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The Anti-Inflammatory and Anti-Oxidant Activity of Ethanol Extract from Red Rose Petals

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Abstract

Red rose petals are usually disposed but they are an abundant source of phenolics and traditionally used as food supplement and as herbal medicine. Of the Various phenolics, they are known to have anticancer, antioxidant, and anti-inflammatory properties. In this study, we investigated the anti-inflammatory effects of red rose ethanolic extracts (GRP) on lipopolysaccharide (LPS)-activated RAW 264.7 cells. The results demonstrated that pretreatment of GRP (500μg/mL) significantly reduced NO production by suppressing iNOS protein expression in LPS-stimulated cells. Anti-inflammatory effects by red rose petals were observed in the following. Red rose petals inhibited the translocation of NF-κB from the cytosol to the nucleus via the suppression of IκB-α phosphorylation and also inhibited LPS-stimulated NF-κB transcriptional activity. These findings suggest that red rose petals exert anti-inflammatory actions and help to elucidate the mechanisms underlying the potential therapeutic values of red rose petals. Therefore, red rose petals could be regarded as a potential source of natural anti-inflammatory agents.

Keywords*: Anti-Oxidant Activity, Anti-Inflammatory, Lipopolysaccharide, No Production, Red Rose Petal*

1. Introduction

Plant-based sources have increasingly gained the interest of scientists in search of naturally-existing antioxidants. These sources exhibit strong biological activity and are easily available. Moreover, they are better alternatives to several synthetic antioxidants that promote carcinogenesis. Antioxidant com pounds sourced from plants exhibit protective effects in cells against reactive oxygen species (ROS) [1, 2]. They consist of singlet oxygen, peroxyl radicals, hydroxyl radicals, superoxide, and peroxynitrite, which induce oxidative stress, and thereby cause cellular damage. Therefore, it is important to continue evaluating the antioxidant activities and free radical-quenching abilities of plant-based sources. In our study, we investigated the antioxidant properties of red rose petal extracts. We also investigated heat-treated red rose petals extracts that underwent indirect heat treatment, since processed red rose petals extracts have been indicated to exhibit elevated and improved bioactive properties. Therefore, we aimed to investigate the antioxidative effects of heat-treated red rose petals extracts to determine whether increased processing improved their bioactive

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properties [3-5]. Inflammation occurs when an organism combats invasion, either physically or via noxious chemical stimuli. The inflammatory response is a mechanism that inactivates invading pathogens. Since ROS are activators of inflammation. we proceeded to investigate the anti-inflammatory properties of the aforementioned samples. Both nitric oxide (NO) and ROS modulate inflammation. Lipopolysaccharides (LPS), specific ligands to toll like receptors and inducers of inflammation, are chemical moieties that are present in the outer membrane of gram negative bacteria [11, 12]. In our study, we also investigated the anti-inflammatory properties of the samples by treating RAW 264.7 cells with LPS in order to determine NO production and expression of pro-inflammatory mediators (iNOS and COX-2) and cytokines (TNF-α, IL-1β, and IL-6) at the transcriptional level.

2. Experiment Materials and Methods

2.1 Sample Preparation

The heat-treated sample was cooled, and then crushed using a crusher, and a 10-fold volume (v/v) of ethanol was added, followed by extraction at 60℃ for 2 hours. The ethanol extract was filtered, and then freeze-dried before use. The samples were washed, cut into uniform shapes of 0.5 cm×0.5 cm×0.5 cm, freeze-dried, sealed dry to keep moisture away, and stored in -70℃ Raw samples were prepared by extracting the samples at 60℃ for 2 hours. To obtain processed samples, previously freeze-dried samples were heat-treated under pressurized conditions (10 kg/cm^2) using a heating apparatus (Jusco, Seoul, South Korea). Samples were placed in the inner compartment and water was added to the outer compartment of the container. The apparatus was heated according to predetermined temperature and time values (60~70℃ for 2 hour) to prevent carbonization of the samples from direct heat. Finally, samples were weighed for further experiments.

2.2 Reagent

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were obtained from WelGENE (South Korea). Streptomycin and penicillin were obtained from Lonza (MD, USA). TRIzol reagent was sourced from In-vitrogen (Carlsbad, CA, USA). LPS (Escherichia coli 055: B5), 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) were obtained from Sigma-Aldrich. Oligo(dT) and iNOS, COX-2, TNF-α, IL-1β, IL-6, and GAPDH primers were purchased from Bioneer (South Korea).

2.3 DPPH Radical Scavenging Activity

To measure the radical scavenging activity of red rose petal extracts, 20 μL of the samples were mixed with 200 μL of DPPH solution (0.2 mM in ethanol). Ethanol was used as the control for these experiments. After a 30-min reaction at 37℃, the absorbance of the solution was measured at 517 nm. The free radical scavenging activity of each fraction was determined by comparing their absorbance with that of the control groups. Ascorbic acid was used as a positive control sample. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH Radical Scavenging Activity (%) = [1 - (A1 - A2)/(A3 - A4)] × 100

Where, A1 is the absorbance of DPPH and the sample, A2 is the absorbance of 100% ethanol and the sample, A3 is the absorbance of DPPH and the solvent for sample dilution (DMSO/DDW), and A4 is the absorbance of 100% ethanol and the solvent for sample dilution (DMSO/DDW).

2.4 ABTS Radical Scavenging Activity

The ABTS cation radical was produced via a reaction between 5 mL of 14 mM ABTS solution and 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution, which was then stored in the dark for 16 h at room temperature. Before use, this solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Various concentrations of the red rose petal extracts (50 μL) were mixed with 100 μL of ABTS solution in 96-well plates and allowed to stand in the dark for 10 min. Trolox was used as the positive control group for standardizing ABTS activity. The inhibition percentage of the ABTS radical was calculated using the following formula:

ABTS Scavenging Activity (%) = [1 - (A1 - A2)/(A0)] × 100

Where, A1 is the absorbance of the ABTS working solution and sample, A2 is the absorbance of the sample without ABTS working solution, and A0 is the absorbance of the ABTS working solution only.

2.5 Cell culture

This study used RAW 264.7 cells, a murine macrophage cell line obtained from the American Type Culture Collection (ATCC), which were maintained in DMEM (WelGENE, South Korea) supplemented with 5% FBS, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin sulfate. The cells were maintained at 37℃ and 5% CO_2.

2.6 Nitric oxide & MTT cell viability assays

NO assay was carried out using RAW 264.7 cells, which were seeded into 96-well plates for 24 hours. The cells were treated with various sample concentrations, followed by 0.1 μg/mL LPS treatment 30 min later. After 18 hours of incubation, 100 μL of the supernatant was collected, 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 to the cells at a concentration of 0.1 mg/mL. The plates were incubated for 3 hour at 37℃ and 5% CO_2. The resulting crystals were dissolved in DMSO and read at 560 nm using a microplate reader (VersaMax).

2.7 Reverse-transcriptase polymerase chain reaction (RT-PCR)

After seeding RAW 264.7 cells into 6-well plates for 24 hours, they were treated with or without 1 mg/mL of samples followed by 0.1 μg/mL of LPS 30 min later. After 18 hours, TRIzol reagent was used to extract RNA. 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

2.8 Statistical analysis

All data is presented as mean ± SEM. One-way ANOVA and Dunnett's test were applied for statistical evaluation of the data. Statistical analyses with $P < 0.001$ were considered to be significant.

3. Result and Discussion

3.1 Radical scavening activity(DPPH, ABTS) of red rose petal ethanol extracts

ROS generated from these reactions affect cellular components like DNA, proteins, and lipids, resulting in oxidative DNA damage. Hence, there is a need to reduce ROS quantities via the intake of food and nutrients exhibiting high antioxidant activity. DPPH, which has an absorption band of 517 nm and diminishes due to reduction in the presence of dietary antiradical compounds, was used here to investigate the antioxidant activity of compounds and extracts. ABTS cation generation via the ABTS-potassium persulfate reaction was also utilized in this study as a marker for antiradical compounds existing in the samples. DPPH assay results indicated that the heat-treated forms of red rose petal eaxtract showed increased radical scavenging activities, indicating that processed samples exhibited better antioxidant activities when compared to unprocessed samples. The red rose petal extracts showed potent antioxidant activity similar to ascorbic acid (positive control). In the ABTS assay, red rose petal extracts samples showed similar radical scavenging activities via both raw and processed samples. A previous study conducted that the antioxidant activity measured by the ABTS assay highly correlated with total phenolic content while exhibiting a weak correlation towards antioxidant activity and total flavonoid content. This suggested that all the raw and processed samples in this study had relatively high total phenolic content which had reduced phenolic content but increased antioxidant activity and flavonoid content. The ABTS cation readily reacts with molecules that donate hydrogen atoms and electrons, such as phenolic compounds, which causes the appearance of the blue-green color of the radical. ABTS radicals are relatively more re- active than DPPH radicals. DPPH radicals are involved in the transfer of hydrogen atoms, whereas ABTS radicals work via electron transfer. This explains the generally high radical scavenging activity of the samples in the ABTS assay. It also indicates that the samples have high phenolic content. Although heat-treated red rose petal extracts exhibits slightly lower radical scavenging activity in the ABTS assay, its effect in the DPPH assay is quite potent, demonstrating its high antioxidant activity and low phenolic content. ROS are produced during normal metabolic and physiological reactions such as signal transduction, gene expression, and mitochondrial electron transport. ROS generated from these reactions affect cellular components like DNA, proteins, and lipids, resulting in oxidative DNA damage. Hence, there is a need to reduce ROS quantities via the intake of food and nutrients exhibiting high antioxidant activity. DPPH, which has an absorption band of 517 nm and diminishes due to reduction in the presence of dietary antiradical compounds, was used here to investigate the antioxidant activity of compounds and extracts.

The antioxidant properties of red rose petal extracts were compared for ascorbic acid in figure 1 using DPPH assay. Ascorbic acid was used as positive control. Based on the results, heat-treated red rose extracts exhibited a higher percentage of radical scavenging activity as compared to the raw samples (Figure 1). 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[13,14].

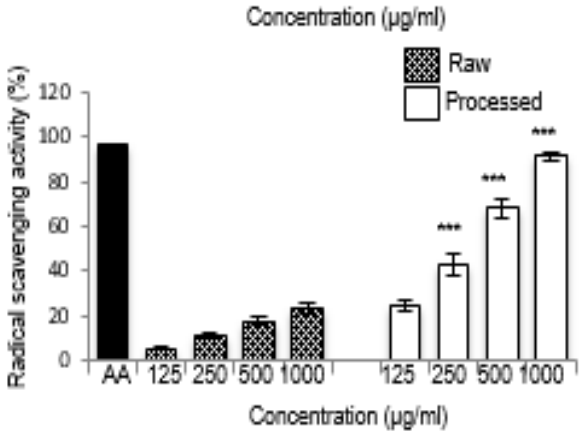

Figure 1. Radical scavening activity of red rose petal ethanol extracts using DPPH ass ay. The antioxidant activity of samples were tested, and compared to 100 µg/mL of ascorbic acid (AA) which was used as a positive control. The absorbance was read at 517 nm using a microplate reader, and the radical scavenging activity was calculated using the formula as mentioned in materials and methods section. The radical scavenging activity of raw and proc essed red rose petal extract was shown in Figure 1. Processed samples show relatively hig her 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 c ompared to ascorbic acid only.

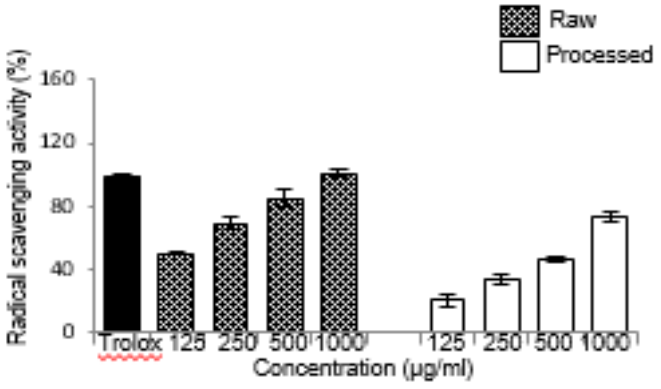

Figure 2. Radical scavening activity of red rose petal ethanol extracts using ABTS assay. The 5 mM of Trolox was used as a positive control. The absorbance was read at 734 nm using a microplate reader, and the radical scavenging activity was calculated. The radical scavenging activity of raw and processed red rose petal extract was shown in Figure 2. Processed samples show relatively higher amounts of scavenged radicals, indicating higher antioxidant activity. Using ABTS assay, all samples show good radical scavenging activity as compared to Trolox. Values in bar graph are mean ± SEM of at least 3 independent experiments. ***p < 0.001, significantly increased as compared to Trolox only.

3.2 Nitric oxide (NO) production inhibitory effect and cytotoxicity of red rose petal extract

Red rose petal extract inhibited the production of NO induced by LPS in RAW264.7 cells. Macrophage RAW264.7 cells were treated with various concentrations of red rose petal ethanol extract (GRP) to confirm whether NO production was inhibited by LPS. There was no significant cytotoxicity from 1.9 μg/mL to 500 μg/mL (Figure 3), and NO production was significantly inhibited, especially at concentrations of 250 and 500 μg/mL (Figure 3). Based on these results, the concentration of RGP was determined to be 50, 100, 250 and 500 μg/mL ((Figure 4).

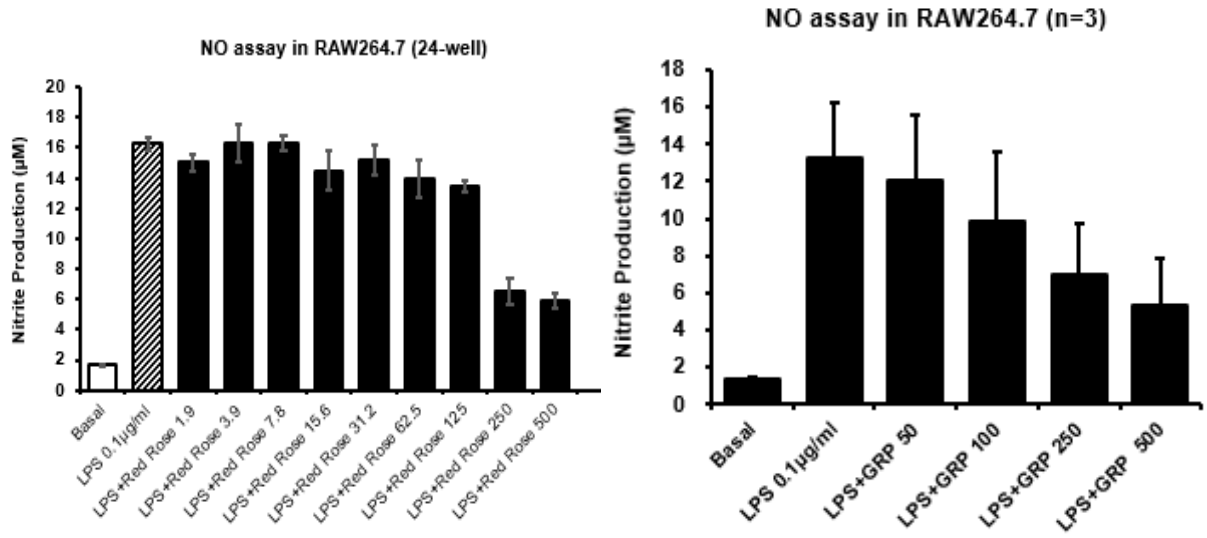

Figure 3. Inhibition of nitric oxide (NO) production of red rose petals ethanol extract screening in inflammatory reaction induced by LPS (lipopolysaccharide) in Raw 264.7 cells. Cells were seeded in 96-well plates, treated with or without samples after 24 hours of incubation and treated with or without 0.1 μg/mL of LPS after 30 minutes. The results of the assay by raw and processed red rose petal extracts was shown in Figure 3. 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.

RAW264.7 cells were treated with 50, 100, 250, and 500 μg/mL red rose petal ethanol extract (GRP) and stimulated with LPS. As shown in Figure 4, GRP inhibited the increase of NO produced by LPS in a concentration-dependent manner, and showed a statistically significant effect especially at a concentration of 500 μg/mL (Figure 4).

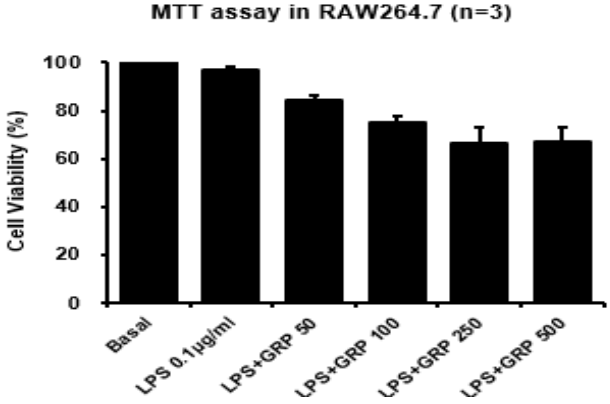

Figure 4. Effect of cell viability of red rose petals extract on Raw 264.7 cells in inflammatory reaction induced by LPS (lipopolysaccharide). Cells were seeded in 96-well plates, treated with or without samples after 24 hours of incubation and treated with or without 0.1 μg/mL of LPS after 30 minutes. The results of the assay by raw and processed red rose petal extracts was shown in Figure 4. 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.

3.3 Measurement of inflammatory effect and cytokine expression levels in Raw264.7 cells

RAW264.7 cells were treated with red rose petal ethanol extracts and stimulated with LPS. Total RNA was extracted from the cells, and mRNA expression levels of iNOS, COX-2, TNF-α, IL-1β, and IL-6 were examined through RT-PCR. As shown in Figure 5, both inflammatory mediators and inflammatory cytokines significantly increased expression by LPS, which was significantly or partially reduced by red rose petal ethanol extract (GRP) treatment. Synthesizing the above results, it was confirmed that the red rose petal ethanol extract (GRP) has a potential anti-inflammatory effect in an in vitro test using RAW264.7 cells.

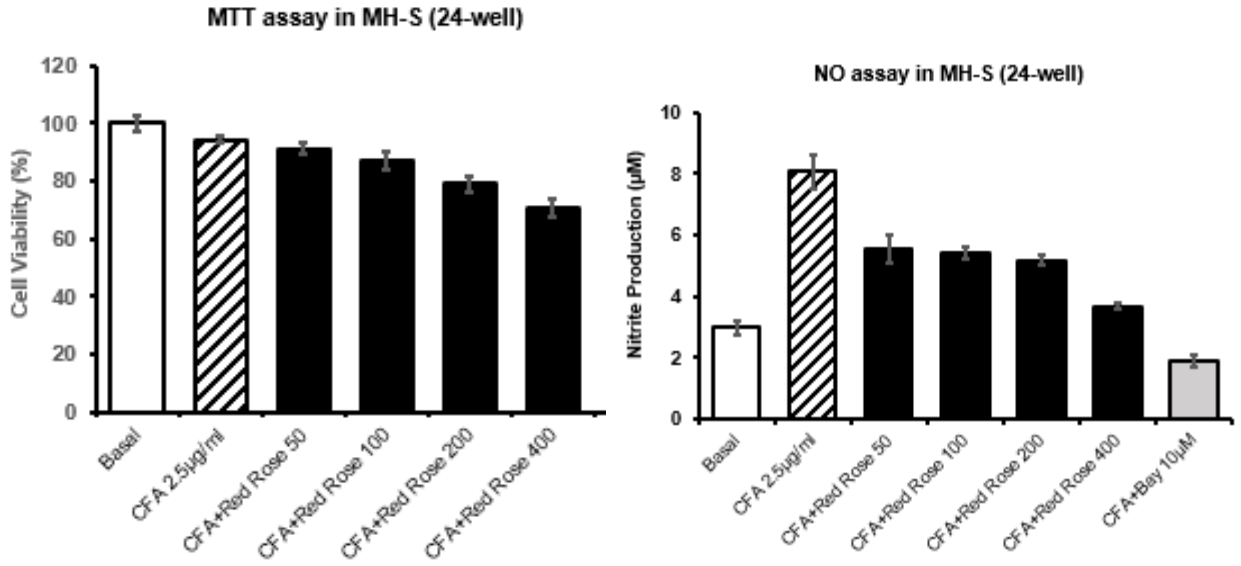

Figure 5. Effect of NO and cell viability of red rose petals extract on MH-S cells in inflammatory reaction induced by LPS (lipopolysaccharide). Cell viability assay in MH-S cells treated with fruit and vegetable samples. Cells were seeded in 24-well plates, treated with or without samples after 24 hours 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 red rose petal extract was shown in Figure 5. Based on the results, all samples that were tested does not exhibit cytotoxicity towards MH-S cells. Values in bar graph are mean ± SEM of at least 3 independent experiments.

3.4 NO production inhibition and inflammatory mediator and cytokine expression level measurement in MH-S cell

MH-S cells were treated with various concentrations of heat-treated red rose petal extracts to confirm whether or not NO was inhibited by LPS. From 1.9 μg/mL to 500 μg/mL concentration, the red rose petal extracts inhibited NO production by LPS treatment in a concentration-dependent dosage, but showed cytotoxicity at 500 μg/mL. Based on these results, the concentration of the red rose petal extracts to be used in the next test was determined to be 500 μg/mL or less. The heat-treated red rose petal extracts inhibited the

production of NO induced by LPS in MH-S cells. MH-S cells were treated with 50, 100, 200, and 400 μg/mL red rose petal extracts, followed by stimulation with LPS. As shown in Figure 5, the heat-treated red rose petal extracts inhibited increased NO production by LPS in a concentration-dependent dosage, and showed a statistically significant inhibitory effect, especially at a concentration of 400 μg/mL (Figure 5).

3.5 Pro-inflammatory mediator and cytokine expression in MH-S cells using RT-PCR

Pro-inflammation mediator (iNOS, COX-2) and inflammatory cytokine (TNF-α, IL-1β, IL-6) mRNA expression of MH-S cells were assessed using RT-PCR. As shown in Figure 6, the red rose petal extract significantly inhibited the expression of iNOS, COX-2, IL-1β and IL-6. Expression of TNF-α was not a significant change between treatment groups. Synthesizing the above results, it was confirmed that the red rose petal ethanol extract has a potential anti-inflammatory effect in an in vitro test using MH-S cells. Red rose petal ethanol extract inhibited inflammatory cytokines and inflammatory mediators induced by CFA in MH-S cells (Figure 6).

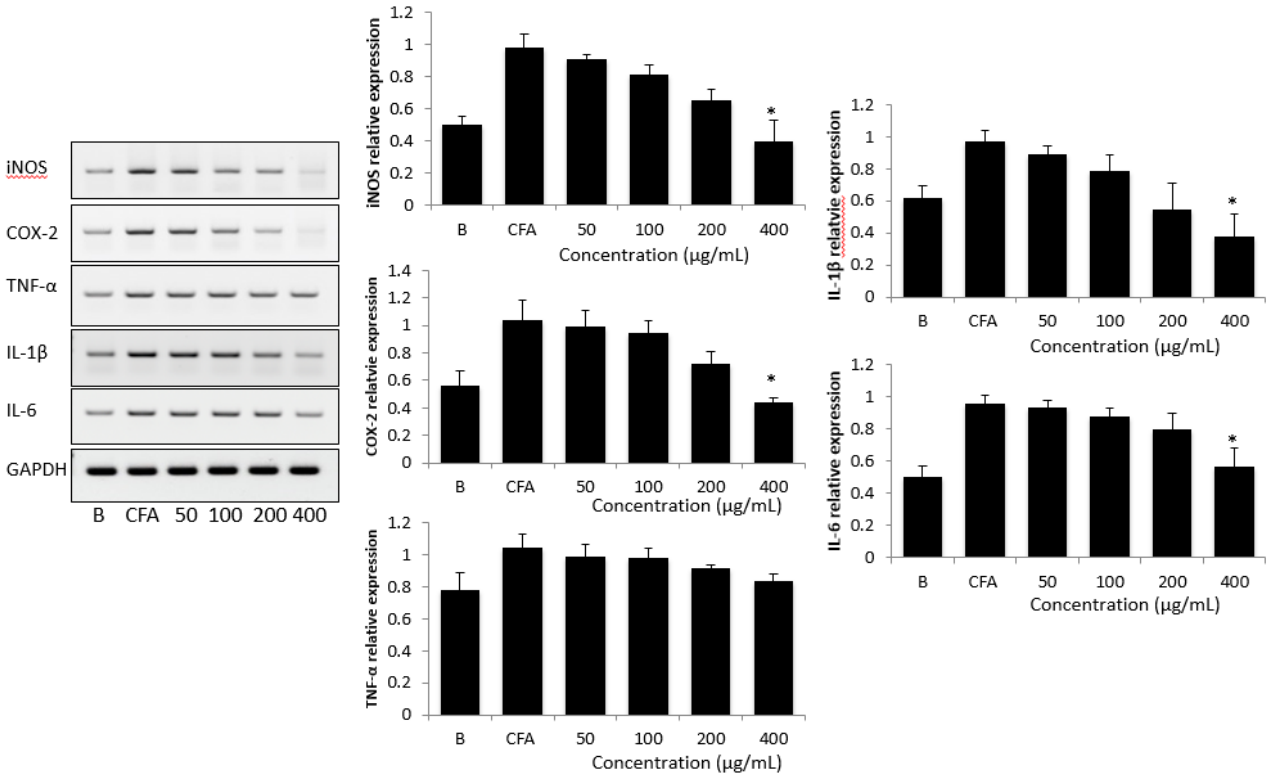

Figure 6. Inhibition of NO production and cytotoxicity screening of red rose petals eth anol extract in MH-S cells. RAW 264.7 cells were seeded in 6-well plates for 24 hours, tr eated with or without samples at 1 mg/mL and treated with or without LPS 30 minutes later. RNA was extracted 18 hours later using TRIzol solution as described, and RT-PCR was car ried out. The expressions of iNOS, COX-2, TNF-α, IL-1β, IL-6 and GAPDH was determined. The quantification of gel images were carried out using ImageJ software in triplicates for the relative expression of cytokines, iNOS, COX-2, TNF-α, IL-1β, and IL-6 against GAPDH. The experiment was done in triplicate. Values in bar graph are mean ± SEM of at least 3 indep endent experiments. ***p < 0.001 compared to LPS only.

4. Conclusion

In our study, we investigated red rose petal extracts for antioxidant and anti-inflammatory activities. Furthermore, raw and heat-treated samples were compared to determine whether additional heat treatment on the raw samples improved their bioactive properties. The heat-treated red rose petal extracts exhibits slightly lower radical scavenging activity in the ABTS assay, its effect in the DPPH assay is quite potent, demonstrating its high antioxidant activity and low phenolic content. Inflammation contributes to the pathophysiology of many chronic ailments. Since NO and ROS play an important role in inflammation, we investigated the antiinflammatory properties of these samples. Despite its beneficial roles, the large quantities of NO generated by iNOS are toxic and pro-inflammatory in nature, making it a marker of inflammation. In this study, we investigated the ability of these samples to inhibit NO production in LPS-treated RAW 264.7 cells. From our results, the red rose petal extracts potently inhibited

NO production. Moreover, their concentrations (up to 1 mg/mL) were not cytotoxic to RAW 264.7 cells. We also proceeded to investigate the mRNA expression levels of pro-inflammatory mediators and cytokines. Based on our results, the red rose petal extracts inhibited the production of iNOS, COX-2, iNOS and IL-1β. The heat-treated samples strongly inhibited iNOS expression when compared to raw samples, thereby establishing their potent anti-inflammatory properties. Overall, our results showed that the Heat-treated samples exhibited better antioxidant and anti-inflammatory properties when compared to their raw forms. They demonstrated higher radical scavenging activities via the DPPH and ABTS assays and stronger NO and iNOS inhibition at the transcriptional level. The heat-treated red rose petal samples showed high antioxidant and flavonoid content (DPPH). The heat-treated red rose petal samples exhibited the strongest anti-inflammatory properties.

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