รายงานวิจัยฉบับสมบูรณ์

โครงการ ฤทธิ์ยับยั้งของสารสกัดจากใบและเมล็ดงาม้อนที่อุดมไปด้วยกรดโรสมารินิก ต่อกระบวนการสร้างเซลล์ทำลายกระดูกที่ถูกกระตุ้นด้วยไซโตคายน์ ผ่านวิถีการส่งสัญญาณของ NF-KB (RANK)

โดย ดร. กนกกาญจน์ พรหมน้อย และคณะ

สิงหาคม 2562

สัญญาเลขที่ MRG5980170

รายงานวิจัยฉบับสมบูรณ์

โครงการ ฤทธิ์ยับยั้งของสารสกัดจากใบและเมล็ดงาม้อนที่อุดมไปด้วยกรดโรสมารินิก ต่อกระบวนการสร้างเซลล์ทำลายกระดูกที่ถูกกระตุ้นด้วยไซโตคายน์ ผ่านวิถีการส่งสัญญาณของ NF-KB (RANK)

ผู้วิจัย	สังกัด
ดร. กนกกาญจน์ พรหมน้อย	มหาวิทยาลัยพะเยา
ศาสตราจารย์เกียรติคุณ ดร. ไมตรี สุทธจิตต์	มหาวิทยาลัยพะเยา

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: MRG5980170

ชื่อโครงการ: ฤทธิ์ยับยั้งของสารสกัดจากใบและเมล็ดงาม้อนที่อุดมไปด้วยกรดโรสมารินิกต่อกระบวนการสร้างเซลล์ทำลายกระดูกที่ถูกกระตุ้นด้วยไซโตคายน์ผ่านวิถีการส่งสัญญาณของ NF-ΚB (RANK)

ชื่อนักวิจัย และสถาบัน: ดร. กนกกาญจน์ พรหมน้อย มหาวิทยาลัยพะเยา

ศาสตราจารย์เกียรติคุณ ดร. ไมตรี สุทธจิตต์ มหาวิทยาลัยพะเยา

อีเมล์: kphromnoi@gmail.com/ kanokkarn@up.ac.th

ระยะเวลาโครงการ: 2 ปี

บทคัดย่อ:

การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบและต้านการสร้างเซลล์สลายกระดูกของสารกัดจากงาม้อนรวมทั้งศึกษากลไกที่เกี่ยวข้อง ใบและกากงาม้อนนำมาสกัดโดย 70% แอลกอฮอล์ และแยกต่อโดยในสารลพลายที่มีขั้วแตกต่างกันตามลำดับดังนี้ เฮกเซน ไดคลอโรมีเทน เอทิลอะซิเตท และน้ำ จากนั้นนำสารสกัดที่ได้ไปวิเคราะห์ฤทธิ์ต้านอนุมูลอิสระ ต้านการอักเสบและสารประกอบออกฤทธิ์ทางชีวภาพที่สัมพันธ์กับการต้านการสร้างเซลล์สลายกระดูก ผลการทดลองชี้ให้เห็นว่า สารสกัดชั้นเอทิลอะซิเตทของทั้งใบและกากงาม้อนมีฤทธิ์ยับยั้งอนุมูลอิสระ การอักเสบและมีกรดโรสมารินิกสูงที่สุด ดังนั้นสารสกัดชั้นเอทิลอะซิเตทจึงเหมาะที่จะเรียกว่า “สารสกัดที่อุดมด้วยกรดโรสมารินิก” และใช้สำหรับศึกษาฤทธิ์ต่อกระบวนการสร้างและกระตุ้นเซลล์สลายกระดูกที่ถูกเหนี่ยวนำด้วยไซโตคายน์ก่อการอักเสบ อนุมูลอิสระชนิด ROS เป็นสัญญาณตัวกลางสำคัญในการสร้างเซลล์สลายกระดูก เพื่อให้ได้ข้อมูลเชิงลึกระดับโมเลกุล การส่งสัญญาณผ่าน RANKL ที่สามารถไปกระตุ้นกระบวนการสร้างเซลล์สลายกระดูกจะถูกนำมาศึกษาด้วย โดยสารสกัดที่อุดมด้วยกรดโรสมารินิกสามารถยับยั้งการสังเคราะห์อนุมูลอิสระชนิด ROS ในเซลล์แมโครฟาจชนิด RAW 264.7 ได้ สารสกัดนี้ยังสามารถลดจำนวนเซลล์สลายกระดูกที่มีหลายนิวเคลียสและลดกัมมันตภาพของเอนไซม์ TRAP การวิเคราะห์โดยวิธี western blot พบว่า สารสกัดที่อุดมด้วยกรดโรสมารินิก ยับยั้งกระบวนการสร้างเซลล์สลายกระดูกที่ถูกกระตุ้นด้วย RANKL ผ่านการควบคุมวิถีสัญญาณของ NF-ΚB c-Jun และ NFATc1 แสดงให้เห็นได้ว่าสารสกัดชั้นเอทิลอะซิเตทของทั้งใบและกากงาม้อนเป็นแหล่งที่อุดมด้วยกรดโรสมารินิกและใช้เป็นส่วนผสมในผลิตภัณฑ์เสริมอาหารและอาจมีประโยชน์สำหรับการป้องกันและรักษาโรคกระดูกเช่น โรคกระดูกพรุน ด้วย

คำหลัก : งาม้อน กระดูกพรุน กระบวนการสลายกระดูก ไซโตคายน์ การส่งสัญญาณผ่าน RANK

Abstract

Project Code: MRG598017

Project Title: Inhibitory effect of rosmarinic acid-rich extracts from leaves and seed of Nga-mon (perilla) on inflammatory cytokines-induced osteoclastogenesis through receptor activator of NF-κB (RANK) signaling pathway

Investigator: Dr. Kanokkarn Phromnoi University of Phayao

Prof. Emeritus Dr. Maitree Suttajit University of Phayao

E-mail Address: kphromnoi@gmail.com/ kanokkarn@up.ac.th

Project Period : 2 years

Abstract:

This study aimed to determine the antioxidant, anti-inflammatory, and anti-osteoclastogenic effects of perilla or Nga-mon extracts and identify the underlying mechanisms *in vitro*. The leaves and seed meal of perilla were extracted with 70% ethanol and sequentially fractionated according to the solvent's polarity with hexane (Hex), dichloromethane (DMC), ethyl acetate (EtOAc), and water (H_2O). The antioxidant, anti-inflammatory activities and bioactive compounds which related to anti-osteoclastogenic effects of these fractions were analyzed. The results indicated that the EtOAc fractions from both leaves and seed meal presented the highest antioxidant, anti-inflammatory activities, including rosmarinic acid (RA) contents. Therefore, these fractions were the most suitable as "RA-rich fractions" and used for study the inflammatory cytokines-induced osteoclast differentiation and activation. Reactive oxygen species (ROS) are crucial signal mediators in osteoclast differentiation. To attain molecular insights, the signaling pathways induced by receptor activator of nuclear factor kappa B ligand (RANKL) which trigger many transcription factors leading to the induction of osteoclastogenesis, were also investigated. RA-rich fractions inhibited RANKL-induced ROS production in RAW 264.7 cells. The fractions also attenuated RANKL-induced tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts and reduced RANKL-induced TRAP activity. Our western blot analysis found that the RA-rich fractions significantly inhibited RANKL-induced osteoclast differentiation by down-regulating nuclear factor kappa B (NF-κB), c-Jun and NFATc1 signaling pathway. It can be demonstrated here that, the EtOAc extract of perilla leaves and seed meal could thus be a valuable source of RA and used as a natural active pharmaceutical ingredient in dietary supplements and may be useful for the prevention and treatment of bone diseases, such as osteoporosis.

Keywords: Perilla (Nga-mon), Osteoporosis, Osteoclastogenesis, Cytokine, RANK signaling

Executive summary

Our preliminary research focus on the polarity-based fractionation of perilla (Nga-mon) leaves and seed meals according to the solvent's polarity with hexane (Hex), dichloromethane (DMC), ethyl acetate (EtOAc), and water (H_2O). HPLC results indicated that the EtOAc fractions presented the highest RA. The perilla fractions were analyzed the scavenging effects on DPPH•, ABTS•+, O2•- and nitric oxide (NO) radicals, FRAP assay, and determination of the inhibition effects on NO, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) production in the cell-based study. The EtOAc fractions had shown the highest antioxidant and anti-inflammatory activities. Therefore, these fractions were called "RA-rich fractions" and used to study the inflammatory cytokines-induced osteoclast differentiation and activation.

The effect of RA-rich fractions on inflammatory cytokines-induced osteoclast in terms of differentiation, activation, and the underlying mechanism had been evaluated. RANKL delivers an important signal for osteoclast differentiation. The generation of intracellular reactive oxygen species (ROS) is considered a crucial step for RANKL-induced osteoclastogenesis. Moreover, the activation of NF-κB, AP-1, and NFATc1 are induced by RANKL signaling in osteoclast precursor cells.

The result found that RA-rich fractions inhibited ROS production in RAW 264.7 cells using DCFH assay. From TRAP staining and TRAP activity assays, the fractions attenuated RANKL-induced the number of TRAP-positive multinucleated osteoclasts and decreased RANKL-induced TRAP activity. The western blot was also investigated and found that the RA-rich fractions significantly inhibited RANKL-induced osteoclast differentiation by downregulating nuclear factor kappa B (NF-κB), c-Jun and NFATc1 signaling pathway.

Therefore, the EtOAc fractions from perilla leaves and seed meal could thus be a valuable source of RA and used as a natural active pharmaceutical ingredient in dietary supplements and nutraceuticals and may be useful for the prevention and treatment of osteoporosis.

วัตถุประสงค์ (Objectives)

1. To study the effect of differential solvents on the polyphenols, flavonoids, rosmarinic acid (RA), anti-inflammatory and antioxidant activities of perilla leave and seed meals.

2. To analyze the inhibitory effect of RA-rich fractions from perilla leaves and seed meal on inflammatory cytokine-induced osteoclastogenesis in RAW 264.7 cells

3. To investigate the molecular mechanism of RA-rich fractions on inflammatory cytokine-induced osteoclastogenesis through RANK signaling pathway

วิธีทดลอง (Material and Methods)

1. Collection and preparation of plants

Nga-mon was collected from Wiang-Sa district, Nan province, Thailand. The voucher specimen code is QSBG-K2 , prepared by Dr.Komsak Pintha and Dr.Payungsak Tantipaiboonwong, and certified by the Queen Sirikit Botanic Garden Herbarium, Chiang Mai, Thailand. The seed of Nga-mon will be extracted for oil by Cold Press Oil Machine, and seed oil will be then centrifuged until be made transparent yellow oil. The seed meal, by produced had been collected for our experiment.

2. Extraction and Partial purification of RA-rich fraction from perilla leaves or seed meal

Dried perilla leaves and seeed meal were extracted with 70% ethanol (EtOH) to obtain the EtOH crude powder which was then sequentially partitioned with hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc), and residue aqueous phase is water (H2O) (**Figure 1)**. The percent yield of extracts and fractions were calculated as % w/w dry base. Each fraction was stored at 20°C and suspended in dimethyl sulfoxide (DMSO) before use.

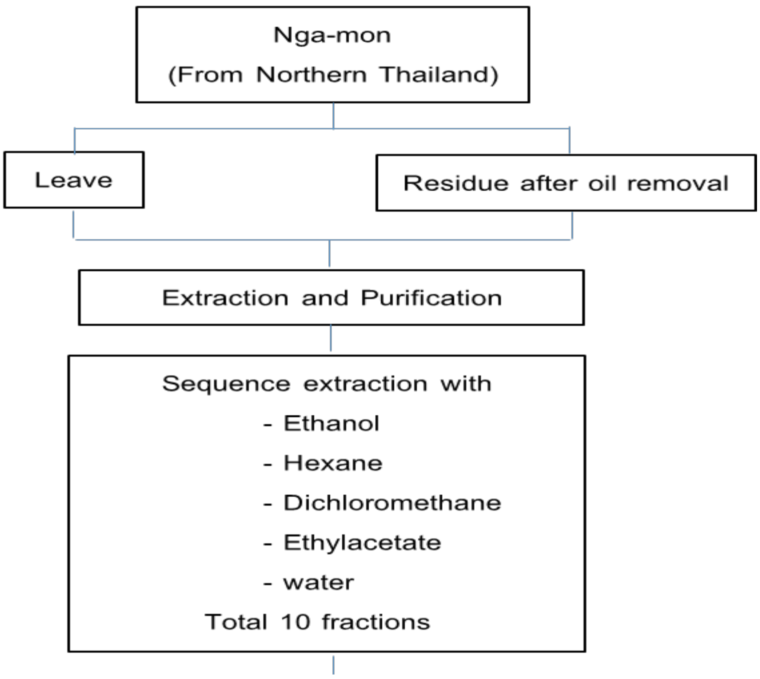

Figure 1 Polarity-based fractionation of perilla

3. Total phenolic content (TPC)

TPC was determined using the Folin–Ciocalteu method of Hossain *et al*. (1). Briefly, 20 μL of the sample was mixed with 100 μL of 10% Folin–Ciocalteu reagent and 80 μL of 7.5% Na_2CO_3. After 30 mins at room temperature, the absorbance at 765 nm was measured, and TPC was estimated using a standard curve of gallic acid. TPC was expressed as milligram gallic acid equivalent per 1 g fraction (mg GAE/g fraction).

4. Total flavonoid content (TFC)

TFC was examined using the aluminum chloride colorimetric method with slight modification (2). Initially, 25 μL of the fraction and 125 μL deionized water were mixed with 7.5 μL of 5% $NaNO_2$ solution and incubated at room temperature for 6 min. Then, 15 μL of 10% $AlCl_3$ was added and incubated for another 6 min. Color development was performed by adding 50 μL of 1 M NaOH. The final volume of the reaction mixture was adjusted to 250 μL using deionized water. The absorbance was measured at 510 nm. TFC was calculated using a standard curve of catechin and expressed as milligram of catechin equivalent per 1 g fraction (mg CE/g fraction).

5. Measurement of RA

RA was determined by reversed-phase HPLC using the Agilent 1200 equipped with the multi-wavelength and fluorescence detectors. The assay was carried out using a Symmetry Shield RP18 column (4.6 mm × 250 mm, 5 μm particle diameters, Waters Co., Ltd.), and 30% acetonitrile in 0.1% acetic acid and H_2O was used as a mobile phase with a flow rate 1.0 mL/min. The peaks were detected using a UV detector at 330 nm. The rosmarinic acid content was rechecked with a fluorescence detector with excitation of wavelength at 330 nm and emission of wavelength at 400 nm. All samples were measured in triplicates.

6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging assay was performed as previously described (3). Various concentrations of each sample (20 μL) were mixed with 180 μL of freshly prepared DPPH methanolic solution and kept in the dark for 30 mins before measuring with an AccuReader microplate reader (Metertech Taiwan) at 540 nm. The standard curve was of ascorbic acid. Results were expressed as 50% DPPH decolorization (IC_{50}).

7. 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging assay

The ABTS free radical-scavenging assay was also performed using the method explained above (4) but with slight modifications. The ABTS solution was dissolved in potassium persulfate and kept in the dark for 12–14 h. Before use, this solution was diluted with distilled water to get an absorbance at 734 nm of approximately 0.70. The various concentrations of each fraction (10 μL) were mixed with 990 μL of working diluted ABTS and incubated for 6 min in the dark. The absorbance was measured at 734 nm. The standard curve was of ascorbic acid and Trolox. Results showed 50% ABTS decolorization (IC_{50}).

8. Superoxide anion radical scavenging assay

The scavenging effects on superoxide anion of samples were assayed according to the method of Saenjum et al. (5). This method is based on the power of the samples to inhibit formazan formation in a phenazine methosulfate (PMS) - β-nicotinamide adenine dinucleotide (NADH) system. Superoxide anion radicals were generated in an NADH-PMS system through the oxidation of NADH and then analyzed by the reduction of nitroblue tetrazolium (NBT). The reaction mixture was made in PBS buffer (pH 7.4), which contained 78 μM of NADH, 25 μM of NBT and 45 μM of EDTA, and combined with different concentrations of the tested samples or positive controls (L-ascorbic acid and RA). PMS was added to initiate the reaction, and after 5 min of incubation in the dark, the absorbance was measured at 560 nm. All samples were tested in triplicate. The decreased absorbance indicated increased superoxide anion scavenging activity.

9. Nitric oxide radical scavenging assay

In vitro NO-scavenging activity is analyzed using Griess reaction. The reaction mixture is composed of 6.25 M sodium nitroprusside in PBS buffer and positive control (curcumin and RA) or tested samples. The reaction mixtures were incubated at 37^{o}C for 150 min. Then, the reaction mixture was transferred to a 96-well plate. The Griess reagent, a mixture of naphthylethylene diamine and sulphanilamide was added and incubated at room temperature for 5 min. The absorbance was measured at 540 nm. All samples were tested in triplicate.

10. Ferric reducing/antioxidant power (FRAP) assay

40 μL of each extract was mixed with 3 mL of FRAP reagent, and the reaction mixture was incubated at 37°C for 4 min before it was measured at 593 nm using a spectrophotometer. The blank solution consisted of 40 μL distilled water in 3 mL FRAP reagent and was incubated at 37°C for 1 h. The standard solutions consisted of $FeSO_4 \cdot 7H_2O$ in different concentrations. The results were expressed as mg Fe (II) per 1 g fraction (mg Fe(II)/g fraction).

11. Cell viability assay

Cells were seeded into each well of a 96-well plate and incubated with fractions at different concentrations for 48 h. Then, 15 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was added and the samples were incubated at 37°C for 4 h. The excess MTT dye solution was removed, and only MTT formazan that stained living cells were re-dissolved in DMSO. The color intensity was measured at 540 and 630 nm using a microplate reader.

12. Inhibition of NO, iNOS, and COX-2 production

Briefly, RAW 264.7 cells were cultured with DMEM in 24-wells plate for 24 h. Then, cells were replaced with a new medium containing various concentrations of tested samples and incubated for 12 h. After that, lipopolysaccharide (LPS) and interferon-γ (IFN-γ) was added and incubated for 48 h, the culture supernatants were collected to measure for NO production using Griess reaction, and the cell lysates were measured for iNOS and COX-2 using immunoassay kit. The results were represented as 50% inhibitory concentration values (IC_{50}). Curcumin and RA were used as a positive control (6).

13. ROS activity using Dichlorofluorescin (DCFH) assay

The inhibitory effect of the extracts on intracellular ROS production was investigated using the DCFH-DA method as previously described (7) with minor modifications. Cells will be seeded in 96-well cell culture plate and treated with cytokines or the fractions for the indicated time. Remove media from all wells. Wash cells gently with DPBS or HBSS 2-3 times. Remove the last wash and discard. 100 µL of DCFH-DA solution final concentration 40 µM was loaded 1 hour before the incubation time will be terminated.The cells were measured for the green fluorescent intensity by a fluorescent microplate reader with 525 nm excitation and 480 nm emission wavelength. 50 μM N-acetylcysteine and 250 μM ascorbic acid were used as anti-oxidant control

14. Tartrate-resistant acid phosphatase (TRAP) staining assay

RAW 264.7 cells will be seeded on a 96-well plate at a density of 5.0×10^3 cells/well after that incubated with RANKL at various concentration and subsequently cultured for 9 days. The cells were subjected to TRAP staining using the leucocyte acid phosphatase kit (sigma Procedure No.387). TRAP-positive osteoclasts with more than 3 nuclei will be considered to be osteoclasts. The number of osteoclasts will be counted under a microscope.

15. Tartrate-resistant acid phosphatase (TRAP) activity assay

After treatment, the cells were subjected to TRAP activity using acid phosphatase kit (sigma Procedure No CS0740) with some modification. Plate RAW 264.7 cell 1x 10^4 cell/1ml/well of 24 well plates and incubate for 24 h. Remove ole medium and treat cell with RANKL(50 ng/ml) or extracts (various concentration) in 300 ul alpha-MEM+C-MSF 30 ng/ml /well and change media every 3 days. After treatment for 6 days, remove culture supernatant, add 1 ml PBS in each well for flush cell with autopipette and collect cell in 1.5 or 2 ml microcentrifuge tube. Centrifuge 12,000 rpm, 4^oC, 5 mins and collect pellet. Add 100 ul 0.1% TritonX-100 (in citrate buffer), keep in -80oC >2h and defrost at 37 ^oC. Afterthat, mix **25** ul of lysate with **25** ul of 1 mg/ml pNPP (in citrate buffer: freshly prepare) and incubate 37 oC at dark (CO_2 incubator) for 4 h. Stop reaction with 0.5 M NaOH **25** ul/well. The colour intensity was measured at 405 nm using a microplate reader. PNP was use as standard. RA was used as anti-osteoclastogenesis controls.

Acid phasphatase

RXN : pNPP -------------------> PNP (OD 405 nm)

(citrate buffer)

16. Western blot analysis

Equal amounts of treated cells will be resuspended in sample buffer and separated by SDS-PAGE. After electrophoresis, proteins were electroblotted to a Hybond–C Extra nitrocellulose membrane. The membrane was blocked at room temperature (RT) with 4% BSA. Membranes will be further probed with primary antibodies. The blots will be washed and probed with secondary antibodies. After incubation, the immunoreactive material was visualized by enhanced chemiluminescence.

17. Statistical analysis

Data are shown as the mean ± standard deviation of three independent experiments. The statistical analysis was determined using one-way analysis of variance. Significant differences at the level of $p < 0.05$ and < 0.01 were determined by Tukey's multiple comparison test, and data correlation was obtained by Pearson correlation test, using IBM SPSS Statistics 22

ผลการทดลอง (Results and discussion)

1. Extraction & Purification of Nga-mon (Perilla)

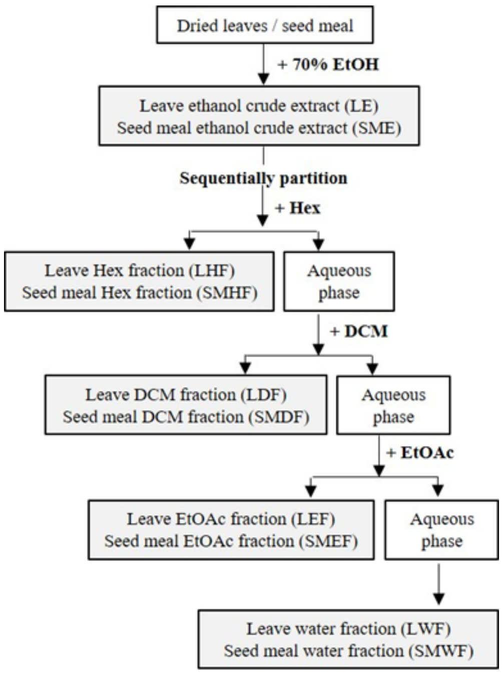

Figure 2 Perilla extraction and partition.

2. Yield and physical properties of perilla fractions

The percent yields of ethanolic crude extract of leaves (LE) and seed meal (SME) were 13.43 and 5.64% w/w, respectively. The crude extract was sequentially partitioned with Hex, DCM, EtOAc, and H_2O fractions. The percent yields by the above solvents from leaves were 42.2, 14.5, 12.2, and 30.8%, respectively, and from seed meal were 15.97, 1.95, 2.77, and 54.46%, respectively. Each fraction produced different yields according to the solvent used and its PI (8). The results showed that the lower yield was noted in EtOAc and DCM fractions compared with the Hex and H_2O fractions. This may be due to the presence of polyphenols in the fractions. Moreover, various colours and appearance of fractions were observed (**Table 1**), which may indicate different ingredients such as polyphenols, chlorophyll, resin, tannin, nutrients, and minerals (9, 10).

Table 1 Percent yield and physical properties of perilla fraction.

Solvents	Polarity index (PI)	Leaves Abbr.[1]	Leaves % Yield	Seed meal Abbr.[1]	Seed meal % Yield	Physical Properties
Ethanol (EtOH)	**5.2**	**LE**	13.4	**SME**	5.6	Green-brownish powder
Hexane (Hex)	**0**	**LHF**	42.5	**SMHF**	16.0	Dark-brownish sticky
Dichloromethane (DCM)	**3.7**	**LDF**	14.5	**SMDF**	2.0	Dark green-brownish sticky
Ethyl acetate (EtOAc)	**4.4**	**LEF**	12.2	**SMEF**	2.8	Yellow-brownish sticky
Water (H_2O)	**9**	**LWF**	30.8	**SMWF**	54.5	Brown powder

[1] Abbr. = Abbreviation

3. Total phenolic, flavonoid content of perilla fractions

Phenolic compounds are the most ubiquitous groups of plant secondary metabolites and known for their antioxidant activities (11). The chain reaction of lipid peroxidation can be stopped through the donation of a hydrogen from phenolic compounds to a free radical, which acts as an antioxidant. The total amount of the phenolic compounds was evaluated by using the regression equation of the calibration curve and expressed in gallic acid equivalents (GAE).

The TPC values of LF (from 399.0 ± 1.5 to 5225.9 ± 5.7 mg GAE/g fraction) were significantly higher than those of SMF (from 2.3 ± 0.0 to 55.0 ± 0.3 GAE/g fraction). This result is consistent with previous studies that found that the TPC of crude extracts of leaves was greater than the seed crude extracts (12) and in seed EtOH extract compared with the seed meal EtOH extract (3). The highest TPC was detected in the leaf and seed meal EtOAc fractions (5225.9 ± 5.7 and 55.0 ± 0.3 GAE/g fraction, respectively) and the lowest in leaf DCM fraction and seed meal hexane fraction (399.0 ± 1.5 and 2.3 ± 0.0 GAE/g fraction, respectively).

Inconsistent with the TPC results, LF exhibited a greater TFC (218.8 ± 4.1 to 4,012.6 ± 15.9 mg CAE/g fraction) compared with SMF (0.9 ± 0.0 to 41.8 ± 1.1). The highest TFC was found in the leaf and seed meal EtOAc fractions (4,012.6 ± 15.9 and 41.8 ± 1.1 mg CAE/g fraction, respectively). The declining level of LF was sequenced by leaf EtOH extract (1,293.3 ± 9.7 mg CAE/g fraction), leaf water fraction (1,010.6 ± 1.4 mg CAE/g fraction), leaf hexane fraction (305.0 ± 7.6 mg CAE/g fraction), and leaf DCM fraction (218.8 ± 4.1 mg CAE/g fraction). The seed meal EtOH extract had the second highest TFC (6.5 ± 0.0 mg CAE/g fraction), followed by the seed meal water fraction (5.4 ± 0.1 mg CAE/g fraction), seed meal DCM fraction (3.2 ± 0.2 mg CAE/g fraction), and seed meal hexane fraction (0.9 ± 0.0 mg CAE/g fraction). As shown in **Table 2**.

The polarity of a solvent is a primary determinant in the amount of extracted compounds, especially polyphenolics and flavonoids, which are commonly present in rosemary and tea (13). In this study, ethyl acetate (PI = 4.4) was found to be the most effective solvent for the extraction of polyphenolics and flavonoids. Normally, polar solvents contain hydroxyl groups that can hydrogen bond with polyphenolics and flavonoids (14). Reasonably, the TPC and TFC was high in the H_2O, EtOH, and EtOAc fractions, which have higher PI than the Hex and DCM fractions (15).

4. RA levels in the perilla fractions

RA, an ester of caffeic acid and 3-(3,4-dihydroxyphenyl) lactic acid, is found in several plants of the Lamiaceae family, such as perilla, balm mint, and rosemary (16-18). RA is known to have a number of potentially beneficial biological effects including antioxidant, anti-inflammation, anti-allergy, anti-glycation, and protective effects on the nervous system and the liver (19-21). It was reported that polyphenolic compounds in the ethanolic extract of perilla leaves are primarily composed of RA by ultra-HPLC (22). Perilla parts also contain other polyphenolics such as luteolin, apigenin, caffeic acid, and their glucosides, as well as ferulic acid, vanillic acid, chlorogenic acid, and 4',5,7-trimethoxyflavone (23, 24). However, the sequential fractionation and purification of RA from perilla leaves and seed meal have not been extensively investigated and reported.

RA levels in all of the fractions were therefore determined by HPLC. The EtOAc fraction of leaves and seed meal contained the highest amount of RA (303.3 ± 6.4 and 376.8 ± 1.9 mg/g, respectively) **(Figure 3 and Table 2)**. Korean and Chinese investigators (24, 25) also recently demonstrated that the EtOAc fraction of perilla leaves and seed flour had the highest content of polyphenolics and flavonoids and the most abundant phenolic acid was RA.

RA contains carboxyl and hydroxyl groups, which easily form hydrogen bonds with polar solvents (15, 26); therefore, H_2O, EtOH, and EtOAc can extract more RA than Hex and DCM. The results in this study also showed that the purity of RA from the seed meal EtOAc fraction was approximately 18-fold higher than that of the EtOH fraction. Thus, we can conclude that the solubilisation of RA in the EtOAc fraction is related to the PI of the solvent. Moreover, we demonstrated that the oil-removed perilla seed meal is rich in RA, which could be isolated and purified by sequential extraction.

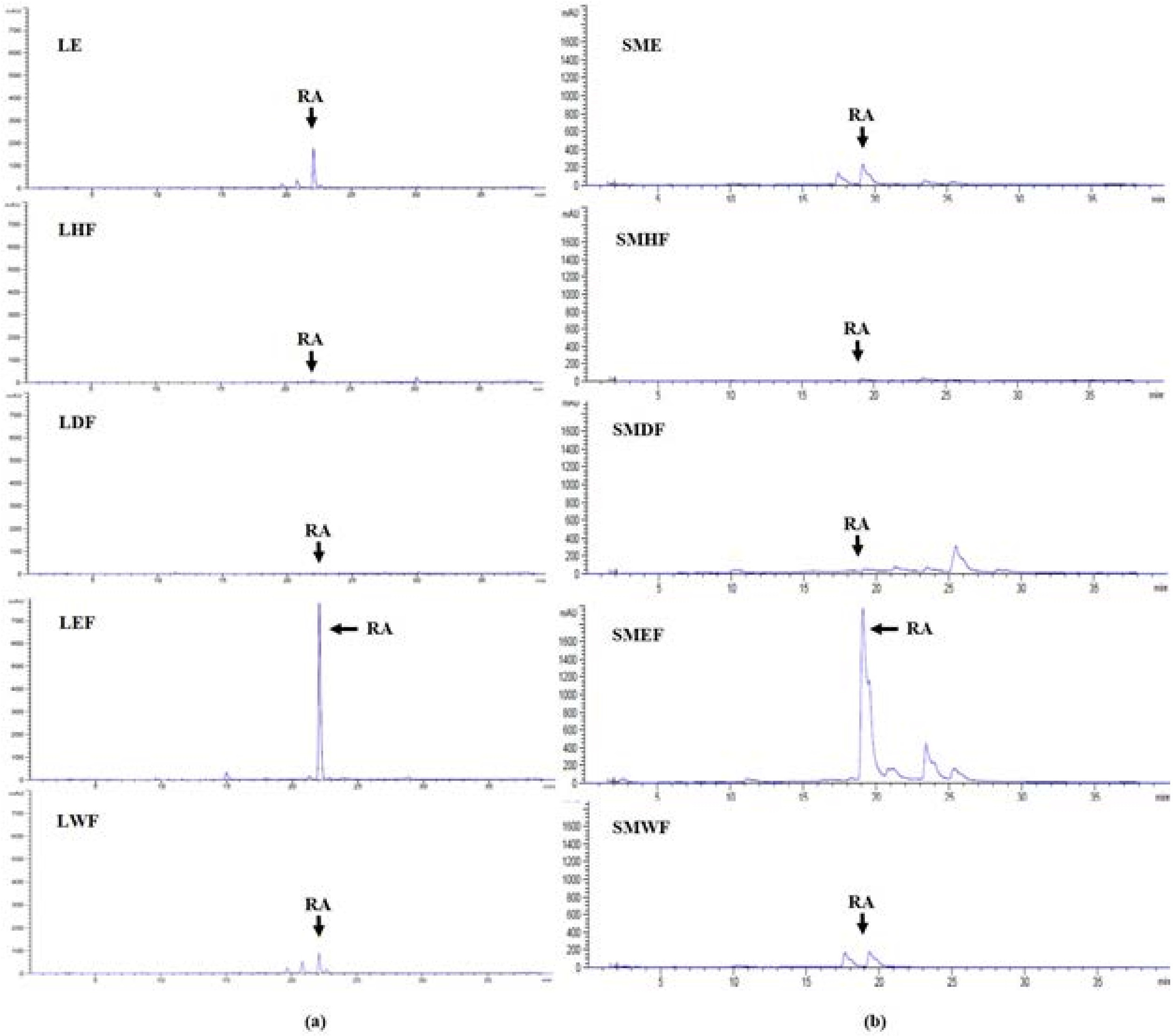

Figure 3 High performance liquid chromatography (HPLC) chromatograms of rosmarinic acid (RA) in the leaf fraction **(a)** and seed meal fraction **(b).** Detection was at 280 nm. Retention times (min) of RA peaks of each fraction are shown.

Table 2 Total phenolic, flavonoid, and RA content of perilla fractions.

Extract & fractions	TPC mg GAE/g fraction	TFC mg CAE/g fraction	RA mg /g fraction
LE	2022.1 ± 9.2 [b]	1293.3 ± 9.7 [b]	69.1 ± 1.8 d
LHF	447.5 ± 0.7 [d]	305.0 ± 7.6 [d]	6.6 ± 0.7 b
LDF	399.0 ± 1.5 [e]	218.8 ± 4.1 [e]	2.9 ± 0.6 a
LEF	5225.9 ± 5.7 [a]	4012.6 ± 15.9 [a]	303.3 ± 6.4 e
LWF	1753.4 ± 11.0 [c]	1010.6 ± 1.4 [c]	45.2 ± 0.9 c
SME	10.8 ± 0.0 [c]	6.5 ± 0.0 [b]	42.2 ± 0.3 [b]
SMHF	2.3 ± 0.0 [e]	0.9 ± 0.0 [e]	4.5 ± 0.0 [d]
SMDF	11.2 ± 0.0 [b]	3.2 ± 0.2 [d]	3.8 ± 0.0 [e]
SMEF	55.0 ± 0.3 [a]	41.8 ± 1.1 [a]	376.8 ± 1.9 [a]
SMWF	8.0 ± 0.0 [d]	5.4 ± 0.1 [c]	32.0 ± 0.5 [c]

All values are expressed as mean ± standard deviation (SD; n = 3).

Different letters in each group (leaves and seed meal) indicate a significant difference ($p < 0.05$).

5. ***In vitro*** antioxidant activities of perilla fractions

The antioxidant activity of perilla fractions was measured by DPPH, ABTS radical scavenging assay. In the DPPH assay, the EtOAc fractions, LEF and SMEF, possessed the highest antioxidant activity with IC_{50} values of 9.14 ± 0.54 and 9.20 ± 0.69 μg/mL, respectively. The Hex fractions, LHF and SMHF, exhibited less scavenging activity with IC_{50} values > 200 and 86.1 ± 5.9 μg/mL (**Table 3**). Consistent with a previous study (27), the data showed the scavenging abilities of DPPH radicals at 0.5 mg fraction/mL were highest in the EtOAc fractions and lowest in the Hex fractions of the perilla leaf.

In the ABTS assay, the LEF and SMEF also had the highest antioxidant activity with IC_{50} values of 4.2 ± 0.3 and 3.6 ± 0.1 μg/mL. The LHF and SMHF also exhibited less scavenging activity with IC_{50} values of 48.2 ± 1.4 and 86.1 ± 5.9 μg/mL (**Table 3**). Similar results have been reported previously (23-25).

The ability of perilla fractions to scavenge $O_2^{\bullet -}$ and NO radicals were also analyzed using NADH-PMS system and Griess reaction assay. The calculated IC_{50} values denote the concentrations of the fraction required to decrease the scavenging activity by 50%. Moreover, the results are shown in **Table 3**. The radical scavenging showed that the EtOAc fraction possessed the highest antioxidant activity. The DCM fraction exhibited less scavenging activity. Surprisingly, nitric oxide radical scavenging activity in EtOAc fraction was almost equal to rosmarinic acid, which was used as a positive control. Beside scavenging assay, antioxidant activity was also measured by ferric reducing/antioxidant power assay; the highest and lowest antioxidant capacity was also observed in the EtOAc and DCM fractions, respectively.

Table 3 Antioxidant activities of perilla fractions

Fractions & standards	Reducing power assay (mg Fe (II)/g fraction)	Radical scavenging assay IC_{50} (μg/mL)			
	FRAP	DPPH$^{\bullet}$	ABTS$^{\bullet+}$	$O_2^{\bullet-}$	NO
PE	1,326.6 ± 67.4 ^{b}	23.0 ± 3.2 ^{a}	5.6 ± 0.1 ^{b}	24.5 ± 0.82 ^{d}	30.64 ± 1.24 ^{c}
PHF	279.1 ± 12.2 ^{a}	110.6 ± 5.9 ^{c}	48.2 ± 1.4 ^{d}	45.2 ± 2.45 ^{e}	66.64 ± 1.25 ^{d}
PDF	213.0 ± 12.7 ^{a}	157.6 ± 7.7 ^{d}	48.8 ± 1.6 ^{d}	87.7 ± 2.54 ^{f}	99.17 ± 2.77 ^{e}
PEF	4,759.1 ± 183.6 ^{c}	9.1 ± 0.5 ^{a}	4.2 ± 0.3 ^{b}	18.4 ± 0.57 ^{c}	17.44 ± 4.68 ^{b}
PWF	1,328.9 ± 16.2 ^{b}	99.4 ± 6.4 ^{c}	12.2 ± 0.7 ^{c}	24.9 ± 0.93 ^{d}	36.46 ± 0.93 ^{c}
SME	667.6±14.7 ^{a}	76.6 ± 5.1 ^{b}	20.7 ± 0.4 ^{c}	28.7 ± 1.39 ^{c}	40.63 ± 1.32 ^{d}
SMHF	156.7±6.4 ^{a}	>200 ^{d}	86.1 ± 5.9 ^{e}	81.9 ± 3.25 ^{d}	95.86 ± 3.04 ^{f}
SMDF	294.3±5.9 ^{a}	162.9 ± 11.7 ^{c}	16.2 ± 0.2 ^{b}	92.8 ± 3.37 ^{e}	100.05 ± 3.20 ^{g}
SMEF	17,086.6±313.2 ^{b}	9.2 ± 0.7 ^{a}	3.6 ± 0.1 ^{a}	19.5 ± 1.13 ^{b}	26.55 ± 0.87 ^{c}
SMWF	610.6±17.7 ^{a}	25.6 ± 1.4 ^{a}	30.6 ± 0.5 ^{d}	32.0 ± 1.79 ^{c}	43.89 ± 1.24 ^{e}
L-ascorbic acid		13.6 ± 1.3 ^{b}	2.1 ± 0.0 ^{a}	6.9 ± 0.3 ^{a}	
RA				13.1 ± 0.5 ^{b}	17.7 ± 1.0 ^{b}
Curcumin					9.6 ± 0.6 ^{a}

All values are expressed as mean ± standard deviation *(*SD; n = 3*).*

Different letters indicate a significant difference *(*$p < 0.05$*).*

6. Cell-based study of anti-inflammatory activities of perilla fractions

To evaluate the effect of the perilla fractions on the LPS and IFN-γ-stimulated the production of NO, iNOS, and COX-2 in RAW 264.7 cells, Griess reaction assay and ELISA were measured. As displayed in **Table 4**, amongst the fractions, the most active response was seen in the EtOAc fraction in all inflammatory proteins. The weak inhibition was found in the DCM fraction.

Table 4 Inhibition of NO, iNOS, and COX-2 production

Fractions & standards	IC_{50} (µg/mL)		
	NO	iNOS	COX-2
PE	26.4 ± 1.0^{d}	34.3 ± 1.5^{d}	39.4 ± 1.7^{d}
PHF	33.3 ± 1.4^{e}	38.3 ± 1.6^{e}	42.6 ± 2.1^{d}
PDF	44.3 ± 1.5^{f}	53.9 ± 1.3^{g}	> 100
PEF	17.9 ± 0.7^{c}	24.2 ± 1.8^{c}	26.9 ± 1.4^{c}
PWF	24.1 ± 1.1^{d}	42.2 ± 1.6^{f}	49.6 ± 2.8^{e}
SME	28.1 ± 1.6 d	34.4 ± 1.5 d	39.4 ± 1.7 d
SMHF	43.0 ± 1.6 e	38.3 ± 0.6 e	42.6 ± 0.1 d
SMDF	54.2 ± 1.7 f	53.9 ± 2.3 g	> 100
SMEF	21.2 ± 1.4 c	24.2 ± 2.7 c	26.9 ± 2.4 c
SMWF	29.7 ± 2.0 d	42.2 ± 7.6 f	49.6 ± 7.8 e
RA	13.2 ± 0.6^{b}	17.5 ± 1.3^{b}	21.3 ± 1.8^{b}
Curcumin	7.5 ± 0.7^{a}	8.9 ± 0.6^{a}	9.4 ± 0.7^{a}

All values are expressed as mean ± standard deviation (SD; n = 3).

Different letters in each group (leaves and seed meal) indicate a significant difference ($p < 0.05$).

7. Effect of perilla fractions on cells viability

In the present study, cellular cytotoxicity was measured using the MTT assay. Our results demonstrated that the perilla fractions at concentrations 100-200 µg/mL were safe and could be used for the further experiments (**Figure 4-5**).

(A) (B)

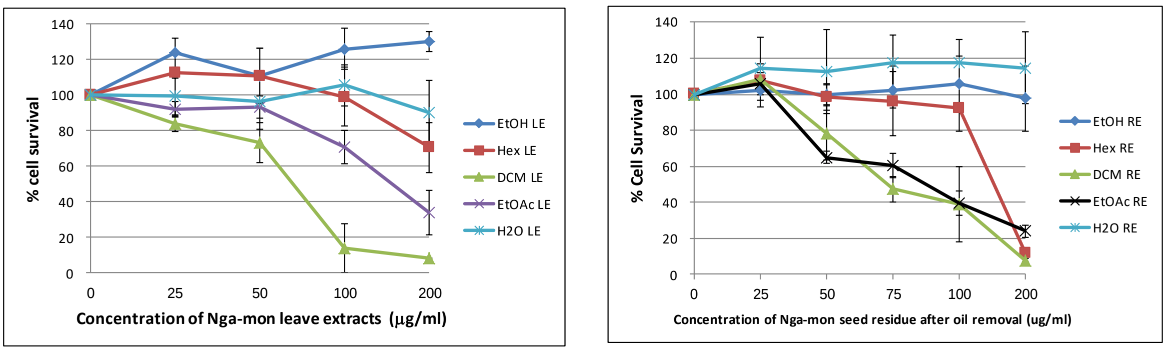

Figure 4 Cytotoxicity of perilla fractions on murine macrophage/osteoclast precursor cell line (RAW 264.7) using MTT assay. **(A)** Perilla leave extract **(B)** Perilla seed meal extract. The intensity was determined by microplate reader (540/630). Data are expressed as percentages of the value of the control cells. Each value represents mean ± SD in triplicate.

(A) (B)

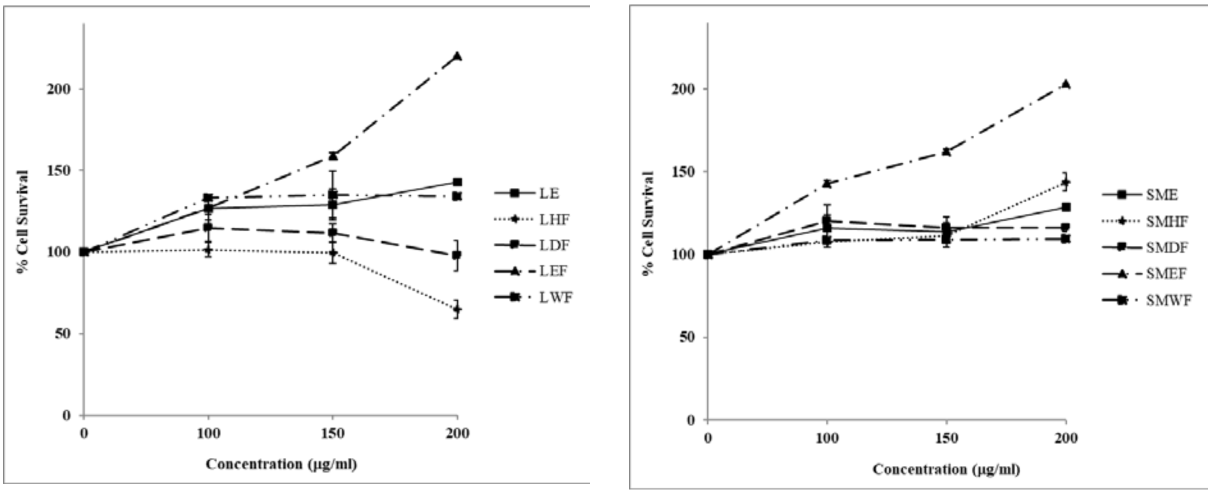

Figure 5 Cytotoxicity of perilla fractions on peripheral blood mononuclear cells (PBMCs) using MTT assay. **(A)** Perilla leave extract **(B)** Perilla seed meal extract. The intensity was determined by microplate reader (540/630). Data are expressed as percentages of the value of the control cells. Each value represents mean ± SD in triplicate.

8. Effects of perilla fractions on H_2O_2-induced ROS in RAW 264.7 macrophages and PBMCs.

Hydrogen peroxide (H_2O_2) is an important indicator of ROS accumulation and is used in many studies to induce ROS. Treatment with H_2O_2 increased ROS generation and set as 100%. But ROS generation was significantly reduced in the presence of the perilla fractions as the dose-dependent manner (**Figure 6-7**).

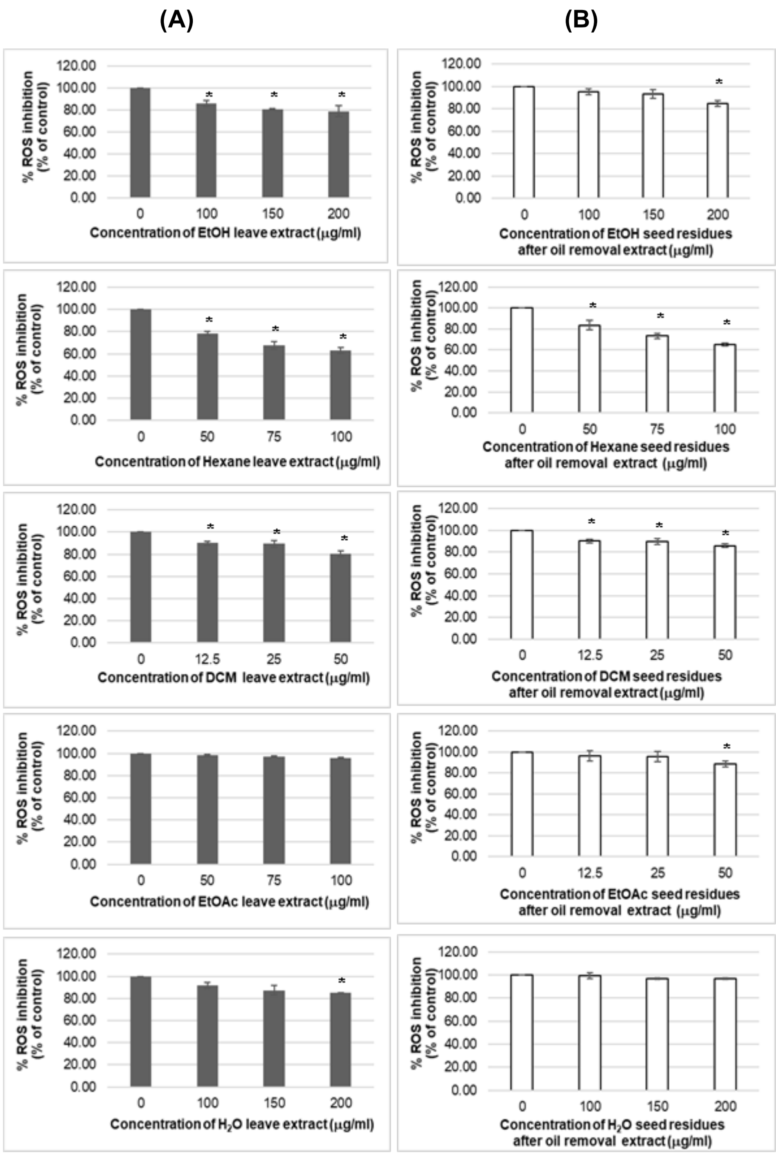

Figure 6 Effect of perilla fractions on ROS production induce by H_2O_2 using DCFH assay in RAW 264.7 cells. ROS production was measured by a fluorescent plate reader (485/525). Data are expressed as percentages of the value of the control cells. Each value represents mean ± SD in triplicate. (* P<0.05, vs. control).

(A)

(B)

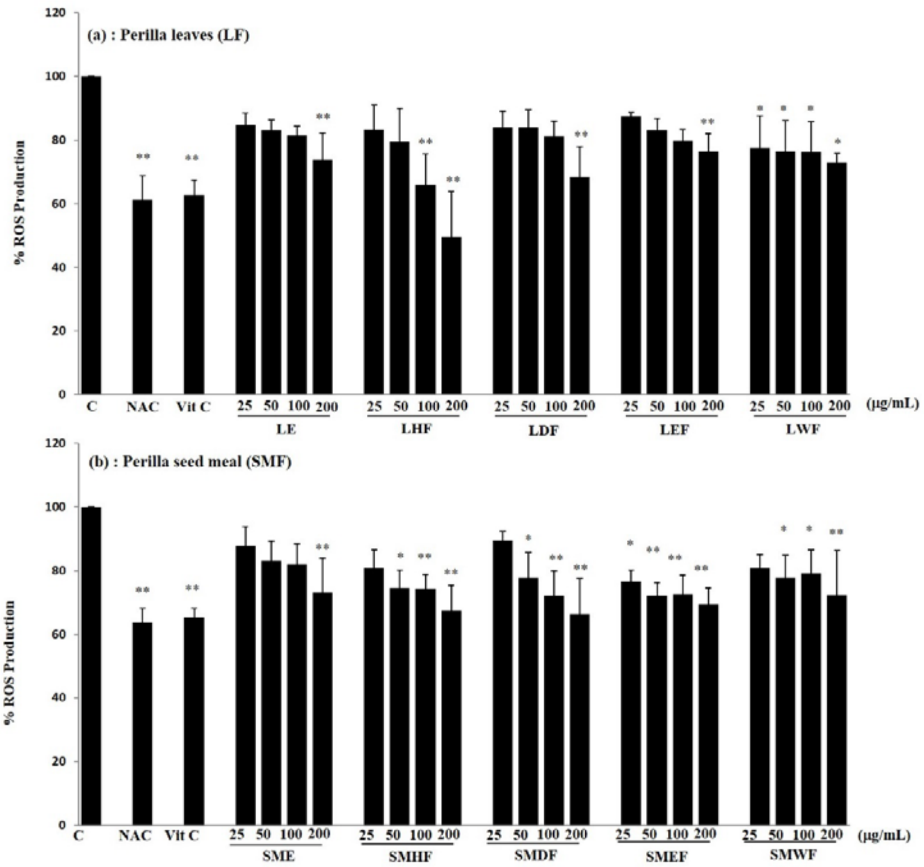

Figure 7 Effects of perilla fractions on H_2O_2-induced reactive oxygen species (ROS) in PBMCs. Untreated cells were used as a negative control. N-acetylcysteine (80 μM) and ascorbic acid (250 mM) were used as positive controls. Data represent the mean ± SD of three independent experiments. *p < 0.05, ** p < 0.001 versus untreated cells. Error bars indicate SD. LE, leaf EtOH extract; LHF, leaf hexane fraction; LDF, leaf DCM fraction; LEF, leaf EtOAc fraction; LWF, leaf water fraction; SME, seed meal EtOH extract; SMHF, seed meal hexane fraction; SMDF, seed meal DCM fraction; SMEF, seed meal EtOAc fraction; and SMWF, seed meal water fraction.

9 . Effects of perilla fractions on RANKL-induced ROS in RAW 264.7 macrophages and PBMCs.

The sustained generation of intracellular ROS is considered an important step for RANKL-induced osteoclastogenesis. In various receptor signaling pathways, ROS acts as a second messenger. It is reported that the production of ROS in response to RANKL is increased and acts as an intracellular mediator for the ERK signaling pathway in osteoclast differentiation and activation (28). Therefore, it was determined if the fractions affected RANKL-induced ROS generation.

The intracellular ROS production in RAW264.7 cells after the treatment with RANKL as 100% (control group). The generation of intracellular ROS was dose-dependently attenuated by the treatment of perilla fractions within the concentration range from 25-200 μg/mL (**Figure 8**).

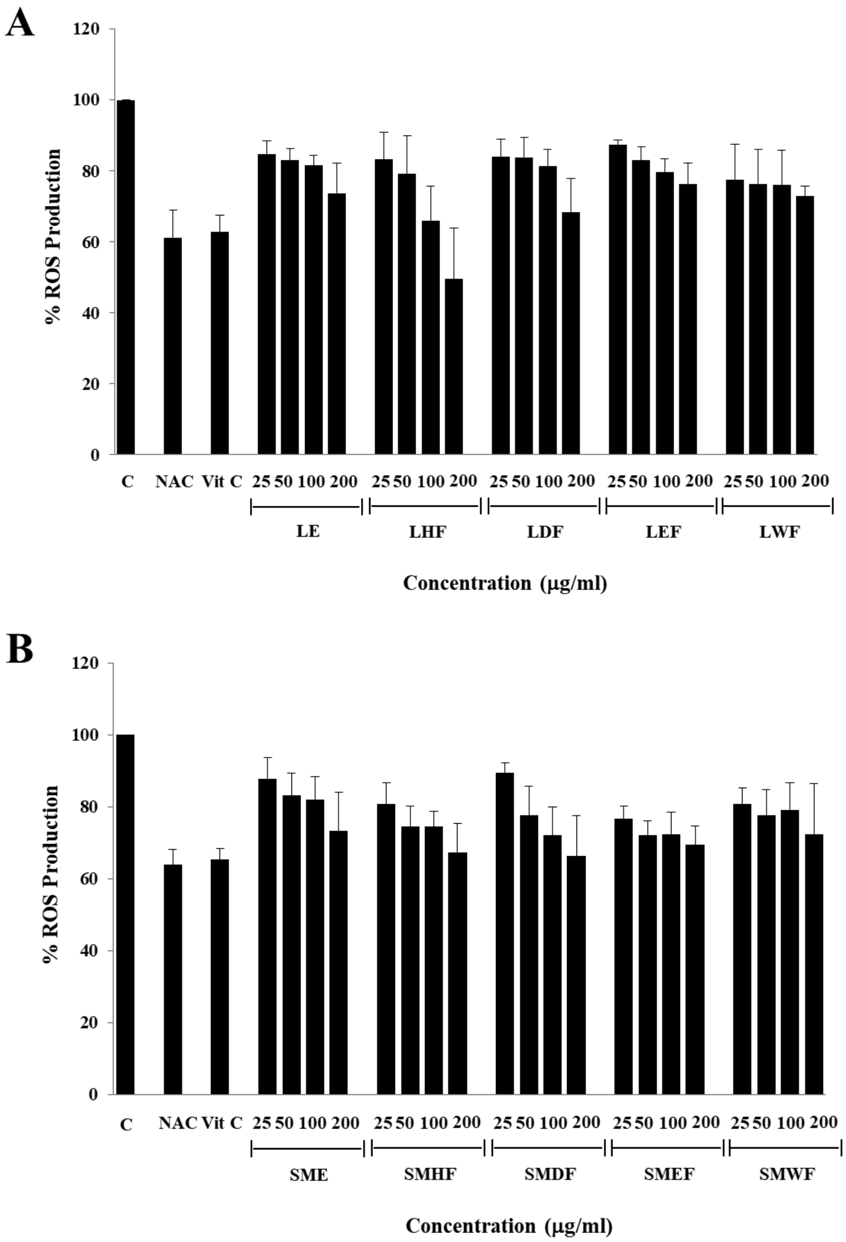

Figure 8 Effects of perilla fractions on RANKL-induced ROS in RAW 264.7 cells. Untreated cells were used as a negative control. N-acetylcysteine (80 μM) and ascorbic acid (250 mM) were used as positive controls. Data represent the mean ± SD of three independent experiments. *$p < 0.05$, ** $p < 0.001$ versus untreated cells. Error bars indicate SD. LE, leaf EtOH extract; LHF, leaf hexane fraction; LDF, leaf DCM fraction; LEF, leaf EtOAc fraction; LWF, leaf water fraction; SME, seed meal EtOH extract; SMHF, seed meal hexane fraction; SMDF, seed meal DCM fraction; SMEF, seed meal EtOAc fraction; and SMWF, seed meal water fraction.

10. Effect of RA-rich fractions from perilla leaves and seed meal on RANKL-induced osteoclastogenesis in RAW 264.7 cells using TRAP staining and TRAP activity assay

To evaluate whether the RA-rich fractions (LEF and SMEF) inhibited osteoclastogenesis, its effects on osteoclast differentiation in RANKL-induced RAW 264.7 cells were investigated. Osteoclast precursor cells of monocyte-macrophage lineage fuse to form TRAP-positive multinucleated cells. The multinucleated osteoclasts reorganize the actin cytoskeleton to attach to the bone surface and to resorb the bone (29).

The degree of osteoclast differentiation was indicated by the number of TRAP-stained cells according to TRAP staining analysis. The TRAP staining showed that RA-rich fractions inhibited the formation of mononuclear and multinuclear osteoclasts as the concentration of the extracts was increased. RA were use as TRAP inhibitors (**Figure 9**).

Furthermore, we investigated whether the fractions were effective on RANKL-stimulated TRAP activity in RAW 264.7 cells. The TRAP activity increased by up to 160% after treatment with RANKL compared with that of the control. However, treatment with LEF 100mg/ml, SMEF 50mg/ml reduced the TRAP activity. RA were use as TRAP inhibitors. This result was well correlated with the analysis of TRAP staining. As shown in **Figure 10**.

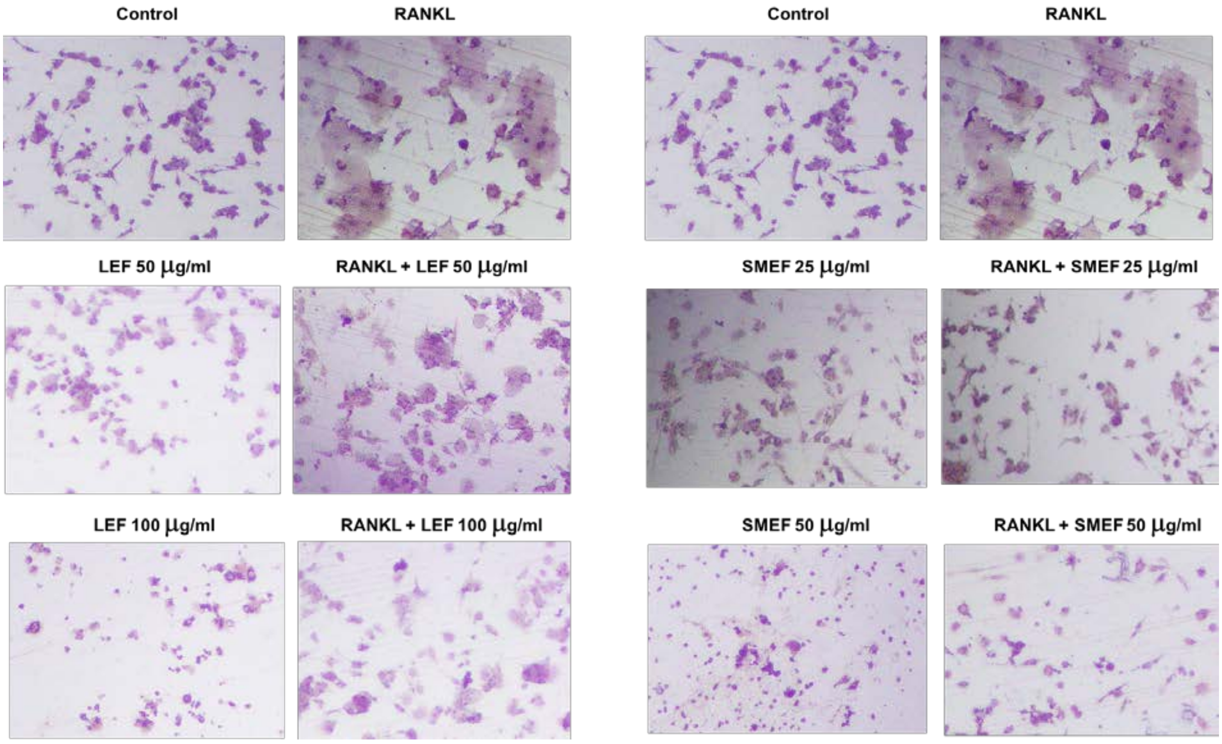

Figure 9 Effect of RA-rich fractions on RANKL-induced osteoclast differentiation. Cells were exposed to RANKL (100 ng/mL) in the presence and absence of RA-rich fractions (**A**: LEF and **B**: SMEF) for 10 days. The cells were fixed and stained using a leukocyte acid phosphatase (TRAP) kit. Con; positive control, (which was not treated), RANKL; negative control, (which was treated with only RANKL), Sample treated group; RANKL+ sample. TRAP positive multinucleated osteoclasts were visualized in 10× magnification under light microphotography

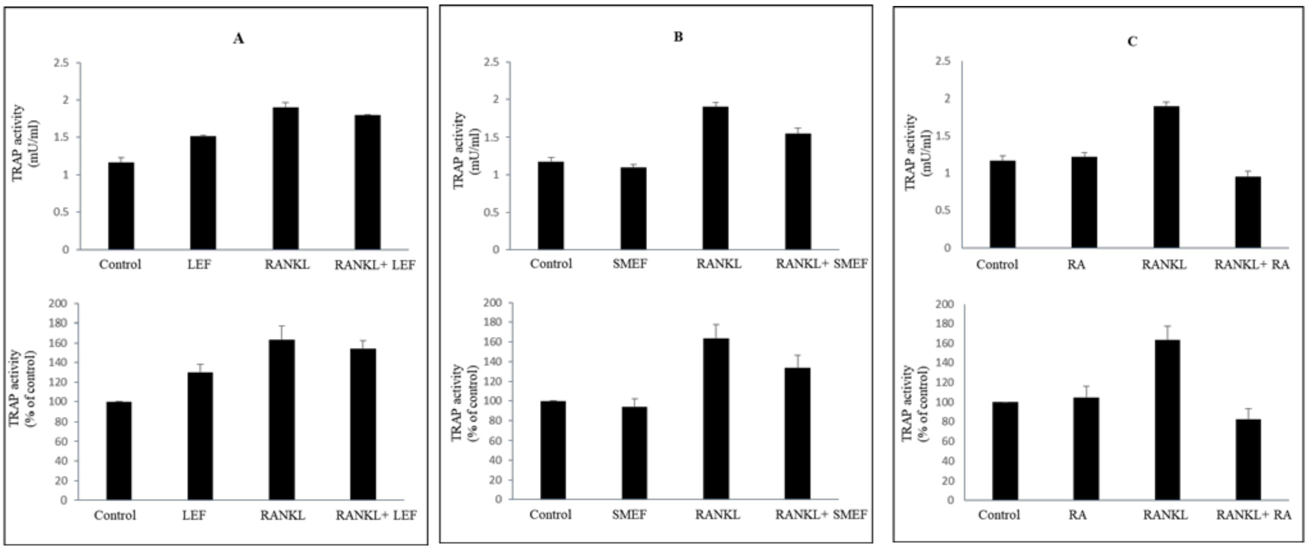

Figure 10 Effect of RA-rich fractions from perilla leaves and seed meal on RANKL-stimulated TRAP activity in RAW 264.7 cells. Cells were exposed to RANKL (100 ng/mL) in the presence of LEF (A), SMEF (B), RA (C) for 6 days. TRAP activity was measured by TRAP solution assay. All data are presented as the mean ± SD of three independent experiments performed with n =3. Statistical analysis were done by Comparing, Con (positive control, which was not treated) vs. RANKL (negative control, which was treated with only RANKL, ### $p < 0.05$) and RANKL vs. sample treated group (*** $p < 0.05$). Result of TRAP activity were express as mU/ml (**Upper**) and % of control (**Lower**).

11. The molecular mechanism of RA-rich fractions on RANKL-induced osteoclastogenesis through RANK signaling pathway using western blot analysis

The RANKL/RANK/OPG system is essential for osteoclast differentiation as well as fever response in inflammation. RANKL delivers important signals for osteoclast differentiation, whereas osteoclasts and their precursor cells receive survival signals from M-CSF. The activation of three major mitogen-activated protein kinases, ERK, JNK and p38, are induced by RANKL signaling in osteoclast precursor cells. Subsequently, MAPKs initiate the induction and activation of many transcription factors associated with the expression of osteoclast-specific genes—namely, NFATc1, activator protein 1 (AP-1) and NF-kB. Activated ERK and JNK can directly phosphorylate c-Fos and c-Jun, respectively. Therefore, transcription factor AP-1, which is a heterodimer of proteins from the Fos and Jun families, could be a target of ERK and JNK in RANKL-induced osteoclast precursor cells. A previous study reported that c-Fos and NFATc1 expression was induced by RANKL in osteoclast precursor cells (30). NF-kB is also considered a crucial transcription factor for osteoclast differentiation induced by RANKL. In response to RANKL signaling, the proteasome-mediated degradation of inhibitory kappa B (IκB) characterized the activation and nuclear translocation of NF-kB in osteoclast precursor cells. Then, the effect of RA-rich fractions on RANK signaling pathway through NFATc1, NF-kB, and AP-1 expression will be studied.

Our results found that RA-rich fractions can inhibit IκBα degradation (**Figure 11**), NF-κB activation (**Figure 12-13**), and translocation (**Figure 14**). In addition, RA-rich fractions can also decrease c-Jun (AP-1) (**Figure 15**) and NFATc1 expression (**Figure 16-17**) in time and dose-dependent manner.

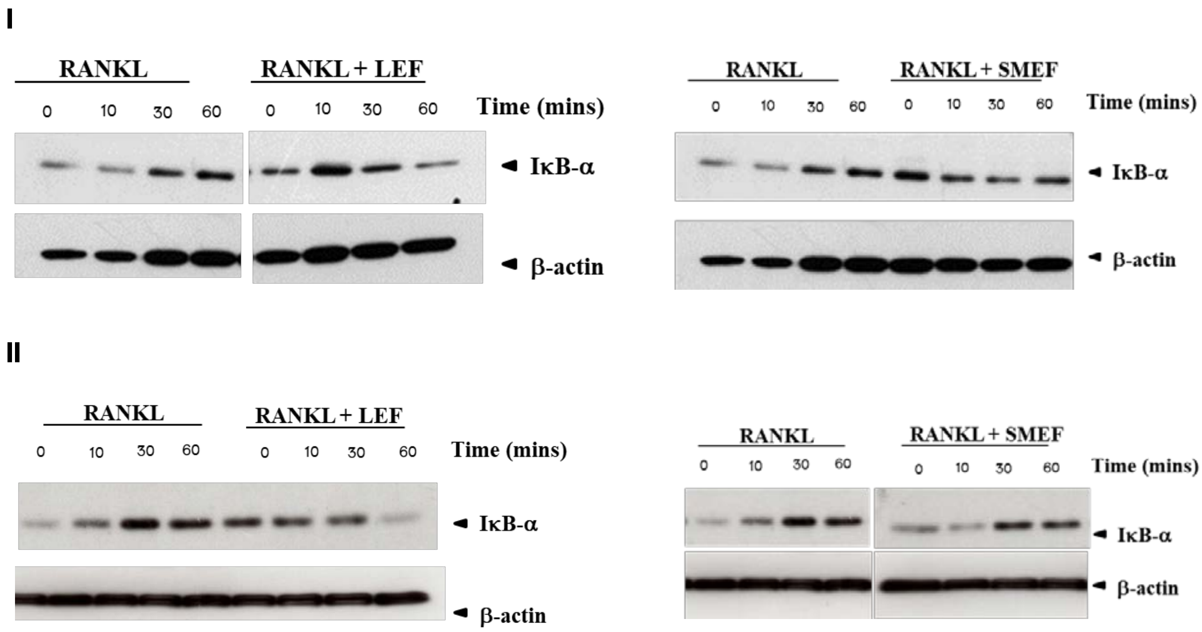

Figure 11 Effect of RA-rich fractions on IκBα degradation. Cytoplasmic extract from RAW264.7 treated with LEF 100 μg/ml (**Left**) and SMEF 50 μg/ml (**Right**) for 12 h, follow by RANKL (100 ng/mL) for 0, 10, 30, and 60 mins. The expression of the proteins was determined by Western blot analysis, and β-actin was used as a loading control.

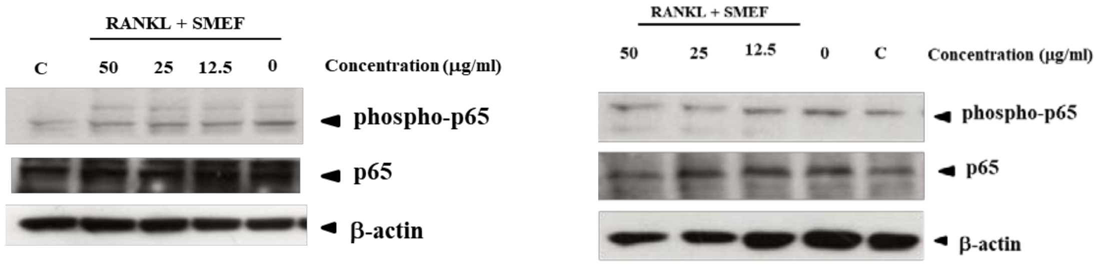

Figure 12 Effect of SMEF on the phosphorylation of NF-κB. Whole cell extract from RAW264.7 exposed to RANKL (100 ng/mL) in the presence and absence of SMEF 12.5, 25, and 50 µg/ml for 24 h (**Left**) and 48 h (**Right**). The expression of the proteins were determined by Western blot analysis and β-actin was used as loading control.

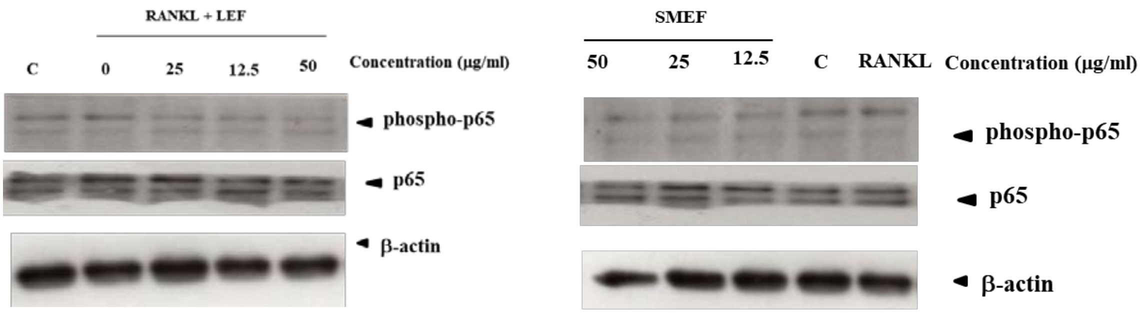

Figure 13 Effect of LEF and SMEF on the phosphorylation of NF-κB. Whole cell extract from RAW264.7 exposed to RANKL (100 ng/mL) in the presence and absence of LEF 25, 50, and 100 µg/ml (**Left**) and SMEF 12.5, 25, and 50 µg/ml (**Right**) for 48 h. The expression of the proteins were determined by Western blot analysis and β-actin was used as loading control.

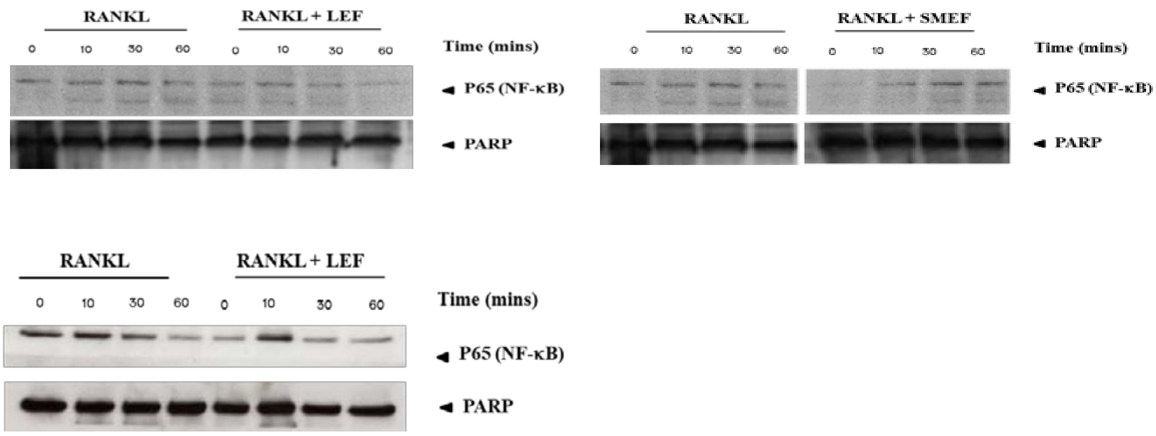

Figure 14 Effect of RA-rich fractions on nuclear translocation of NF-κB. Nuclear extract from RAW264.7 treated with LEF 100 µg/ml (**Left**) and SMEF 50 µg/ml (**Right**) for 12 h, followed by RANKL (100 ng/mL) for 0, 10, 30, and 60 mins. The expression of the proteins was determined by Western blot analysis, and PARP was used as a loading control.

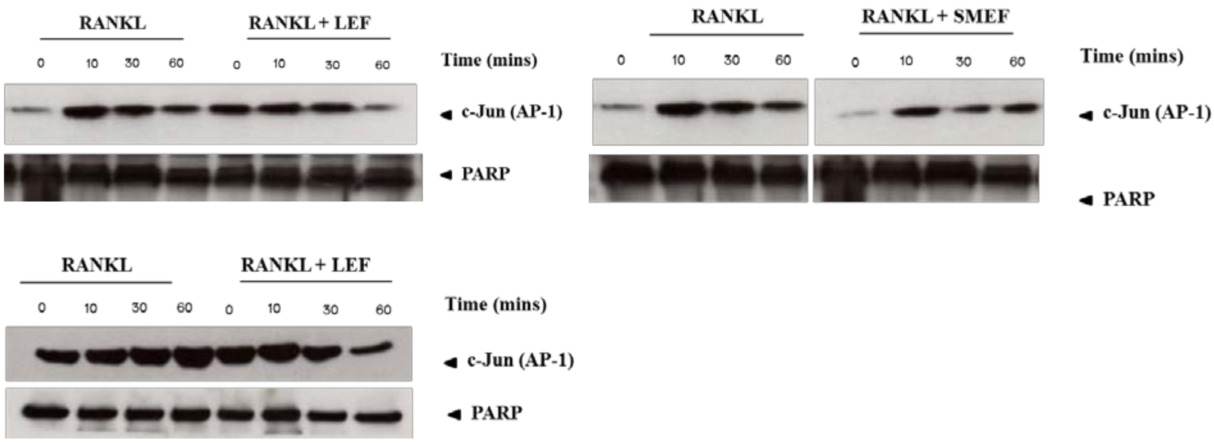

Figure 15 Effect of RA-rich fractions on c-Jun expression. Nuclear extract from RAW264.7 treated with LEF 100 μg/ml (**Left**) and SMEF 50 μg/ml (**Right**) for 12 h, follow by RANKL (100 ng/mL) for 0, 10, 30, and 60 mins. The expression of the proteins was determined by Western blot analysis, and PARP was used as a loading control.

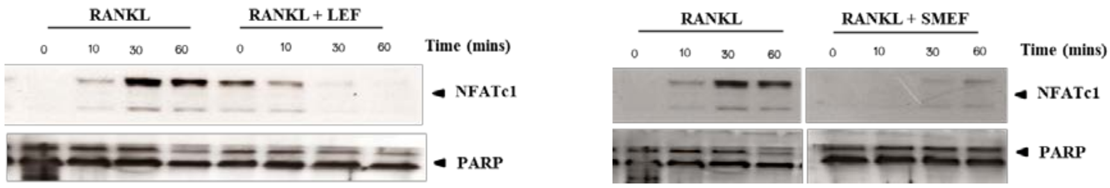

Figure 16 Effect of RA-rich fractions on NFATc1 expression (vary time). Nuclear extract from RAW264.7 exposed to LEF 100 μg/ml (**Left**) and SMEF 50 μg/ml (**Right**) for 12 h, follow by RANKL (100 ng/mL) for 0, 10, 30, and 60 mins. The expression of the proteins was determined by Western blot analysis, and PARP was used as a loading control.

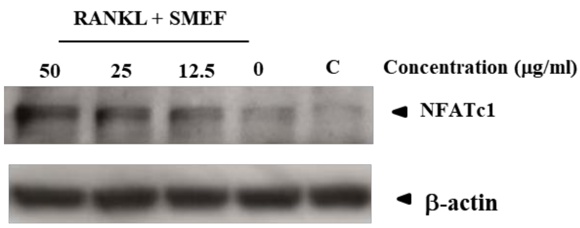

Figure 17 Effect of SMEF on NFATc1 expression (vary dose). Whole cell extract from RAW264.7 treated with RANKL (100 ng/mL) in the presence and absence of SMEF 12.5, 25, and 50 μg/ml for 24 h. The expression of the proteins was determined by Western blot analysis, and β-actin was used as a loading control.

สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

(Conclusions and suggestion for future research)

Perilla Frutescens (perilla or Nga-mon in Thai) is a herb that belongs to the mint family, traditionally grown in East Asia, mainly Northern Thailand (31). It is used in local wisdom for various purposes including medicine and cosmetics, edible oil, as a herb in salads, sushi, soups, and as a spice, as well as a food decoration or a colorant. The perilla leaves have a sweet taste, containing about 3.1% protein, 0.8% fat, 4.1% carbohydrate, and 1.1% ash. The young leaves are used as a spice, older leaves used as a food decoration or flavoring and wound healing. In particular, perilla leaves exhibit several health benefits due to a high content of polyphenols, flavonoids, including rosmarinic acid (32). Recently, it was shown that Thai perilla leaf inhibited the invasion and migration of human breast cancer (33).

Our studies found that the EtOAc fraction of perilla leaves and seed meal had the highest content of polyphenols, flavonoids, and RA. Typically, antioxidants are compounds that can delay or inhibit the oxidation of lipids and other molecules, and act by one or more of the following mechanisms; reducing activity, free radical scavenging, possible complexation of pro-oxidant metals, and quenching of singlet oxygen (34). As seen in the present study, the various scavenging capacities by different assays may be due to the principle and mechanism (35-37). The results from the different antioxidant activities demonstrated a similar trend that the EtOAc fractions showed the highest DPPH, ABTS, $O_2^{\bullet-}$ and NO radical scavenging activity. Also, the EtOAc fractions showed the highest antioxidant capacity through ferric reducing/antioxidant power assay. These results were consistent with that reported in the literature (9, 25, 38). Moreover, a cell-based anti-inflammatory activity found that perilla can also inhibit LPS and IFN-γ-stimulated the production of NO, iNOS, and COX-2 in RAW 264.7 mouse macrophage cells. From all the above data, the EtOAc fraction from perilla leaves and seed meal were the most suitable as RA-riched fractions to use for study the inflammatory cytokines-induced osteoclast differentiation and underlying molecular mechanisms.

Osteoclasts are unique cells that degrade the bone matrix. These large multinucleated cells differentiate from the monocyte/macrophage lineage upon stimulation by two essential cytokines, macrophage colony-stimulating factor (M-CSF) and RANKL (39). In addition, RANKL activates various transcription factors such as NF-κB, c-Jun, and NFATc1, including ROS, which are responsible for osteoclast differentiation (40).

The present studies showed that RA-rich fractions inhibited RANKL-induced ROS production in RAW 264.7 cells. The fractions also attenuated RANKL-induced the number of TRAP-positive multinucleated osteoclasts and reduced RANKL-induced TRAP activity. To attain molecular insights, our results found that the RA-rich fractions significantly inhibited RANKL-induced osteoclast differentiation by down-regulating nuclear factor kappa B (NF-κB), c-Jun and NFATc1 signaling pathway.

It can be concluded here that, the EtOAc fractions from perilla leaves and seed meal showed the highest in phenolics, flavonoids, and RA, which were strongly related to antioxidant capacity and anti-inflammatory activity. Moreover,these RA-riched fractions can also inhibit RANKL-induced ROS generation and reduce osteoclast differentiation by down-regulating nuclear factor kappa B (NF-κB), c-Jun and NFATc1 signaling pathway. Finally, RA-rich fractions may provide therapeutic strategies for bone diseases associated with excessive osteoclast differentiation and function.

Future research should focus on the isolation of their active compounds and their *in-vivo* biological activities. Their beneficial applications need to be warranted by such evidence.

References

1. Hossain MA, Shah MD. A study on the total phenols content and antioxidant activity of essential oil and different solvent extracts of endemic plant Merremia borneensis. Arabian Journal of Chemistry. 2015;8(1):66-71.
2. Abarca-Vargas R, Peña Malacara CF, Petricevich VL. Characterization of Chemical Compounds with Antioxidant and Cytotoxic Activities in Bougainvillea x buttiana Holttum and Standl, (var. Rose) Extracts. Antioxidants (Basel, Switzerland). 2016;5(4):45.
3. Chumphukam O. PK, Khanaree C., Chewonarin T., Chaiwangyen W., Tantipaiboonwong P., Suttajit M., Khantamat O. Potential anti-mutagenicity, antioxidant, and anti-inflammatory capacities of the extract from perilla seed meal. Journal of Food Biochemistry. 2018.
4. Limtrakul P, Yodkeeree S, Thippraphan P, Punfa W, Srisomboon J. Anti-aging and tyrosinase inhibition effects of Cassia fistula flower butanolic extract. BMC Complementary and Alternative Medicine. 2016;16(1):497.
5. Saenjum C, Chaiyasut C, Kadchumsang S, Chansakaow S, Suttajit M. Antioxidant activity and protective effects on DNA damage of Caesalpinia sappan L. extract. Journal of Medicinal Plants Research. 2010;4(15):1594-608.
6. Saenjum C, Chaiyasut C, Chansakaow S, Suttajit M, Sirithunyalug B. Antioxidant and anti-inflammatory activities of gamma-oryzanol rich extracts from Thai purple rice bran. Journal of medicinal plants Research. 2012;6(6):1070-7.
7. Banjerdpongchai R, Wudtiwai B, Khaw-on P, Rachakhom W, Duangnil N, Kongtawelert P. Hesperidin from Citrus seed induces human hepatocellular carcinoma HepG2 cell apoptosis via both mitochondrial and death receptor pathways. Tumor Biology. 2016;37(1):227-37.
8. Do QD, Angkawijaya AE, Phuong LTN, Huynh LH, Soetaredjo FE, Ismadji S, et al. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. Journal of Food and Drug Analysis. 2014;22(3):296-302.
9. Al-Snafi AE. PHARMACOLOGICAL AND THERAPEUTIC EFFECTS OF JASMINUM SAMBAC- A REVIEW. Indo American Journal of Pharmaceutical Sciences. 2018;5(3):1766-78.
10. Li HZ, Zhang ZJ, He DL, Xia YY, Liu QY, Li XJ. Ultrasound-assisted aqueous enzymatic extraction of oil from perilla seeds and determination of its physicochemical properties, fatty acid composition and antioxidant activity. Food Science and Technology. 2017;37:71-7.
11. Brown JE, Rice-Evans CA. Luteolin-rich artichoke extract protects low density lipoprotein from oxidation in vitro. Free radical research. 1998;29(3):247-55.
12. Lin ES, Li, C.C., Chou J.J. Evaluation of the antioxidant and antiradical activities of perilla seed, leaf and stalk extracts. Journal of Medicinal Plants Research. 2014;8(2):109-15.
13. Rababah TM, Hettiarachchy NS, Horax R. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. Journal of Agricultural and Food Chemistry. 2004;52(16):5183-6.

14. Tomsone L, Kruma, Z., Galoburda, R. Comparison of different solvents and extraction methods for isolation of phenolic compounds from horseradish roots (Armoracia rusticana). International Journal of Agriculturaland Biological Engineering. 2012;6(4):236-41.
15. Farhan BM, Razak, A., Yong, P.K., Shah, Z.M., Abdullah, L.C., Soh Sin Yee, S.S., Yaw, T.C.S. The Effects of Varying Solvent Polarity on Extraction Yield of *Orthosiphon stamineus* Leaves. Journal of Applied Sciences. 2012;12(11):1207-10.
16. Caleja C, Barros L, Prieto MA, Barreiro MF, Oliveira M, Ferreira I. Extraction of rosmarinic acid from *Melissa officinalis* L. by heat-, microwave- and ultrasound-assisted extraction techniques: A comparative study through response surface analysis. Separation and Purification Technology. 2017;186:297-308.
17. Li HZ, Zhang ZJ, Xue J, Cui LX, Hou TY, Li XJ, et al. Optimization of ultrasound-assisted extraction of phenolic compounds, antioxidants and rosmarinic acid from perilla leaves using response surface methodology. Food Science and Technology. 2016;36(4):686-93.
18. Oliveira GDR, de Oliveira AE, da Conceicao EC, Leles MIG. Multiresponse optimization of an extraction procedure of carnosol and rosmarinic and carnosic acids from rosemary. Food Chemistry. 2016;211:465-73.
19. Liang ZM, Xu YF, Wen XM, Nie HY, Hu TJ, Yang XF, et al. Rosmarinic Acid Attenuates Airway Inflammation and Hyperresponsiveness in a Murine Model of Asthma. Molecules (Basel, Switzerland). 2016;21(6).
20. Wu L, Wang HM, Li JL, Feng HX, Zhao WM, Zhang HY. Dual anti-ischemic effects of rosmarinic acid n-butyl ester via alleviation of DAPK-p53-mediated neuronal damage and microglial inflammation. Acta Pharmacologica Sinica. 2017;38(4):459-68.
21. Zhu FX, Asada T, Sato A, Koi Y, Nishiwaki H, Tamura H. Rosmarinic Acid Extract for Antioxidant, Antiallergic, and alpha-Glucosidase Inhibitory Activities, Isolated by Supramolecular Technique and Solvent Extraction from Perilla Leaves. Journal of Agricultural and Food Chemistry. 2014;62(4):885-92.
22. Pintha K, Tantipaiboonwong P, Yodkeeree S, Chaiwangyen W, Chumphukam O, Khantamat O, et al. Thai perilla (Perilla frutescens) leaf extract inhibits human breast cancer invasion and migration. Maejo International Journal of Science and Technology. 2018;12(2):112-23.
23. Kwon SH, Wang Z, Hwang SH, Kang YH, Lee JY, Lim SS. Comprehensive evaluation of the antioxidant capacity of Perilla frutescens leaves extract and isolation of free radical scavengers using step-wise HSCCC guided by DPPH-HPLC. International Journal of Food Properties. 2017;20:S921-S34.
24. Zhou XJ, Yan LL, Yin PP, Shi LL, Zhang JH, Liu YJ, et al. Structural characterisation and antioxidant activity evaluation of phenolic compounds from cold-pressed Perilla frutescens var. arguta seed flour. Food Chemistry. 2014;164:150-7.
25. Jun HI, Kim BT, Song GS, Kim YS. Structural characterization of phenolic antioxidants from purple perilla (Perilla frutescens var. acuta) leaves. Food Chemistry. 2014;148:367-72.
26. Kloetzer L, Bompa AS, Blaga AC, Galaction AI, Cascaval D. Study on rosmarinic acid separation by synergic extraction. Separation Science and Technology. 2018;53(4):645-54.

27. Hong E, Park KH, Kim GH. PHENOLIC-ENRICHED FRACTIONS FROM PERILLA FRUTESCENS VAR. ACUTA: DETERMINATING ROSMARINIC ACID AND ANTIOXIDANT ACTIVITY. Journal of Food Biochemistry. 2011;35(6):1637-45.

28. Lee NK, Choi YG, Baik JY, Han SY, Jeong DW, Bae YS, et al. A crucial role for reactive oxygen species in RANKL-induced osteoclast differentiation. Blood. 2005;106(3):852-9.

29. Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423(6937):337-42.

30. Ghosh M, Kim IS, Lee YM, Hong SM, Lee TH, Lim JH, et al. The Effects of Aronia melanocarpa 'Viking' Extracts in Attenuating RANKL-Induced Osteoclastic Differentiation by Inhibiting ROS Generation and c-FOS/NFATc1 Signaling. Molecules (Basel, Switzerland). 2018;23(3).

31. Siriamornpun S. LD, Yang L., Suttajit S, Suttajit M. Variation of lipid and fatty acid compositions in Thai Perilla seeds grown at different locations. Songklanakarin Journal of Science and Technology 2006;28 (Suppl.1):17-21.

32. Asif M. Phytochemical study of polyphenols in Perilla Frutescens as an antioxidant. Avicenna J Phytomed. 2012;2(4):169-78.

33. Pintha K, Yodkeeree S, Limtrakul P. Proanthocyanidin in Red Rice Inhibits MDA-MB-231 Breast Cancer Cell Invasion via the Expression Control of Invasive Proteins. Biological & Pharmaceutical Bulletin. 2015;38(4):571-81.

34. Gutierrez RMP, Cotera, L.B.F., Gonzalez, A.M.N. Evaluation of the antioxidant and anti-glication effects. . Molecules (Basel, Switzerland). 2012;17:11897-919.

35. Huang D, Ou, B., Prior, R.L. The Chemistry behind Antioxidant Capacity Assays. J Agric Food Chem. 2005;53(6):1841-56.

36. Magalhaes LM, Segundo, M. A., Reis, S., Lima, J. L. F. C. . Methodological aspects about in vitro evaluation of antioxidant properties. Analytica Chimica Acta. 2008;613:1-19.

37. Shah P, Modi, H.A. . Comparative Study of DPPH, ABTS and FRAP Assays for Determination of Antioxidant Activity. International Journal for Research in Applied Science & Engineering Technology 2015;3(VI):636-41.

38. Lee JH, Park KH, Lee MH, Kim HT, Seo WD, Kim JY, et al. Identification, characterisation, and quantification of phenolic compounds in the antioxidant activity-containing fraction from the seeds of Korean perilla (Perilla frutescens) cultivars. Food Chemistry. 2013;136(2):843-52.

39. Kim JH, Kim N. Regulation of NFATc1 in Osteoclast Differentiation. Journal of bone metabolism. 2014;21(4):233-41.

40. Ikeda F, Nishimura R, Matsubara T, Hata K, Reddy SV, Yoneda T. Activation of NFAT signal in vivo leads to osteopenia associated with increased osteoclastogenesis and bone-resorbing activity. Journal of immunology (Baltimore, Md : 1950). 2006;177(4):2384-90.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

---

1. **Kanokkarn Phromnoi** , Puksiri Sinchaiyakij , Chakkrit Khanaree , Piyawan Nuntaboon , Yupa Chanwikrai , Thida Chaiwangsri and Maitree Suttajit. Anti-inflammatory and antioxidant activities of medicinal plants used by traditional healers for antiulcer treatment. ***Scientia Pharmaceutica****, 2019, 87, x; doi:*
Scopus Q2

2. **Kanokkarn Phromnoi**, Maitree Suttajit and Chalermpong Saenjum. Polyphenols and rosmarinic acid contents, antioxidant and anti-inflammatory activities of different solvent fractions from Nga-Mon (Perilla frutescens) leaf. ***Journal of Pharmacy and Nutrition Sciences***, 2019, 9, 000-000
Scopus Q3

3. **Kanokkarn Phromnoi**, Komsak Pintha, Chalermpong Saenjum, Suphachai Yodkeeree, Pornngarm Dejkriengkraikul, Maitree Suttajit**.** Effect of Nga-mon (perilla) extracts on DPPH radical scavenging activity and ROS production in RAW 264.7 macrophages. The First Innovation of Functional Foods in Asia (IFFA2018). January 22 th-24th, 2018 University of Phayao, Muang, Phayao, Thailand. (Poster presentation)

4. **Kanokkarn Phromnoi**, Komsak Pintha, Chalermpong Saenjum, Suphachai Yodkeeree, Pornngarm Dejkriengkraikul, Maitree Suttajit**.** Inhibitory effect of rosmarinic acid-rich extracts from leaves and seed of Nga-mon (perilla) on inflammatory cytokines-induced osteoclastogenesis through receptor activator of NF-kB (RANK) signaling pathway. TRF-OHEC Annual Congress 2018 (TOAC 2018). January 10th-12th, 2018. The Regent Cha Am Beach Resort. Phetchaburi, THAILAND**.** (Poster presentation)