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Antioxidant Activity and Anti-inflammatory Effects of Raw and Processed Fruits and Vegetables

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Abstract

In this study we investigated antioxidant and anti-inflammatory activities of Malus Domestica (apple), Pyrus Communis L. (pear), Daucus carota L. (carrot), Brassica oleracea var. (broccoli), Brassica oleracea var. capitata (cabbage) and Raphanus sativus L. (radish), that were obtained from local market. As these are common fruits and vegetables that are widely consumed, we aimed to investigate their beneficial properties especially the antioxidant and anti-inflammatory. The samples were processed by an indirect heating method and their properties were compared to their raw forms. Based on DPPH and ABTS assay, processed samples showed better antioxidant activity compared to raw samples, and processed pear sample had the best antioxidant activity. The anti-inflammatory activities of the samples were also investigated in LPS-treated RAW 264.7 cells. The mRNA expressions of pro-inflammatory mediators and cytokines (iNOS, COX-2, TNF-α, IL-1β and IL-6) were assessed by RT-PCR. Processed samples exhibited better inhibition of iNOS, compared to the raw forms. Processed broccoli and cabbage samples exhibited outstanding anti-inflammatory effects. The samples did not exhibit cytotoxicity against RAW 264.7 cells up to 1mg/ mL as shown in the cell viability assay. Taken together, processed broccoli and cabbage samples exhibited the strongest anti-inflammatory properties.

Keywords: *Antioxidant, Anti-inflammation, RAW 264.7 cells, Fruits, Vegetables*

1. Introduction

An increasing interest in and use of plants including fruits, vegetables and herbs is developed these days among scientific communities as antioxidants[1, 2]. This is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants which have possible activity as promoters of carcinogenesis. Therefore, the need exits for safe, economic, powerful, and natural antioxidants to replace these synthetic ones[3, 4]. Many plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage[5]. Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of

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chronic diseases. Therefore, in recent years it is considered an important task to evaluating plant's antioxidant activity and their free radical quenching ability.

Therefore in our study we have geared to investigate the antioxidant properties of Malus Domestica (apple), Pyrus Communis L. (pear), Daucus carota L. (carrot), Brassica oleracea var. (broccoli), Brassica oleracea var. capitata (cabbage) and Raphanus sativus L. (radish). Besides the raw samples, we have also investigated on the processed samples, which have undergone indirect heat treatment because processed fruits and vegetables may have elevated and improved bioactive properties[6]. Therefore, we aimed to investigate the anti-oxidative properties of the raw samples, and whether increased processing improves their bioactive properties.

Inflammation occurs when an organism combats invasion, physically, or by noxious chemical stimuli. The inflammatory response is a mechanism to inactivate invading pathogens[7]. Reactive oxygen species(ROS) are an activator of inflammation[8]. Therefore, we proceeded to investigate the anti-inflammatory properties of the aforementioned samples. Both nitric oxide(NO) and ROS modulates inflammation. Lipopolysaccharides (LPS) is a chemical moiety that is present in the outer membrane of gram-negative bacteria which is a specific ligand to toll-like receptor, and an inducer of inflammation[9]. In our study, we investigated the anti-inflammatory properties of the samples by treating RAW 264.7 cells with LPS, and determined the Nitric oxide secretion (NO), and the expression of pro-inflammatory mediators (iNOS and COX-2) and cytokines (TNF-α, IL-1β and IL-6) at the transcriptional levels. Our results have shown that additional processing of these fruits and vegetables extracts had elevated their antioxidant and anti-inflammatory properties.

2. Experiment Materials

2.1 Reagents

Apples Dulbecco's modified Eagle medium(DMEM) and fetal bovine seru (FBS) were obtained from Welgene, South Korea. Streptomycin and penicillin were obtained from Lonza, MD, USA. TRIzol reagent was sourced from Invitrogen(Carlsbad, CA, USA), and LPS(*Escherichia coli* 055:B5) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) from Sigma-Adrich. Oligo(dT), and primers iNOS, COX-2, TNF-α, IL-1β, IL-6, and GAPDH were purchased from Bioneer (South Korea).

2.2 Sample Preparation

The samples Malus Domestica (apple), Pyrus Communis L. (pear), Daucus carota L. (carrot), Brassica oleracea var. (broccoli), Brassica oleracea var. capitata (cabbage) and Raphanus sativus L. (radish) were agricultural products grown in South Korea and locally purchased from traditional market. The samples were washed; then cut into uniform shapes of 0.5 cm x 0.5 cm x 0.5 cm, freeze-dried, sealed dry to keep away from moisture and stored in -70°C. Raw samples were prepared by extracting the samples at 60°C for 2 hours.

For the processed samples, previously freeze-dried samples underwent heat treatment using an apparatus, with a pressure of 10 kg / cm^2 (Jisco, Seoul, South Korea). The samples were placed in the inner compartment container, and water was added in the outer compartment of the container. The apparatus was heated up according to predetermined temperature and time (140°C - 150°C for 6 hours) to prevent carbonization of samples from direct heat. Samples were weighed accordingly for proceeding experiments.

3. Experiments Method

3.1. DPPH Radical Scavenging Activity

For measuring the radical scavenging activity of fruits and vegetables, 20μl samples was mixed with 200 μl of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.2 mM in ethanol). Simultaneously, ethanol was taken as

control group. After a 30 min of reaction at 37°C, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of the control groups. Ascorbic acid was taken as positive control sample. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = $[1-(A_1 - A_2)/ (A_3-A_4)] \times 100$

Where, A_1 is the absorbance of the DPPH and the sample, A_2 is the absorbance of ethanol 100% and the sample, A_3 is the absorbance of DPPH and solvent for sample dilution (DMSO/DDW), and A_4 is the absorbance of ethanol 100% and solvent for sample dilution (DMSO/DDW).

3.2 ABTS radical scavenging activity

The ABTS cation radical was produced by the reaction between 5 ml of 14 mM ABTS solution and 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, stored in the dark at room temperature for 16 hour. Before use, this solution was diluted with ethanol to get an absorbance of 0.700 ± 0.020 at 734 nm. In the 96 well plates, the fruits and vegetables extracts at various concentrations (50 µl) were mixed with 100 µl of ABTS solution and allowed to stand in dark for 10 min. Trolox was taken as positive control group for standardizing the ABTS activity. The inhibition percentage of ABTS radical was calculated using the following formula.

ABTS scavenging activity (%) = $[1-(A_1 - A_2)/ (A_0)] \times 100$

Where A_1 is the absorbance of ABTS working solution and sample, A_2 is the absorbance of the sample without ABTS working solution, and A_0 is the absorbance of only ABTS working solution.

3.3 Cell culture

Murine macrophage cell line, RAW 264.7 cells originating from American Type culture collection (ATCC) were used in this study, and were maintained in Dulbecco's Modified Eagle Medium (Welgene, South Korea). DMEM was supplemented with 5% FBS, 100 IU/mL penicillin and 100 µg/mL streptomycin sulfate. Cells were maintained at 37°C and 5% CO_2.

3.4 Nitric oxide & MTT cell viability assay

Nitric oxide assay was carried out using RAW 264.7 cells. Cells were seeded in 96-well plates for 24 hour. Samples with respective concentrations were treated and 30 min later, 0.1 µg/mL of LPS was treated. After 18 hour of incubation, supernatant (100 µL) was collected and mixed with an equal amount of Griess reagent, and the absorbance was measured using a microplate reader (VersaMax, Molecular Devices, USA) at 540 nm. Cell viability was determined using MTT reagent, which was added at a concentration of 0.1 mg/mL, and the plates were incubated for 3 hour at 37°C and 5% CO_2. The resulting crystals formed were dissolved in DMSO, and read at 560 nm using a microplate reader (VersaMax, Molecular Devices, USA)

3.5 Reverse transcriptase polymerase chain reaction (RT-PCR)

24 hour after plating RAW 264.7 cells in 6-well plates, cells were treated with or without 1mg/mL samples and 0.1 µg/mL of LPS 30 min later. 18 hour later, TRIzol reagent was used to extract the RNA and proceeding steps were described as previously reported. Sequence of primers used is given in Table 1.

Table 1. Primer sequences used for RT-PCR.

Gene	Primer sequence
GAPDH	F: 5'-CACTCACGGCAAATTCAACGGCAC-3'
	R: 5'-GACTCCACGACATACTCAGCAC-3'

iNOS	F: 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' R: 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F: 5'-CACTACATCCTGACCCACTT-3' R: 5'-ATGCTCCTGCTTGAGTATGT-3
TNF-α	F: 5'-TTGACCTCAGCGCTGAGTTG-3' R: 5'-CCTGTAGCCCACGTCGTAGC-3'
IL-1β	F: 5'-CTGTGGAGAAGCTGTGGCAG-3' R: 5'-GGGATCCACACTCTCCAGCT-3'
IL-6	F: 5'-GTACTCCAGAAGACCAGAGG-3' R: 5'-TGCTGGTGACAACCACGGCC-3

3.6 Statistical analysis

All data is presented as mean ± SEM. One way ANOVA and Dunnett's test were applied for statistical evaluation of data. Statistical analyses with $p<0.001$ were considered significant (***$p<0.001$.)

4. Result and Discussion

4.1. Processed samples exhibited stronger antioxidant activity than raw samples

The antioxidant properties of raw and processed samples were compared for apple (A), carrot (B), pear (C), broccoli (D), cabbage (E) and radish (F) in figure 1 using DPPH assay. Ascorbic acid was used as positive control. Based on the results, processed samples exhibited a higher percentage of radical scavenging activity as compared to the raw samples, especially in the case of apple, pear, broccoli and cabbage. However, in the case of ABTS assay, both the raw and processed samples exhibited strong radical scavenging activity as compared to Trolox, which was used as positive control (Figure 2). Collectively, all processed samples showed comparatively stronger antioxidant activity[10,11,12].

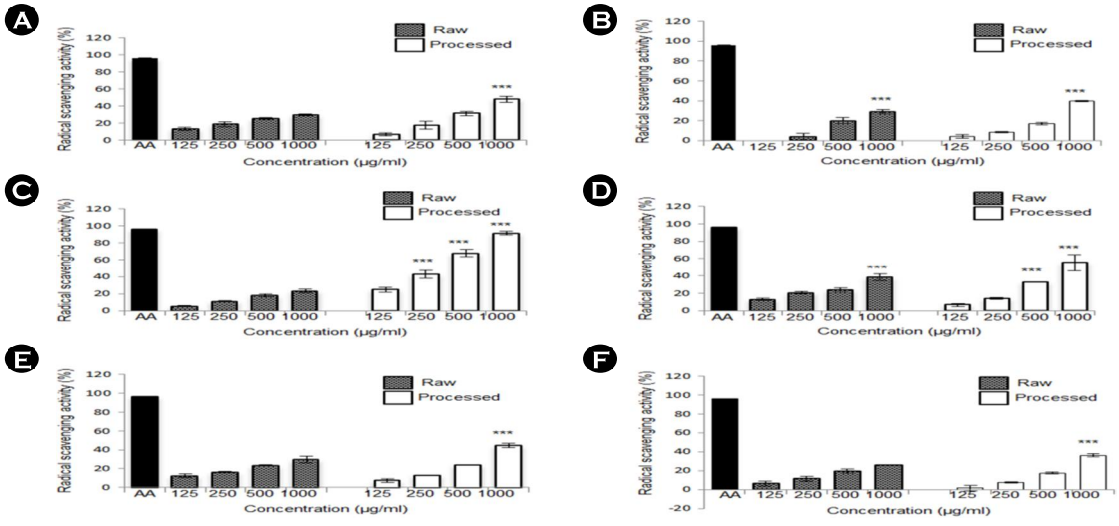

Figure 1. Radical scavenging activity of fruit and vegetable samples using DPPH assay.

The antioxidant activity of samples were tested, and compared to 100 µg/mL of ascorbic acid (AA) which was used as a positive control. The absorbance was read at 517 nm using a microplate reader, and the radical scavenging activity was calculated using the formula as mentioned in materials and methods section. The radical scavenging activity of raw and processed apple was shown in (A), raw and processed carrot in (B), raw and processed pear in (C), raw and processed broccoli in (D), raw and processed cabbage in (E) and raw and processed radish in (F). Processed samples show relatively higher amounts of scavenged radicals, indicating higher antioxidant activity. Values in bar graph are mean ± SEM of at least 3 independent experiments. *** $p < 0.001$, significantly lesser as compared to ascorbic acid only.

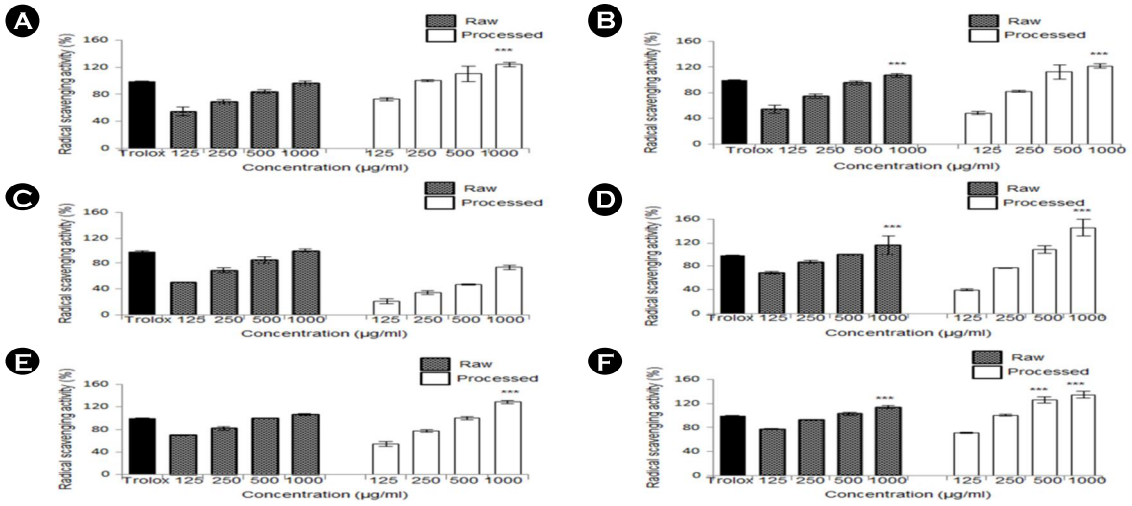

Figure 2. Radical scavenging activity of fruits and vegetable samples using ABTS assay.

5 mM of Trolox was used as a positive control. The absorbance was read at 734 nm using a microplate reader, and the radical scavenging activity was calculated. The radical scavenging activity of raw and processed apple was shown in (A), raw and processed carrot in (B), raw and processed pear in (C), raw and processed broccoli in (D), raw and processed cabbage in (E) and raw and processed radish in (F). Using ABTS assay, all samples show good radical scavenging activity as compared to Trolox. Values in bar

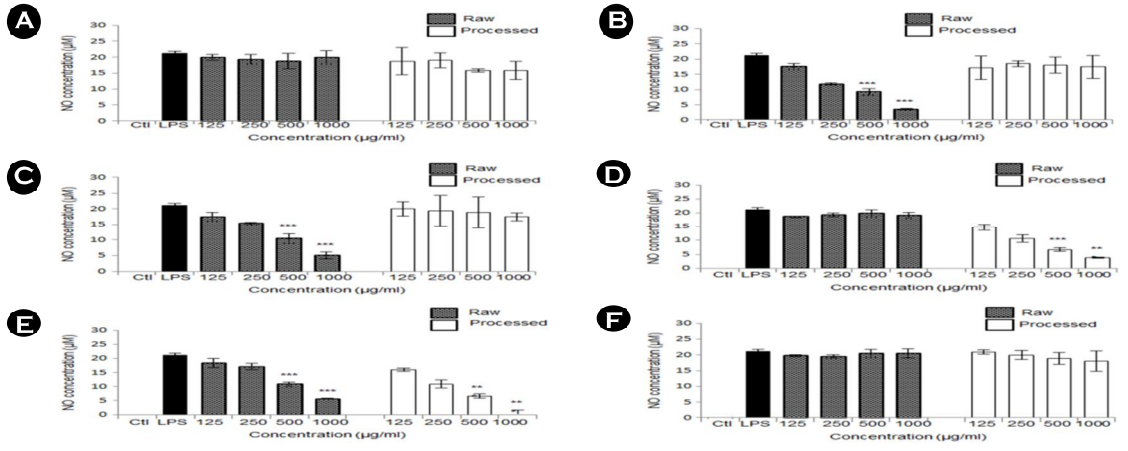

Figure 3. Nitric oxide inhibition induced by LPS. RAW 264.7 cells were seeded in 96-well plates.

graph are mean ± SEM of at least 3 independent experiments. *** $p < 0.001$, significantly increased as compared to Trolox only.

After 24 hours, cells were treated with or without samples, and 0.1 μg/mL of LPS after 30 minutes. The NO production was analyzed using Griess reagent. The NO inhibition induced by LPS of raw and processed apple was shown in (A), raw and processed carrot in (B), raw and processed pear in (C), raw and processed broccoli in (D), raw and processed cabbage in (E) and raw and processed radish in (F). Values in bar graph are mean ± SEM of at least 3 independent experiments. *** $p < 0.001$ compared to LPS only.

4.2 Variable anti-inflammatory activity of raw and processed samples

NO production was induced with the treatment of LPS in RAW 264.7 cells. Raw and processed samples were treated and the ability of the samples to inhibit NO production, which is correlated to its anti-inflammatory properties, was assessed using Griess reaction. Based on figure 3, raw carrot and pear samples have stronger anti-inflammatory effects, whereas processed broccoli and cabbage was more potent compared to its raw counterparts. Cabbage had strong anti-inflammatory effects whether it was raw or processed, with the latter being more potent. Moreover, since the dosages we used in our study was

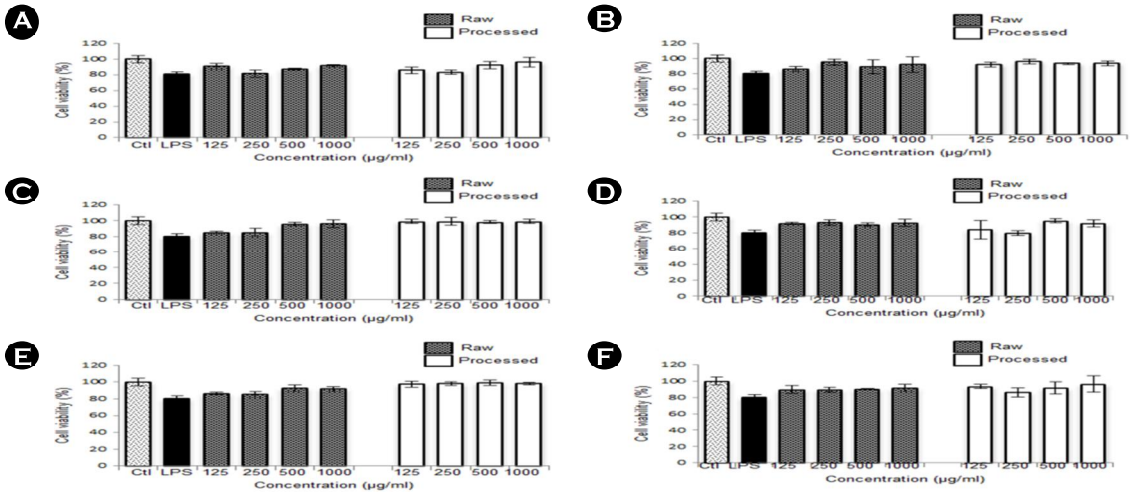

Figure 4. Cell viability assay for RAW 264.7 cells treated with fruit and vegetable samples.

scientifically very high for cells (1mg/ mL); we sought to determine the cytotoxicity and using the MTT reagent as shown in figure 4, none of the concentrations used in our studies showed cytotoxicity[13,14].

Cells were seeded in 96-well plates, treated with or without samples after 24 hours of incubation and treated with or without 0.1 μg/mL of LPS after 30 minutes. The results of the assay by raw and processed apple was shown in (A), raw and processed carrot in (B), raw and processed pear in (C), raw and processed broccoli in (D), raw and processed cabbage in (E) and raw and processed radish in (F). Based on the results, all samples that were tested does not exhibit cytotoxicity towards RAW 264.7 cells. Values in bar graph are mean ± SEM of at least 3 independent experiments.

4.3 Pro-inflammatory mediator and cytokine expressions in RAW 264.7 cells using Reverse-transcriptase Polymerase Chain Reaction (RT-PCR)

The expressions of pro-inflammatory mediators (iNOS and COX-2) and cytokines (TNF-α, IL-1β and IL-6) was assessed using RT-PCR. GAPDH was used as a loading control. Based on figure 5, LPS had induced

increased expression of all pro-inflammatory mediators and cytokines. Samples 1 – 6 are raw samples, whereas samples 7 – 12 are processed samples. Raw and processed samples of apple, broccoli, cabbage and

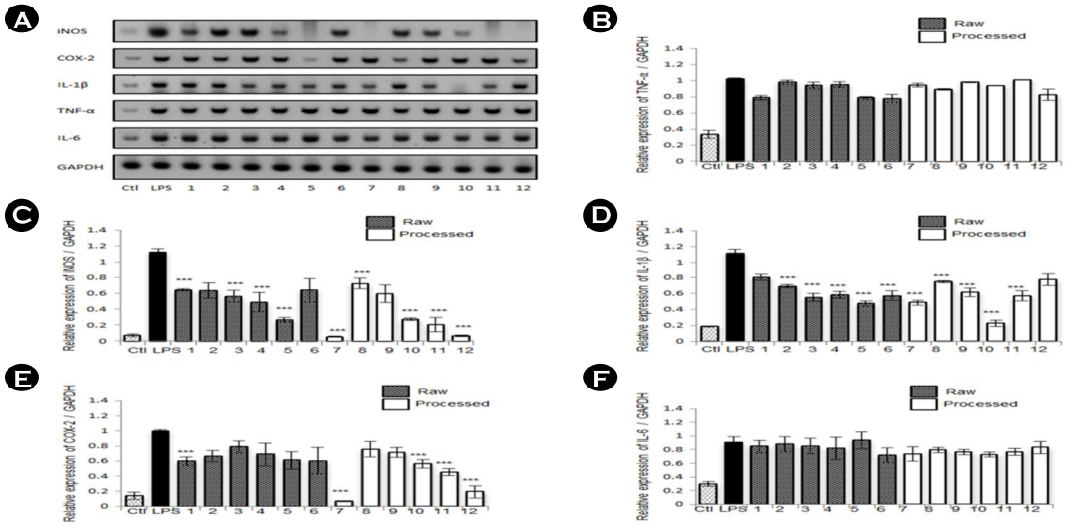

Figure 5. Reverse-transcriptase polymerase chain reaction of pro-inflammatory cytokines in RAW 264.7 cells treated with or without fruit and vegetable samples.

radish showed potent inhibition of iNOS. COX-2 had also been inhibited potently by raw cabbage, whereas processed cabbage has potently inhibited IL-1β. TNF-α and IL-6 were not inhibited by any of the samples. [15,16]. This has been confirmed with the quantification of gel images using ImageJ software (Fig. 5).

RAW 264.7 cells were seeded in 6-well plates for 24 hours, treated with or without sa*m*ples at 1 mg/mL and treated with or without LPS 30 minutes later. RNA was extracted 18 hours later using TRIzol solution as described, and RT-PCR was carried out. The expressions of iNOS, COX-2, TNF-α, IL-1β, IL-6 and GAPDH was determined. Samples 1 - 6 are raw samples of (1) Apple, (2) Carrot, (3) Pear, (4) Broccoli, (5) Cabbage and (6) Radish. Samples 7 – 12 are processed samples of (7) Apple, (8) Carrot, (9) Pear, (10) Broccoli, (11) Cabbage and (12) Radish, as shown in (A). The quantification of gel images were carried out using ImageJ software in triplicates for the relative expression of cytokines (B) iNOS, (C), COX-2, (D), TNF-α, (E) IL-1β, and (F) IL-6 against GAPDH. The experiment was done in triplicate. Values in bar graph are mean ± SEM of at least 3 independent experiments. *** $p < 0.001$ compared to LPS only.

5. Conclusion

Results obtained using DPPH assay in our study has indicated that the processed forms of apple, carrot, pear, broccoli and cabbage showed increased radical scavenging activity, indicating that processed samples have better antioxidant activity compared to the unprocessed samples. Among the samples, processed pear sample has the potent antioxidant activity, similar to the positive control, which is ascorbic acid. In the ABTS assay, apple, carrot, broccoli, cabbage and radish samples showed similar radical scavenging activity for both raw and processed samples. However, processed radish samples showed lower radical scavenging activity as compared to its raw counterpart, contradicting with the results obtained in the DPPH assay. A previous study has found that the antioxidant activity measured by ABTS assay highly correlates to the total phenolic contents in plums, with a weaker correlation towards antioxidant activity and total flavonoids content. This suggests that all raw and processed samples, except for processed pear, have relative high total

phenolic content, and processing of pear has reduced its phenolic content, but has increased its antioxidant activity and flavonoid content. ABTS cation reacts readily with molecules that are able to donate hydrogen atoms and electrons, like phenolic compounds. This causes the disappearance of the blue / green color of the radical. ABTS radicals are relatively more reactive than DPPH radicals. DPPH radicals involves the transfer of hydrogen atoms whereas ABTS radicals work by the transfer of electrons. This explains the generally high radical scavenging activity shown by the samples in the ABTS assay, also indicating that the samples have high phenolic content. Although processed pear has slightly lower radical scavenging activity in the ABTS assay, it has potent effect in DPPH assay, which indicates that it has high antioxidant activity, but lower phenolic content. Further research can be carried out on the processed form of pear to identify its bioactive contents, compared to its raw form. Inflammation contributes to the pathophysiology of many chronic ailments. NO and ROS play an important role in inflammation, therefore, we investigated the anti-inflammatory properties of these samples. Despite the beneficial roles of NO, large amounts generated by iNOS are toxic and pro-inflammatory in nature. Therefore, NO is a marker of inflammation. We have investigated the ability of these samples to inhibit NO production in LPS-treated RAW 264.7 cells. From our results, raw carrot, pear and cabbage, and processed broccoli and cabbage potently inhibited NO production. Moreover, their concentrations were not cytotoxic towards RAW 264.7 cells.

We then proceeded to investigate the mRNA expression levels of pro-inflammatory mediators and cytokines. Based on the results, raw cabbage inhibited the production of both iNOS and COX-2, while processed broccoli inhibited production of iNOS and IL-1β, both samples which were the most potent among all other samples. Raw broccoli, processed apple, processed cabbage and processed radish had also potently inhibited iNOS production. When comparing raw and processed samples, processed samples have strongly inhibited iNOS expression as compared to raw samples, concluding that processed samples had potent their anti-inflammatory properties. Based on the results, broccoli and cabbage have potent anti-inflammatory effects, aligned with the results obtained in the inhibition of NO.

Overall, our results showed that processed samples have better antioxidant and anti-inflammatory properties as compared to their raw forms.

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