

## Physiological Activity of Coffee Beans and Roasted Black Beans (*Rhynchosia nulubilis*) Mixture Extracts for Coffee Alternative Beverage Development

<sup>†</sup>Ae-Jung Kim, Hankyu Lee\*, Hyuk Wan Ko\*, Seong Hee Ko\*\* and Nariyah Woo\*\*\*

*The Graduate School of Alternative Medicine, Kyonggi University, Seoul 03752, Korea*

*\*College of Pharmacy, Dongguk University, Goyang 10326, Korea*

*\*\*The Graduate School of Cultural Industry and Arts., Sungsin Women's University, Seoul 01133, Korea*

*\*\*\*Dept. of Food Science and Technology, Hoseo University, Choongnam 31499, Korea*

### Abstract

This study was designed to develop and to qualify a coffee alternative beverage using a mixture of coffee beans and roasted black beans (*Rhynchosia nulubilis*). Therefore, the total isoflavone content (TIC), total phenol content (TPC), antioxidant activity, anti-inflammatory activity, NFATc1 (Nuclear factor of activated T-cells c1) expression in RANKL (receptor activator of nuclear factor kappa-B ligand)-stimulated RAW264.7 cells and sensory evaluation were measured for 5 different Cb (coffee bean)-RoS (roasted *seomoktae*) mixture extracts (Cb100RoS0, Cb75RoS25, Cb50RoS50, Cb25RoS75, and Cb0RoS100). Cb0RoS100 had the highest TIC (516.83±36.61 mg/100 g) and TPC (18.11±1.77 mg TAE/100 g) along with the highest antioxidant activity as measured by DPPH radical scavenging activity (73.55±8.11%) and ABTS radical scavenging activity (63.27±7.27%). Also, Cb0RoS100 showed the highest anti-inflammatory activity as measured by NO production (13.57±2.21 μM) and PGE2 production (3.25±0.21 ng/mL). The more the RoS ratio was increased in the mixtures of Cb-RoS, the more the NFATc1 protein expression was decreased in RANKL-stimulated RAW264.7 cells. In case of sensory evaluation, Cb50RoS50 had the highest scores for flavor, delicate flavor and overall quality, which were similar to those in Cb alone (Cb100RoS0). We suggest that the use of RoS replacement instead of Cb in/as a coffee alternative beverage may help to reduce the risk of caffeine-related bone loss and/or bone disease by effectively blocking NFATc1 expression in RANKL-stimulated RAW264.7 cells compared with Cb alone.

Key words: coffee bean, roasted *seomoktae*, antioxidant activity, anti-inflammatory activity, RAW264.7 cell

### Introduction

Coffee beans, tea leaves, and cocoa beans are the primary natural sources of caffeine (Nawrot et al. 2003), which is added to numerous foods and beverages (e.g., soft drinks and energy/sports drinks). Coffee is the principal source of caffeine and contains hundreds of biologically active compounds (Heckman et al. 2010). Caffeine is obtained mainly from dietary fluid sources, such as coffee, tea, soda, and energy/sport drinks. It is consumed by more than 85% of adults in the United States (US); a 71% of American adults drink coffee on a daily basis (Lopez et al.

2015).

Health benefits associated with coffee intake include reduction of the risk of type 2 diabetes mellitus (Bhupathiraju et al. 2014), cardiovascular disease (Revuelta-Iniesta & Al-Dujaili 2014), and weight loss (Onakpoya et al. 2011). However, negative effects have also been reported, such as those related to bone health. Lee et al. (2014) suggested that coffee promoted increased calcium extraction, inhibition of osteoblast proliferation, and increased osteoclast proliferation. Hallstrom et al. (2013) mentioned that high coffee consumption was associated with a small reduction in bone density. The low bone density constitutes a large problem

<sup>†</sup> Corresponding author: Ae-Jung Kim, The Graduate School of Alternative Medicine, Kyonggi University, Seoul 03752, Korea. Tel: +82-2-390-5044, Fax: +82-2-390-5078, E-mail: aj5249@naver.com

in both women and men worldwide, with a major influence on life quality and mortality rate.

Soybean is rich in protein, and fat, and is a good source of energy, vitamins, and minerals, such as phosphorous, calcium and iron (Phommalath et al. 2014). One type of soybean known as black soybean, or *seomoktae* is traditionally used in Korea because its seed coat contains natural phenolic compounds (Zhao et al. 2015). *Seomoktae* exerts protective effects against a number of disease such as cardiac diseases, metabolic syndromes, osteoporosis, and cancer (Levis et al. 2011; Jung et al. 2013). *Seomoktae* contains a high level of glutamine and asparagine residues, whose negatively charged carboxyl groups may bind calcium and, hence, may increase calcium bioavailability. In addition, *seomoktae*'s isoflavones are structurally similar to estrogen and bind to estrogen receptors, suggesting that they exhibit estrogen activity (Setchell et al. 2005).

Recently the consumption of high caffeine beverages has been increased, but the effect of caffeine on calcium metabolism remains unknown. This study was designed to evaluate the effects of Cb-RoS mixture extracts on antioxidant, anti-inflammatory activity and the inhibitory effect on RANKL-mediated RAW 264.7 cells differentiation compared with that of Cb extract alone with sensory evaluation to develop coffee alternatives.

## Materials and Methods

### 1. Plant material and chemicals

Black bean (*seomoktae*, *Rhynchosia nulubilis*) and coffee bean (Cb) were purchased from Cholokmayl Co. (Seoul, Korea). All reagents used were analytical grade. Isoflavone standards, Na<sub>2</sub>CO<sub>3</sub>, tannic acid, vitamin C, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) (ABTS), phosphate buffered saline (PBS), and Folin Ciocalteu's phenol reagent were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Cellgro (Manassas, VA, USA).

### 2. Roasting and preparation of the mixture sample's extract

Before preparation of Cb-RoS mixture, black bean (*seomoktae*) was roasted based on condition (at 110°C for 20 min) of our previous study (Jeong SO 2015) using a roaster (Proaster THCR-01, Taehwan Automation Industry Co., Gyeonggi-do, Korea). All

Cb-RoS mixture samples, which were Cb100RoS0 (Cb 100% : RoS 0%), Cb75RoS25 (Cb 75% : RoS 25%), Cb50RoS50 (Cb 50% : RoS 50%), Cb25RoS75 (Cb 25% : RoS 75%), and Cb0RoS100 (Cb 0% : RoS 100%) were ground and the resultant powder was extracted with water (1:20) for 3 h at 80°C using a heating mantle (HM250C; Lab Tech, Seoul, Korea). The final extract was filtered through a 0.22 µm PIFE filter unit and was concentrated using a rotary vacuum evaporator (HS-2005S-N; Hahn Shin Scientific Co., Gyeonggi, Korea). Next, the samples were freeze-dried by storage in a deep freezer (WUF-500; Daihan Scientific Co., Gangwon, Korea) and were stored until analysis.

### 3. The total isoflavone content

The total isoflavone content (TIC) of Cb-RoS mixture extract samples were determined using a method described by Coward et al. (1996), with some modifications. The concentrations of total isoflavone were determined using a SUPELCO™ LC-18 column (particle size: 5 µm, L×id: 250×4.6 mm, Bellefonte, PA, USA) with a flow rate of 1.0 mL/min. The linear gradient mobile phase was 1% (v/v) aqueous acetic acid solution. This was replaced by using a 510 HPLC pump with a gradient controller of acetonitrile for 20 min in the linear gradient (Waters, Milford, MA, USA). In all, 20 µL of the sample was injected (Rheodyne injector, Rheodyne LLC; Rohnert Park, CA, USA) through the column, and the concentrations of total isoflavone was qualified by measuring their absorbance at 254 nm with a UV/Vis detector (UV-2077; Jasco, Tokyo, Japan). Fluorescein was used as an internal standard.

### 4. The total polyphenol contents

The total phenol content (TPC) of the mixture extract samples were determined according to the method described by Arnou et al. (2001) with modifications. Briefly, 500 µL of each extract were each transferred to a test tube and mixed with 50 µL of Folin-Ciocalteu reagent. The mixture extracts were left for 3 min at room temperature, and 500 µL of 20% (w/v) Na<sub>2</sub>CO<sub>3</sub> was added to the extracts. The absorbance was measured at 725 nm. A standard curve was established using tannic acid. All measurements were performed in triplicate.

### 5. The radical scavenging activity of DPPH and ABTS

The radical scavenging activity of the mixture extract samples were determined using DPPH according to the method of described by Naik et al. (2004). Briefly, 100 µL of 0.1 mM DPPH ethanol

solution was mixed with 100  $\mu$ L of each Cb-RoS mixture extract samples, respectively and the resultant mixture was left at room temperature for 30 min. The absorbance was measured at 517 nm against a blank lacking the scavenger using an ELISA reader (Tecan Infinite M200 Pro, GreenMate Bio, Seoul, Korea).

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100$$

The radical scavenging activity of the samples was also determined using ABTS according to a method described by Jeong et al. (2010). Briefly, 2.6 mM potassium phosphate was added to 7.4 mM ABTS solution, and the mixture was stored at  $-70^{\circ}\text{C}$  for 24 h. Next phosphate-buffered saline (PBS) was diluted 15-fold and used as the ABTS reagent after absorbance at 732 nm ( $0.700 \pm 0.03$ ) was confirmed. Next, 50  $\mu$ L aliquots of mixture extract samples were added to 950  $\mu$ L aliquots of the prepared ABTS reagent, respectively, and the solutions were left for five min at room temperature. The absorbance of the solutions was measured at 732 nm by using an ELISA reader (Tecan Infinite M200 Pro, GreenMate Bio, Seoul, Korea).

$$\text{ABTS radical scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100$$

## 6. Cell culture and inducement of RAW 264.7 cells' differentiation

Murine macrophage-derived RAW 264.7 cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were grown overnight in MEM (Cellgro, Manassas, VA) containing 10% FBS and 1% penicillin/streptomycin at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ , according to the manufacturer's instructions. To assess the effects of Cb-RoS mixture extracts (100  $\mu\text{g}/\text{mL}$ ) on RANKL-mediated cells differentiation, the cells were seeded in 6-well plates (cell density:  $1 \times 10^5$  cells/well) and were differentiated in the growth media containing Receptor Activator of NF- $\kappa$ B ligand (RANKL; 40 ng/mL) for 1 day.

## 7. Preparation of mixture extracts for cell treatment

To test the inhibitory effect of the Cb-RoS mixture extracts on RANKL-mediated RAW 264.7 cells differentiation, RAW 264.7 cells were treated with each Cb-RoS mixture extracts at day 0. Fifty gram of each mixture were added to 1 L of distilled water and were extracted for 3 h at  $80^{\circ}\text{C}$  using a heating mantle

attached to a reflux condenser. The extract was filtered through a filter paper (Whatman No. 2) and was concentrated under vacuum using a rotary evaporator. The extracts were lyophilized and stored at  $-70^{\circ}\text{C}$  until further analysis. The extract was dissolved in PBS, and next, filtered and sterilized. For further analysis, the extracts were dissolved in Dulbecco's (D) PBS, filtered through a 0.22  $\mu\text{m}$  filter, and autoclaved.

## 8. Measurement of NO production

NO production was assayed based on measurement of the amount of nitrite in the supernatant of cultured RAW 264.7 cells using Griess reagent. Briefly, cells were cultured at a density of  $1 \times 10^5$  cells/well in a 96-well plate, then stabilized for 24 h under 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . Cells were washed with PBS, replaced with fresh media, and incubated with 1  $\mu\text{g}/\text{mL}$  LPS in the presence or absence of mixture extracts at 100  $\mu\text{g}/\text{mL}$ . After additional 24 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction (Mancocci et al. 1994). The absorbance was measured at 570 nm (Vision Scientific, Daejeon, Korea) and nitrite concentration was calculated.

## 9. Measurement of PGE2 production

Raw 264.7 cells were cultured in 24-well plates (cell density:  $1 \times 10^5$  cells/well) and then incubated with or without 1  $\mu\text{g}/\text{mL}$  LPS in the absence or presence of mixture extracts at 100  $\mu\text{g}/\text{mL}$ . After 24 h incubation, the PGE2 (Prostaglandin E2) concentration in the culture medium was determined using an enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

## 10. Western blot assay

RAW 264.7 cells co-treated with RANKL (R&D system Inc., Minneapolis, MN) (40 ng/mL) and Cb-RoS mixture extracts were lysed in for 24 h modified radioimmunoprecipitation (RIPA) buffer (150 mM NaCl [pH 7.4], 50 mM Tris-HCl, 1 mM EDTA, 1% NP-40, and 0.25% sodium-deoxycholate) supplemented with protease inhibitors, 1 mM phenylmethane-sulfonylfluoride and 1 $\times$ protease inhibitor cocktail tablets (Roche, Indianapolis USA) on ice. The protein extracts were clarified by centrifugation at 13,500 rpm and  $4^{\circ}\text{C}$  for 15 min. The supernatant was transferred to fresh micro-centrifuge tubes, and the protein concentrations were determined using the bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (25

µg) were resolved by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using the Trans-Blot SD semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). The membranes were subsequently washed with Tris-buffered saline solution containing 0.2% Tween 20 (TBST20) and were blocked for 2 h in TBST20 containing 5% skimmed milk. The membranes were incubated overnight with mouse anti-nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1; Santa Cruz, CA, USA) and mouse anti-beta actin (Cell signaling) primary antibodies at 4°C. After the membranes were washed with washing buffer, they were incubated with adequate secondary antibodies for 2 h at room temperature. Signals were detected using a chemiluminescent reagent (Millipore corp., Burlington, MA, USA) according to the manufacturer's instructions.

### 11. Sensory evaluation of Cb-RoS mixture extracts

A sensory evaluation of Cb-RoS mixture extracts with 15 panelists who were well informed of the test method and the evaluation characteristic were performed. Each panelist received 5 individually presented Cb-RoS mixture extracts in a white cup, respectively. The Cb-RoS mixture extracts were coded using a random 3-digit number. The panelists scored for sensory evaluation of color, flavor, delicate flavor, bitterness, and overall quality.

### 12. Statistical analysis

All data (n=3) are expressed as mean ± standard deviation (SD). Differences among the groups were assessed using 1-way ANOVA followed by Duncan's multiple comparison tests. All analyses were conducted using Statistical Package for Social Sciences (SPSS) software for Windows (version 21.0; SPSS Inc., Chicago, USA).

## Result and Discussion

### 1. The phenolic compound content and bioactivity of Cb-RoS mixture extracts

Oxidative stress is characterized by the excessive generation of free radicals during physiological processes. Recent studies provide important evidence that oxidative stress is a primary pathogenic mechanism underlying bone degradation (Almeida et al. 2007; Saglam et al. 2015). One study showed that osteoblastogenesis and bone formation are also related to oxidative stress (Manolagas & Almeida 2007). In this study, the phenolic com-

pounds (TIC and TPC) and biological (antioxidative and anti-inflammatory) activity of the Cb alone extract and, Cb-RoS mixture extracts were compared. The data for TIC, TPC, DPPH radical scavenging activity, and ABTS radical scavenging activity are shown in Table 1, and 2. Cb0RoS100 had the highest TIC (516.83±36.61 mg/100 g), TPC (18.11±1.77 mg TAE 100 g), DPPH radical scavenging activity (73.55±8.11%), and ABTS

**Table 1. The total isoflavone content and polyphenol content of Cb-RoS mixture extracts**

Groups <sup>1)</sup>	Total isoflavone content (mg/100 g)	Total polyphenol content (mg TAE/100 g)
Cb100RoS0	ND <sup>2)</sup>	9.02±1.31 <sup>c</sup>
Cb75RoS25	172.28±28.14 <sup>3)c4)</sup>	13.77±1.22 <sup>b</sup>
Cb50RoS50	344.56±64.32 <sup>b</sup>	15.33±1.03 <sup>ab</sup>
Cb25RoS75	451.44±56.22 <sup>ab</sup>	16.99±1.23 <sup>ab</sup>
Cb0RoS100	516.83±36.61 <sup>a</sup>	18.11±1.77 <sup>a</sup>

<sup>1)</sup> Cb100RoS0: Extract of coffee bean 100% alone, Cb75RoS25: Mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: Mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: Mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: Extract of roasted *seomoktae* 100% alone

<sup>2)</sup> ND: Not detectable

<sup>3)</sup> Data are presented as Mean±S.D. (n=3)

<sup>4)</sup> <sup>a-c</sup> Means with different letters in the same column are significantly different at  $p<0.05$  by Duncan's multiple range test (a>c).

**Table 2. The antioxidant activities of Cb-RoS mixture extracts**

Groups <sup>1)</sup>	DPPH radical scavenging activity (%)	ABTS radical scavenging activity (%)
Positive control	87.86±7.18 <sup>2)a3)</sup>	81.67±5.84 <sup>a</sup>
Cb100RoS0	56.55±4.79 <sup>c</sup>	50.30±4.02 <sup>d</sup>
Cb75RoS25	58.25±3.85 <sup>c</sup>	56.12±2.65 <sup>c</sup>
Cb50RoS50	68.23±6.16 <sup>b</sup>	60.42±3.73 <sup>b</sup>
Cb25RoS75	70.33±7.90 <sup>b</sup>	61.20±4.22 <sup>b</sup>
Cb0RoS100	73.55±8.11 <sup>b</sup>	63.27±7.27 <sup>b</sup>

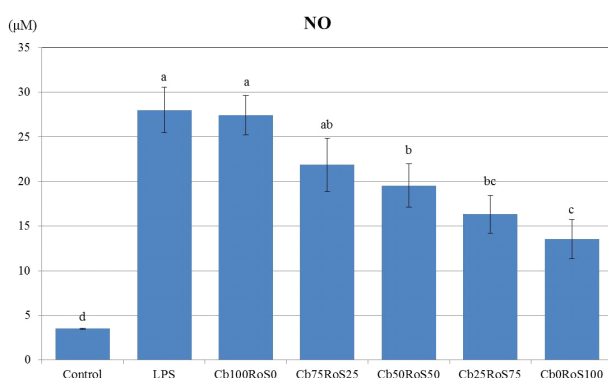
<sup>1)</sup> Positive control: vitamin C, Cb100RoS0: Extract of coffee bean 100% alone, Cb75RoS25: Mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: Mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: Mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: Extract of roasted *seomoktae* 100% alone

<sup>2)</sup> Mean±S.D.

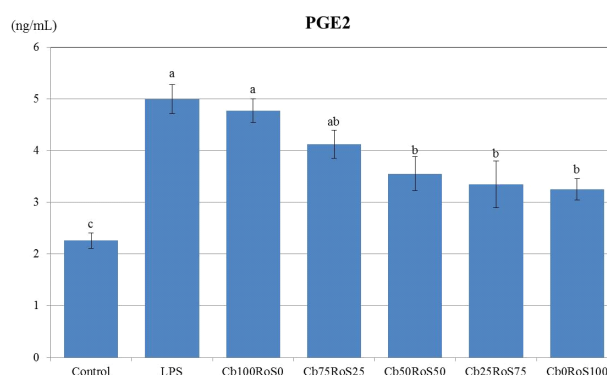
<sup>3)</sup> <sup>a-d</sup> Means with different letters in the same column are significantly different at  $p<0.05$  by Duncan's multiple range test (a>d).

radical scavenging activity ( $63.27 \pm 7.27\%$ ). In particular, its TIC and TPC were significantly higher than that of Cb100RoS0 ( $p < 0.05$ ) and its DPPH and ABTS radical scavenging activity was higher respectively, than that of Cb alone (Cb100RoS0) ( $p < 0.05$ ). The more RoS ratio in the Cb-RoS mixture extracts was increased, the more antioxidative activity was increased due to its improved high phenolic compounds (TIC and TPC) (Table 1, Table 2, Fig. 1, Fig. 2, and Fig. 3).

Inflammation is a complex process mediated by the activation of various immune cells. Macrophages play a central role in mediating a number of different immunopathological phenomena during inflammation by the overproduction of inflammatory mediators, known as Nitric oxide (NO) and prostaglandins (PGs) (Ricciotti et al. 2011; Lee et al. 1999). Therefore, the inhibition of NO and PGE2 production may have potential therapeutic value when related with inflammation. In this study, the production of



**Fig. 1. Inhibitory effects of Cb-RoS mixture extracts of NO production on LPS-stimulated RAW 264.7 cells.** Cells were cultured at a density of  $1 \times 10^5$  cells/well in a 96-well plate, then stabilized for 24 h under 5% CO<sub>2</sub> at 37°C. Cells were washed with PBS, replaced with fresh media, and incubated with 1 μg/mL LPS in the presence or absence of mixture extracts at 100 μg/mL. After additional 24 h incubation, the media were collected and analyzed for nitrite accumulation as an indicator of NO production by the Griess reaction. Control: not treated LPS, Cb100RoS0: treated with extract of coffee bean 100% alone, Cb75RoS25: treated with mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: treated with mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: treated with mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: treated with extract of roasted *seomoktae* alone, Means with different letters (a~d) indicate significant ( $p < 0.05$ ) differences based on 1-way ANOVA followed by Duncan's multiple comparison test (a>d).



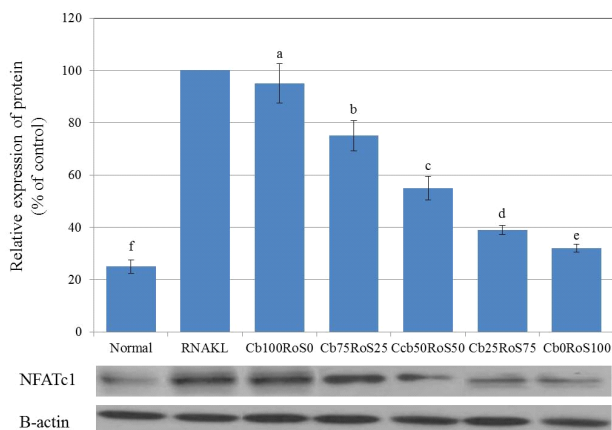
**Fig. 2. Inhibitory effects of Cb-RoS mixture extracts of PGE2 production on LPS-stimulated RAW 264.7 cells.** RAW 264.7 cells were treated with different concentrations of Cb-RoS mixture extracts (100 μg/mL) extract, followed by LPS (1 μg/mL) for 24 h. The culture supernatant was analyzed for PGE2 production using an enzyme linked immunosorbent assay (ELISA) kit, Control: not treated LPS, Cb100RoS0: treated with extract of coffee bean 100% alone, Cb75RoS25: treated with mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: treated with mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: treated with mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: treated with extract of roasted *seomoktae* alone, Means with different letters (a~c) indicate significant ( $p < 0.05$ ) differences based on 1-way ANOVA followed by Duncan's multiple comparison test (a>c).

NO and PGE2 were tested in LPS stimulated RAW264.7 cells (Fig. 1, 2) treated with LPS and 5 different Cb-RoS mixture extracts. The Cb0RoS100 in the mixture extracts showed the highest suppressive effect on NO ( $13.57 \pm 2.21$  μM) and PGE2 ( $3.25 \pm 0.21$  ng/mL) production in LPS stimulated RAW264.7 cells. But the suppressive effect on NO and PGE2 production on LPS stimulated RAW264.7 cells of Cb0RoS100 has not significant difference compared with Cb50RoS50 and Cb25RoS75.

## 2. The inhibitory effects of the Cb-RoS mixture extracts on the RANKL-induced NFATc1 expression during cells' differentiation

Osteoclasts play a critical role in bone development and physiological bone remodeling. Osteoclasts formation is affected directly or indirectly by several factors, such as RANKL, tumor necrosis factor (TNF-α), and macrophage colony stimulating factor (M-CSF) (Xing et al. 2012). RANKL signaling stimulates formation of single cell TRAP (Tartrate resistant acid phosphatase)+ osteoclast precursors and induces NFATc1 expression in osteoclast

precursors (Kim et al. 2015). Over expression of NFATc1 may stimulate osteoclast differentiation independent of RANKL signaling, ie, NFATc1 is a major regulator of osteoclast differentiation (Wang et al. 2015). Here, western blotting was used to determine the level of NFATc1 in RANKL-induced RAW 264.7 cells (Fig.



**Fig. 3. The inhibitory effects of the Cb-RoS mixture extracts of coffee bean and roasted *seomoktae* on the expression of NFATc1 in RANKL mediated RAW264.7 cells differentiation.**

Normal control: Not RANKL-mediated RAW264.7 cell, Positive control: RANKL-mediated RAW264.7 cell without mixture extract treatment, Cb100RoS0: treated with extract of coffee bean 100% alone, Cb75RoS25: treated with mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: treated with mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: treated with mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: treated with extract of roasted *seomoktae* alone, Means with different letters (a~f) indicate significant ( $p < 0.05$ ) differences based on 1-way ANOVA followed by Duncan's multiple comparison test (a>f).

3). In the RANKL stimulated RAW 264.7 cells, the differentiation was inhibited by CbRoS mixture extracts treatments. Interestingly, RAW 264.7 cells treated with increased RoS ratio in Cb-RoS mixture extracts showed reduced NFATc1 expression in RANKL induced RAW 264.7 cells differentiation. This raises the possibility that Cb0RoS100 extract may inhibit RANKL-induced NFATc1 expression during RANKL-mediated RAW264.7 cells differentiation. It was concluded that Cb0RoS100 extract contains increased amounts of TIC and TPC.

### 3. Sensory evaluation

Many adults drink coffee on a daily basis even though several studies have shown that coffee has adverse effects on bone health, i.e. bone density reduction, inhibition of osteoblast proliferation, and increased osteoclast proliferation (Hallstrom et al. 2006; Liu et al. 2012). Therefore, it is necessary to seek alternatives to coffee, which taste is similar to coffee.

In this study, we tried to develop coffee alternative beverage using coffee and roasted black bean, which sensory characteristics are shown in Table 3. Cb alone (Cb100RoS0) has the best score of sensory evaluation compared with other mixture extracts but, Cb50RoS50 has similar score of delicate flavor, and overall quality compared with Cb alone. It was concluded that Cb50RoS50 is useful for coffee alternative beverage even though the score of sensory evaluation was decreased by increased RoS extract in the Cb-RoS mixture extracts.

### Conclusion

This study was designed to develop and to qualify coffee alternative beverage using mixture of coffee bean and roasted

**Table 3. The sensory evaluation of Cb-RoS mixture extracts**

Groups <sup>1)</sup>	Color	Flavor	Delicate flavor	Bitterness	Overall quality
Cb100RoS0	6.47±1.23 <sup>2)NS3)</sup>	6.50±1.44 <sup>a</sup>	6.53±1.33 <sup>a</sup>	6.77±1.66 <sup>a</sup>	6.56±1.34 <sup>a</sup>
Cb75RoS25	6.46±0.19	6.00±1.98 <sup>ab</sup>	6.44±0.19 <sup>a</sup>	6.50±0.38 <sup>a</sup>	6.40±0.82 <sup>a</sup>
Cb50RoS50	6.45±1.11	5.90±1.34 <sup>b</sup>	6.00±1.55 <sup>ab</sup>	6.00±1.75 <sup>a</sup>	6.49±1.21 <sup>a</sup>
Cb25RoS75	5.92±0.34	4.62±0.99 <sup>bc</sup>	5.00±1.33 <sup>b</sup>	4.62±0.65 <sup>b</sup>	5.02±0.53 <sup>b</sup>
Cb0RoS100	5.95±1.30	4.00±1.22 <sup>c</sup>	3.55±0.66 <sup>c</sup>	4.00±0.88 <sup>c</sup>	4.05±1.32 <sup>c</sup>

<sup>1)</sup> Cb100RoS0: Extract of coffee bean 100% alone, Cb75RoS25: Mixture extract of coffee bean 75% and roasted *seomoktae* 25%, Cb50RoS50: Mixture extract of coffee bean 50% and roasted *seomoktae* 50%, Cb25RoS75: Mixture extract of coffee bean 25% and roasted *seomoktae* 75%, Cb0RoS100: Extract of roasted *seomoktae* 100% alone,

<sup>2)</sup> Mean±S.D.

<sup>3)</sup> NS: not significantly, <sup>a-c</sup> Means with different letters in the same column are significantly different at  $p < 0.05$  by Duncan's multiple range test (a>c).

black bean. Our results demonstrated that the Cb-RoS mixture extracts used at different RoS ratios influenced the antioxidant, anti-inflammatory activities and RANKL-mediated RAW 264.7 cells differentiation. The high RoS ratio in the respective mixture extracts was responsible for both the increased cellular antioxidant and anti-inflammatory activity due to its high phenolic compounds (TIC and TPC), and the inhibition of RAW 264.7 cells differentiation by reducing NFATc1 expression. Thus, we suggested that the use of RoS replacement instead of Cb may help to reduce the risk of caffeine-related bone loss and/or bone disease. Especially, Cb50RoS50, which has similar sensory evaluation compared with Cb alone, effectively blocked NFATc1 expression in RANKL-stimulated RAW264.7 cells. Therefore, Cb50RoS50 may be an alternative beverage to coffee with beneficial effects on helping to prevent bone loss compared with coffee.

## References

- Almeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC. 2007. Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor- to forkhead box O-mediated transcription. *J Biol Chem* 282:27298-272305
- Almeida M, Han L, Martin-Millan M, Plotkin LI, Stewart SA, Roberson PK, Kousteni S, O'Brien CA, Bellido T, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC. 2007. Skeletal involution by age-associated oxidative stress and its acceleration by loss of sex steroids. *J Biol Chem* 282:27285-27297
- Arnous A, Makris DP, Kefalas P. 2001. Effect of principal polyphenol components in relation to antioxidant characteristics of aged red wines. *J Agric Food Chem* 49:5736-5742
- Bhupathiraju SN, Pan A, Manson JE, Willett WC, van Dam RM, Hu FB. 2014. Changes in coffee intake and subsequent risk of type 2 diabetes: Three large cohorts of US men and women. *Diabetologia* 57:1346-1354
- Coward L, Kirk M, Albin N, Barnes S. 1996. Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. *Clin Chem Acta* 247:121-142
- Hallstrom H, Byberg L, Glynn A, Lemming EW, Wolk A, Michaëlsson K. 2013. Long-term coffee consumption in relation to fracture risk and bone mineral density in women. *Am J Epidemiol* 178:898-909
- Hallstrom H, Wolk A, Glynn A, Michaëlsson K. 2006. Coffee, tea and caffeine consumption in relation to osteoporotic fracture risk in a cohort of Swedish women. *Osteoporos Int* 17:1055-1064
- Heckman MA, Weil J, Gonzalez de Mejia E. 2010. Caffeine (1, 3,7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. *J Food Sci* 75:R77-R87
- Jeong JH, Jung H, Lee SR, Lee HJ, Hwang KT, Kim TY. 2010. Anti-inflammatory activities of the extracts from black berry fruits and wine. *Food Chem* 123:338-344
- Jeong SO. 2015. Quality characteristics of bone diseases alleviation coffee alternative beverage using roasted scoritae. MS Thesis, The Graduate School of Alternative Medicine, Kyonggi Univ. Seoul.
- Jung JH, Kim HS. 2013. The inhibitory effect of black soybean on hepatic cholesterol accumulation in high cholesterol and high fat diet-induced non-alcoholic fatty liver disease. *Food Chem Toxicol* 60:404-412
- Kim J, Lee H, Kang KS, Chun KH, Hwang GS. 2015. *Cordyceps militaris* mushroom and cordycepin inhibit RANKL-induced osteoclast differentiation. *J Med Food* 18:446-452
- Lee DR, Lee J, Rota M, Lee J, Ahn HS, Park SM, Shin D. 2014. Coffee consumption and risk of fractures: A systematic review and dose-response meta-analysis. *Bone* 63:20-28
- Lee HJ, Kim NY, Jang MK, Son HJ, Sohn DH, Lee SH, Ryu JH. 1999. A sesquiterpene, dehydrocostus lactone, inhibits the expression of inducible nitric oxide synthase and TNF-alpha in LPS-activated macrophage. *Planta Med* 65:104-108
- Levis S, Strickman-Stein N, Ganjei-Azar P, Xu P, Doerge DR, Krischer J. 2011. Soy isoflavones in the prevention of menopausal bone loss and menopausal symptoms: A randomized, double-blind trial. *Arch Intern Med* 171:1363-1369
- Liu H, Yao K, Zhang W, Zhou J, Wu T, He C. 2012. Coffee consumption and risk of fractures: A meta-analysis. *Arch Med Sci* 8:776-783
- Lopez DS, Wang R, Tsilidis KK, Zhu H, Daniel CR, Sinha A, Canfield S. 2015. Role of caffeine intake on erectile dysfunction in US men: Results from NHANES 2001-2004. *PLoS One* 10:e0123547
- Manolagas SC, Almeida M. 2007. Gone with the Wnts: Beta-catenin, T-cell factor, forkhead box O, and oxidative stress in age-dependent diseases of bone, lipid, and glucose metabolism. *Mol Endocrinol* 21:2605-2614
- Marcocci L, Maguire JJ, Droy-Lefaix MT, Packer L. 1994. The nitricoxide-scavenging properties of *Ginkgo biloba* extract

- EGB 761. *Biochem Bioph Res Co* 201:748-755
- Naik GH, Priyadarsini KI, Naik DB, Gangabhagirathi R, Mohan H. 2004. Studies on the aqueous extract of *Terminalia chebula* as a potent antioxidant and a probable radioprotector. *Phytomedicine* 20:530-538
- Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M. 2003. Effects of caffeine on human health. *Food Addit Contam* 20:1-30
- Onakpoya I, Terry R, Ernst E. 2011. The use of green coffee extract as a weight loss supplement: a systematic review and meta-analysis of randomised clinical trials. *Gastroenterol Res Pract* 2011:382852
- Phommalath S, Teraishi M, Yoshikawa T, Saito H, Tsukiyama T, Nakazaki T, Tanisaka T, Okumoto Y. 2014. Wide genetic variation in phenolic compound content of seed coats among black soybean cultivars. *Breed Sci* 64:409-415
- Revuelta-Iniesta R, Al-Dujaili EA. 2014. Consumption of green coffee reduces blood pressure and body composition by influencing 11 $\beta$ -HSD1 enzyme activity in healthy individuals: A pilot crossover study using green and black coffee. *Biomed Res Int* 2014:482704
- Ricciotti E, FitzGerald GA. 2011. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* 31:986-1000
- Saglam M, Koseoglu S, Hatipoglu M, Esen HH, Koksall E. 2015. Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. *J Appl Oral Sci* 23:33-41
- Setchell KD, Clerici C, Lephart ED, Cole SJ, Heenan C, Castellani D, Wolfe BE, Nechemias-Zimmer L, Brown NM, Lund TD, Handa RJ, Heubi JE. 2005. S-equol, a potent ligand for estrogen receptor beta, is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr* 81:1072-1079
- Wang Y, Dong G, Jeon HH, Elazizi M, La LB, Hameedaldeen A, Xiao E, Tian C, Alsadun S, Choi Y, Graves DT. 2015. FOXO1 mediates RANKL-induced osteoclast formation and activity. *J Immunol* 194:2878-2887
- Xing L, Xiu Y, Boyce BF. 2012. Osteoclast fusion and regulation by RANKL-dependent and independent factors. *World J Orthop* 3:212-222
- Zhao D, Shah NP. 2015. Tea and soybean extracts in combination with milk fermentation inhibit growth and enterocyte adherence of selected foodborne pathogens. *Food Chem* 180:306-316

---

Received 1 April, 2016

Revised 4 April, 2016

Accepted 6 April, 2016