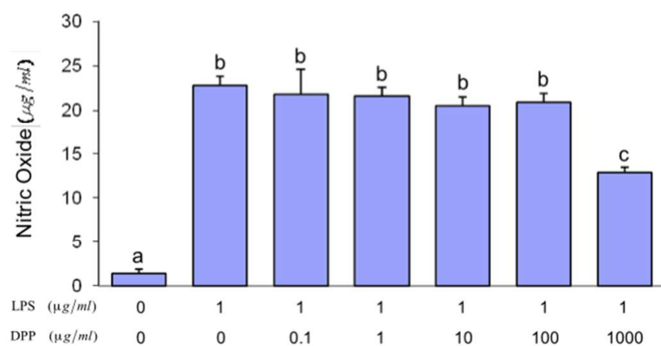

Figure 2. Effect of dried plum polyphenols on production of nitric oxide (NO) in macrophage RAW 264.7 cells. Cells were cultured in the absence or presence of LPS (1 μg/ml) with 0.0, 0.1, 1.0, 10, 100, 1000 μg/ml of dried plum polyphenols for 12 hours. NO production was measured by the Griess reagent and was represented as mean ± standard error (SE) in the bars. Different letters indicate significant different values at $P \leq 0.05$ (n = 4).

3.4. Effects of Dried Plum Polyphenols on COX-2 Expression in LPS-induced Macrophage RAW 264.7 Cells

Treatment with LPS (10 ng/ml) significantly increased the protein level of COX-2 in macrophage RAW 264.7 cells. Dried plum polyphenols at doses of 100 μg/ml and 1000 μg/ml significantly (P < 0.05) decreased protein level of COX-2 by 68% and 81.25%, respectively, in comparison with the LPS-treated control group. Treatment with 1000 μg/ml dried plum polyphenols tended to further suppress expression of COX-2 although the inhibitory effect did not differ significantly from that of treatment with 100 μg/ml dose (Fig. 3).

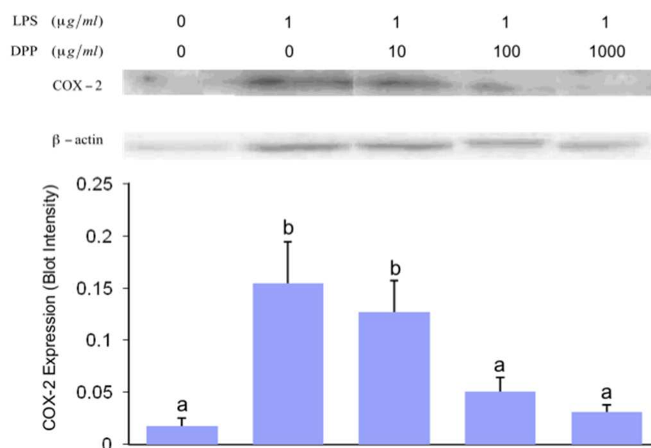

Figure 3. Effect of dried plum polyphenols on expression of cyclooxygenase-2 (COX-2) protein level in macrophage RAW 264.7 cells. Cells were cultured in the absence or presence of LPS (10 ng/ml) with 0.0, 10, 100, 1000 μg/ml of dried plum polyphenols for 6 hours. The expression of COX-2 was measured by the Western blot method and was represented as mean ± standard error (SE) in the bars. Different letters indicate significant different values at $P \leq 0.05$ (n = 4).

3.5. Effects of Dried Plum Polyphenols on Malondialdehye Production in $FeSO_4$ and H_2O_2-induced Macrophage RAW 264.7 Cells

The combination of $FeSO_4$ (100 μg/ml) and H_2O_2 (1000 μg/ml) significantly increased the malondialdehyde level in cells and dried plum polyphenols at a dose of 1000 μg/ml prevented this oxidation-induced increase by 30.7% in comparison to the LPS-induced group (Fig. 4).

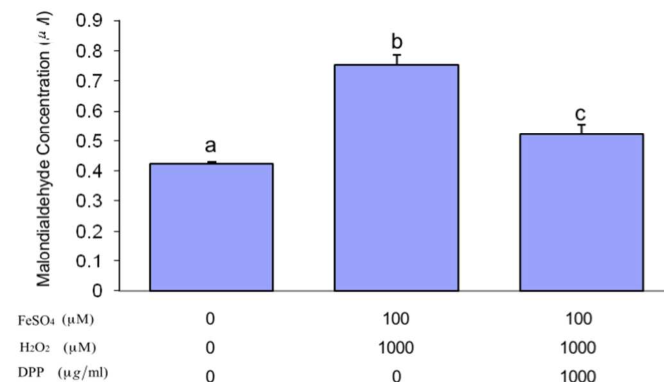

Figure 4. Effect of dried plum polyphenols on malondialdehyde production in macrophage RAW 264.7 cells. Cells were cultured in the absence or presence of $FeSO_4$ (100 μg/ml), H_2O_2 (1000 μg/ml), and dried plum polyphenols (1000 μg/ml) for 4 hours. The concentration of malondialdehyde was measured by colorimetric microplate assay and was represented as mean ± standard error (SE). Different letters indicate significant different values at $P \leq 0.05$ (n = 3). Bars that do not share the same letter are significantly different from each other ($P \leq 0.05$).

4. Discussion

The findings of the present study indicate that dried plum polyphenols suppress the production of NO and COX-2 as well as malondialdehyde in LPS- and $FeSO_4$ + H_2O_2-stimulated macrophages and we therefore accept our hypothesis. These findings support the earlier study by Bu *et al.*[17] where the authors reported that ethanol extracted dried plum polyphenols decreased NO production in LPS-induced macrophages RAW 264.7 cells. We tested dried plum polyphenols in doses of 0.1, 1.0, 10, 100, 1000 and 10,000 μg/ml and only the dose of 10,000 μg/ml reduced cell viability in macrophage RAW 264.7 cells. The higher doses of polyphenols may alter the pH of the media as well as its osmolarity and hence would have adverse effect on cell viability. The 1000 μg/ml dose was the most effective dose in reducing NO production. Dried plum polyphenols were able to reduce the expression of COX-2 protein levels at the dose of 100 μg/ml. The 1000 μg/ml dose further reduced expression of COX-2 protein levels but this reduction was not significantly different from the dose of 100 μg/ml. Although our findings are in agreement with those of Bu *et al.*,[17] our effective doses of dried plum polyphenols were much higher and we cannot offer an explanation for these differences. Though polyphenols refer to a number of compounds, it is known that dried plum polyphenols mainly include chlorogenic acid, caffeic acid and cinnamic acid.[35] Each of these phenolic compounds may suppress the production of pro-inflammatory molecules at different concentrations and whether these polyphenols act additively or synergistically is

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not known. Hence, there is a need to standardize what is called "total phenolic compounds" extracted from dried plum or other fruits and vegetables in order to evaluate their efficacies. Some studies have examined the anti-inflammatory effects of these polyphenols individually. For instance, Lee *et al.*,[36] showed that chlorogenic acid is capable of completely inhibiting NO production in LPS-induced macrophage RAW 264.7 cells at a dose of 40 ug/ml. In another study, it was shown that 2-hydroxycinnamaldehyde inhibited NO production, COX-2 and nuclear factor kappa-B (NF-κB) at a dose of 40 µg/ml in LPS-stimulated macrophages.[37] However, the authors suggest that in order to get the full benefit of polyphenols it would be best to use total polyphenols rather than individual polyphenols. Although the exact mechanism of action of polyphenols on cells is not known, it is suggested that polyphenols exert their anti-inflammatory effects through inhibiting NF-κB activation. NF-κB is a transcription factor located at the cytoplasm and stimulated by degradation of its inhibitor, inhibitor of kappa B (IκB). Inflammatory agents degrade IκB and release the NF-kB molecule to move freely into nucleus, which causes expression of several inflammatory mediators.[38] Pro-inflammatory agents such as LPS are known to stimulate NF-kB through toll-like receptors (TLR). Polyphenols have been found to modulate the TLR-derived inflammatory molecules. Youn *et al.*[39] showed that resveratrol inhibits NF-kB activation by inhibition of TLR 3 and TLR 4 in LPS-induced macrophages. Evidence suggests that dried plum polyphenols inhibit NF-kB activation in macrophages.[17] However, these studies were limited to a single inflammatory agent, LPS. In general, there is a wide variety of inflammatory agents present in the human body and their behavior and mechanism of action may be different. Therefore, further studies are needed to investigate the effectiveness of dried plum polyphenols in animal models of inflammation.

Our study showed that dried plum polyphenols can reduce oxidative stress in macrophages. Oxidation of membrane lipids can induce the formation of products of lipid peroxidation such as 4-hydroxy-2-nonenal which has been shown to up-regulate inflammation through stimulation of COX-2.[40] Zarrouki *et al.*[41] showed that the lipid peroxidation end product 4-hydroxy alkenals increase COX-2 expression by increasing phosphorylation of p38 MAP kinase in 3T3-L1 adipocytes. Malondialdehyde is a well-known product of lipid peroxidation which may disrupt the function of other molecules and impair their functions.[42] We tested the malondialdehyde production in macrophage RAW 264.7 cells stimulated by $FeSO_4$ and H_2O_2 and dried plum polyphenols inhibited the malondialdehyde production by 30%. Ozer *et al.*[43] demonstrated that caffeic acid phenyl ester (a polyphenolic derivative) inhibited malondialdehyde production significantly in ischemic reperfused myocardium of rats in a dose of 50 µM/kg rat weight.

Although the findings of the present study are promising, there are several possible limitations to consider. First, these findings are from a cell culture model of inflammation and cannot be directly extrapolated to *in vivo* conditions. Second, a whole dried plum polyphenol extract was used and not compared to individual polyphenols. Therefore, these findings do not provide insight into whether the anti-inflammatory and antioxidative effects observed were due to the action of individual polyphenols or the combination as a whole. Third, assessment of the activation of certain transcription factors involved in the inflammatory and oxidative stress processes (e.g. NF-κB and nuclear factor erythroid 2–related factor 2, Nrf2) was not done and would have provided greater insight into the mechanisms of action. Lastly, these findings cannot be directly extrapolated to bone metabolism since osteoclastogenesis, osteoblastogenesis, and biomarkers of bone metabolism were not assessed.

Conclusions

The findings of the present study suggest that dried plum polyphenols have potent anti-inflammatory as well as antioxidative properties *in vitro*. Nonetheless, these cell culture findings cannot be directly extrapolated to *in vivo* conditions and therefore further studies are needed to explore the bioactivity, metabolism, and tissue distribution and excretion mechanisms of dried plum polyphenols in using an animal model of inflammation to confirm our findings.

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Notes and references

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1 F. Deyhim, B. J. Stoecker, G. H. Brusewitz, L. Devareddy, and B. H. Arjmandi. Dried plum reverses bone loss in an osteopenic rat model of osteoporosis. *Menopause*, 2005, **12**, 755-762.
2 B. J. Smith, S. Y. Bu, Y. Wang, E. Rendina, Y. F. Lim, D. Marlow, S. L. Clarke, D. M. Cullen, and E. A. Lucas. A comparative study of the bone metabolic response to dried plum supplementation and PTH treatment in adult, osteopenic ovariectomized rat. *Bone*, 2014, **58**, 151-159.
3 B. H. Arjmandi, C. D. Johnson, S. C. Campbell, S. Hooshmand, S. C. Chai, and M. P. Akhter. Combining fructooligosaccharide and dried plum has the greatest effect on restoring bone mineral density among select functional foods and bioactive compounds. *J. Med. Food.*, 2010, **13**, 312-319.
4 C. D. Johnson, E. A. Lucas, S. Hooshmand, S. C. Campbell, M. P. Akhter, and B. H. Arjmandi. Addition of fructooligosaccharides and dried plum to soy-based diets reverses bone loss in the ovariectomized rat. *Evid. Based Complement. Alternat. Med.*, 2011, 836267.

Food & Function Accepted Manuscript

5 E. Rendina, K. D. Hembree, M. R. Davis, D. Marlow, S. L. Clarke, B. P. Halloran, E. A. Lucas, and B. J. Smith. Dried plum's unique capacity to reverse bone loss and alter bone metabolism in postmenopausal osteoporosis model. *PLoS One*, 2013, **8**, e60569.

6 E. Rendina, Y. F. Lim, D. Marlow, Y. Wang, S. L. Clarke, S. Kuvibidila, E. A. Lucas, and B. J. Smith. Dietary supplementation with dried plum prevents ovariectomy-induced bone loss while modulating the immune response in C57BL/6J mice. *J. Nutr. Biochem.*, 2012, **23**, 60-68.

7 S. Y. Bu, E. A. Lucas, M. Franklin, D. Marlow, D. J. Brackett, E. A. Boldrin, L. Devareddy, B. H. Arjmandi, and B. J. Smith. Comparison of dried plum supplementation and intermittent PTH in restoring bone in osteopenic orchidectomized rats. *Osteoporos. Int.*, 2007, **18**, 931-942.

8 M. Franklin, S. Y. Bu, M. R. Lerner, E. A. Lancaster, D. Bellmer, D. Marlow, S. A. Lightfoot, B. H. Arjmandi, D. J. Brackett, E. A. Lucas, and B. J. Smith. Dried plum prevents bone loss in a male osteoporosis model via IGF-I and the RANK pathway. *Bone*, 2006, **39**, 1331-1342.

9 B. P. Halloran, T. J. Wronski, D. C. VonHerzen, V. Chu, X. Xia, J. E. Pingel, A. A. Williams, and B. J. Smith. Dietary dried plum increases bone mass in adult and aged male mice. *J. Nutr.*, 2010, **140**, 1781-1787.

10 S. Hooshmand, S. C. Chai, R. L. Saadat, M. E. Payton, K. Brummel-Smith, and B. H. Arjmandi. Comparative effects of dried plum and dried apple on bone in postmenopausal women. *Br. J. Nutr.*, 2011, **106**, 923-930.

11 S. Hooshmand, J. R. Brisco, and B. H. Arjmandi. The effect of dried plum on serum levels of receptor activator of NF-κB ligand, osteoprotegerin and sclerostin in osteopenic postmenopausal women: a randomised controlled trial. *Br. J. Nutr.*, 2014, **112**, 55-60.

12 B. H. Arjmandi, D. A. Khalil, E. A. Lucas, A. Georgis, B. J. Stoecker, C. Hardin, M. E. Payton, and R. A. Wild. Dried plums improve indices of bone formation in postmenopausal women. *J. Womens Health Gend. Based Med.*, 2002, **11**, 61-68.

13 S. Kayano, H. Kikuzaki, N. F. Yamada, A. Aoki, K. Kasamatsu, Y. Yamasaki, et al. Antioxidant properties of prunes (*Prunus domestica* L.) and their constituents. *Biofactors*, 2004, **21**, 309-313.

14 N. Nakatani. Phenolic antioxidants from herbs and spices. *Biofactors*, 2000, **13**, 141-146.

15 S. Wallach, L. V. Avioli, J. D. Feinblatt. Cytokines and bone metabolism. *Calcif. Tissue Int.*, 1993, **53**, 293-296.

16 S. Y. Bu, T. S. Hunt, and B. J. Smith. Dried plum polyphenols attenuate the detrimental effects of TNF-alpha on osteoblast function coincident with up-regulation of Runx2, Osterix and IGF-I. *J. Nutr. Biochem.*, 2009, **20**, 35-44.

17 S. Y. Bu, M. Lerner, B. J. Stoecker, E. Boldrin, D. J. Brackett, E. A. Lucas, and B. J. Smith. Dried plum polyphenols inhibit osteoclastogenesis by downregulating NFATc1 and inflammatory mediators. *Calcif. Tissue Int.*, 2008, **82**, 475-488.

18 C. Miyaura, M. Inada, C. Matsumoto, T. Ohshiba, N. Uozumi, T. Shimizu, and A. Ito. An essential role of cytosolic phospholipase A2alpha in prostaglandin E2-mediated bone resorption associated with inflammation. *J. Exp. Med.*, 2003, **197**, 1303-1310.

19 L. C. Hofbauer, D. L. Lacey, C. R. Dunstan, T. C. Spelsberg, B. L. Riggs, and S. Khosla. Interleukin-1β and tumor necrosis factor-α, but not interleukin-6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. *Bone*, 1991, **25**, 255-259.

20 S. R. Goldring. Inflammatory mediators as essential elements in bone remodeling. *Calcif. Tissue Int.*, 2003, **73**, 97-100.

21 A. L. Dumitrescu, S. Abd-El-Aleem, B. Morales-Aza, and L. F. Donaldson. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *J. Clin. Periodontol.*, 2004, **31**, 596-603.

22 C. L. Shen, J. K. Yeh, J. J. Cao, O. L. Tatum, R. Y. Dagda, and J. S. Wang. Green tea polyphenols mitigate bone loss of female rats in a chronic inflammation-induced bone loss model. *J. Nutr. Biochem.*, 2010, **21**, 968-974.

23 B. J. Smith, M. R. Lerner, S. Y. Bu, E. A. Lucas, J. S. Hanas, S. A. Lightfoot, R. G. Postier, M. S. Bronze, and D. J. Brackett. Systemic bone loss and induction of coronary vessel disease in a rat model of chronic inflammation. *Bone*, 2006, **38**, 378-386.

24 D. L. Cochran. Inflammation and bone loss in periodontal disease. *J. Periodontol.*, 2008, **79**, 1569-1576.

25 G. Banfi, E. L. Iorio, and M. M. Corsi. Oxidative stress, free radicals and bone remodeling. *Clin. Chem. Lab. Med.*, 2008, **46**, 1550-1555.

26 H. Nakagawa, M. Wachi, J. T. Woo, M. Kato, S. Kasai, F. Takahashi, I. S. Lee, and K. Nagai. Fenton reaction is primarily involved in a mechanism of (-)-epigallocatechin-3-gallate to induce osteoclastic cell death. *Biochem. Biophys. Res. Commun.*, 2002, **292**, 94-101.

27 A. Wattel, S. Kamel, C. Prouillet, J. P. Petit, F. Lorget, E. Offord, and M. Brazier. Flavonoid quercetin decreases osteoclastic differentiation induced by RANKL via a mechanism involving NF kappa B and AP-1. *J. Cell Biochem.*, 2004, **92**, 285-295.

28 Q. Y. Tang, T. Kukita, Y. Ushijima, A. Kukita, K. Nagata, F. Sandra, T. Watanabe, K. Toh, Y. Okuma, S. Kawasaki, L. Rasubala, J. Teramachi, I. Miyamoto, Z. Wu, and T. Iijima. Regulation of osteoclastogenesis by Simon extracts composed of caffeic acid and related compounds: successful suppression of bone destruction accompanied with adjuvant-induced arthritis in rats. *Histochem. Cell Biol.*, 2006, **25**, 215-225.

29 S. Kayano, H. Kikuzaki, T. Ikami, T. Suzuki, T. Mitani, and N. Nakatani. A new bipyrrole and some phenolic constituents in prunes (*Prunus domestica* L.) and their oxygen radical absorbance capacity (ORAC). *Biosci. Biotechnol. Biochem.*, 2004, **68**, 942-944.

30 A. Kroon, M. N. Clifford, A. Crozier, A. J. Day, J. L. Donovan, C. Manach, and G. Williamson. How should we assess the effects of exposure to dietary polyphenols in vitro? *Am. J. Clin. Nutr.*, 2004, **80**, 15-21.

31 Y. C. Chen, S. C. Shen, W. R. Lee, W. C. Hou, L. L. Yang, and T. J. Lee. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J. Cell Biochem.*, 2001, **82**, 537-548.

32 J. M. Lean, J. T. Davies, K. Fuller, C. J. Jagger, B. Kirstein, G. A. Partington, Z. L. Urry, and T. J. Chambers. A crucial role for thiol antioxidants in estrogen-deficiency bone loss. *J. Clin. Invest.*, 2003, **112**, 915-923.

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33 D. O. Kim, K. W. Lee, H. J. Lee, C. Y. Lee. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem.*, 2002, **50**, 3713-3717.
34 S. Hooshmand, D. Y. Soung, E. A. Lucas, S. V. Madihally, C. W. Levenson, and B. H. Arjmandi. Genistein reduces the production of proinflammatory molecules in human chondrocytes. *J. Nutr. Biochem.*, 2007, **18**, 609-614.
35 O. K. Chun, D. O. Kim, and C. Y. Lee. Superoxide radical scavenging activity of the major polyphenols in fresh plums. *J. Agric. Food Chem.*, 2003, **51**, 8067-8072.
36 J. H. Lee, J. H. Park, Y. S. Kim, and Y. Han. Chlorogenic acid, a polyphenolic compound, treats mice with septic arthritis caused by Candida albicans. *Int. Immunopharmacol.*, 2008, **8**, 1681-1685.
37 S. H. Lee, S. Y. Lee, D. J. Son, H. Lee, H. S. Yoo, S. Song, K. W. Oh, D. C. Han, B. M. Kwon, and J. T. Hong. Inhibitory effect of 2'-hydroxycinnamaldehyde on nitric oxide production through inhibition of NF-kappa B activation in RAW 264.7 cells. *Biochem. Pharmacol.*, 2005, **69**, 791-799.
38 J. A. DiDonato, M. Hayakawa, D. M. Rothwarf, E. Zandi, M. Karin. A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature*, 1997, **388**, 548-554.
39 H. S. Youn, J. Y. Lee, K. A. Fitzgerald, H. A. Young, S. Akira, D. H. Hwang. Specific inhibition of MyD88-independent signaling pathways of TLR3 and TLR4 by resveratrol: molecular targets are TBK1 and RIP1 in TRIF complex. *J. Immunol.*, 2005, **175**, 3339-3346.
40 T. Kumagai, Y. Kawamoto, Y. Nakamura, I. Hatayama, K. Satoh, T. Osawa, and K. Uchida. 4-hydroxy-2-nonenal, the end product of lipid peroxidation, is a specific inducer of cyclooxygenase-2 gene expression. *Biochem. Biophys. Res. Commun.*, 2000, **273**, 437-441.
41 B. Zarrouki, A. F. Soares, M. Guichardant, M. Lagarde, and A. Géloën. The lipid peroxidation end-product 4-HNE induces COX-2 expression through p38MAPK activation in 3T3-L1 adipose cell. *FEBS Lett*, 2007, **581**, 2394-2400.
42 J. Chen, D. R. Petersen, S. Schenker, and G. I. Henderson. Formation of malondialdehyde adducts in livers of rats exposed to ethanol: role in ethanol-mediated inhibition of cytochrome c oxidase. *Alcohol Clin. Exp. Res.*, 2000, **24**, 544-552.
43 M. K. Ozer, H. Parlakpinar, Y. Cigremis, M. Ucar, N. Vardi, A. Acet. Ischemia-reperfusion leads to depletion of glutathione content and augmentation of malondialdehyde production in the rat heart from overproduction of oxidants: can caffeic acid phenethyl ester (CAPE) protect the heart? *Mol. Cell. Biochem.*, 2005, **273**, 169-175.

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