Radical scavenging activities of enzyme-treatment *Rhus verniciflua* Stokes via immune-redox on murine macrophage RAW 264.7 cells

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For my beloved family, you have been my inspiration from the beginning until the end. I dedicate all my hard work for my parents and my sisters.

To God, I give you all the honor, glory and thanksgiving!

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ABBREVIATIONS

ANOVA	Analysis of variance
CCK-8	Cell Counting Kit-8
DMEM	Dulbecco's modified eagle's medium
DCFH-DA	2', 7'-dichlorodihydrofluorescein diacetate
ERK1/2	Extracellular signal-regulating kinase
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
IL	Interleukin
NO	Nitric oxide
PBS	Phosphate buffered saline
p-p38 MAPK	Phospho-p38 mitogen-activated protein kinase
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor-alpha

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ABSTRACT

Radical scavenging activities of enzyme-treatment *Rhus verniciflua* Stokes via immune-redox on murine macrophage RAW 264.7 cells

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Rhus verniciflua stokes (RVS), which contains the major bioactive polyphenolic and flavonoids compounds including fustin, fisetin, quercetin, butein, kaempferol, sulfuretin, protocatechuic acid, catechol and ethyl gallate is known to have antioxidant and anti-inflammatory effects. In this study, we investigated the radical scavenging activities and anti-inflammatory effect of enzyme-treated and urushiol free RVS extract via immune-redox system in murine macrophage RAW 264.7 cells. The radical scavenging and anti-inflammatory activities of RVS extract investigated against hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS)-induced oxidative stress and inflammatory response in RAW 264.7 cells. Herein, the potent cytotoxic activity of RVS has been studied by cell viability and cell death on RAW 264.7 cells. Similarly, intracellular ROS and RNS and potential antioxidant enzyme in RAW 264.7 macrophage cells were also investigated. Furthermore, in this study we investigated the effect of RVS on inflammatory markers such as cytokines including interleukin (IL)-1β, IL-6, IL-10, IL-12, interferon-γ (INF-γ) and tumor necrosis factor-alpha (TNF-α) and kinase signaling pathways.

These cells were exposed with RVS extract with different concentration (0, 12.5, 25, 50 and 100%) for 24 hrs under the oxidative stress and we found that the RVS extract significantly reduced the intracellular ROS and NO formation dose-dependently and also prevented the cell death of macrophage RAW 264.7 cells. Furthermore, RVS extract ameliorated LPS-induced pro-inflammatory cytokines IL-1β, IL-6, IL-10, IL-12, INF-γ and TNF-α in the RAW 264.7 cells dose-dependently. Lastly, it was found that MAPK protein and nuclear factor-E2-related factor 2 (Nrf2) were involved in the antioxidant effect of RVS extract against H_2O_2-induced oxidative stress in macrophage RAW 264.7 cells. In conclusion, the present study indicated that urushiol-removed RVS extract is advantageous to reduce the intracellular ROS through its antioxidative properties and anti-inflammatory effect which could be use as a functional food ingredient to improve the immune cells function and its signaling pathways.

Key Words: *Rhus verniciflua* Stokes, Flavonoids, Oxidative stress, Inflammation, Antioxidant

I. INTRODUCTION

Rhus verniciflua Stokes (RVS) is a deciduous tree of the Anacardiaceae family which commonly known as the lacquer tree in Korea, Japan and China and it is the most ancient and widely used plant as a traditional food additive and medicinal properties for its various biological activities such as antioxidant, anti-bacterial, anti-allergic inflammatory effects and other activities [1-3]. Furthermore, recent years experimental studies shown that a diverse range of phenolic and flavonoid compounds from RVS or the whole extract have been reported to exhibit various pharmacological effects including amelioration of oxidative stress and inflammation that may lead to the development of various chronic diseases [4, 5]. These pharmaceutical activities are mediated by the major flavonoids of RVS including fustin, fisetin, quercetin, butein, kaempferol, sulfuretin, protocatechuic acid, ethyl gallate and catechol [4, 6-9]. In spite of these activities, many researchers have been reported to have some allergic reaction with these extracts because of its component known urushiol. Urushiol causes allergies, contact dermatitis, irritation, inflammation and blistering of sensitive individuals [10]. For this reason, many scientific methods and research has been focused on removing urushiol derivatives from RVS by heat treatment, solvent extraction, far-infrared radiation and microbial fermentation [11-13]. The degree of antioxidant activity may depend on the extracting method and solvent polarity [13]. For example, an ethanol extract from RVS was found to have an anti-oxidant effect against oxidative damage by scavenging reactive oxygen species (ROS) or by altering the oxidized forms of chemicals and also prevent oxidation-mediated apoptotic death of thymocytes [4, 14–16]. Previous study revealed that fermentation reduces the urushiol and it has various *in vitro* and *in vivo* biological activities including antioxidant and anti-inflammatory effects [4, 17, 18].

The antioxidant and anti-inflammatory effect of flavonoids are most important biological activities because of their ability to scavenge ROS, thereby reducing oxidative stress. Antioxidants decrease oxygen concentration by scavenging initial free radicals, binding to metal ion catalysts, decomposing primary products to active compounds and chain breaking to prevent continued hydrogen abstraction. Persistent ROS generation causes oxidative stress has been implicated in inflammation and several disorders, including neurodegenerative diseases and immune dysfunction [19, 20]. Consequently, numerous plant-derived flavonoid compounds have been found in recent years to offer a range of health-promoting benefits, including the amelioration of oxidative stress and inflammation that may lead to the development of various chronic diseases [21, 22]. The interest in RVS flavonoids has arisen because the intake of these compounds has been associated with the prevention and treatment of inflammatory diseases by reducing oxidative stress and inflammation, which is translated to benefits in health [23-25]. Activated macrophages produce ROS and NO which causes substantial oxidant injury to surrounding tissue [26-28]. The oxidative stress-induced cytotoxicity and inflammatory response extensively studied in H_2O_2 and LPS-stimulated RAW 264.7 cells respectively, which are very sensitive to LPS stimulation and respond by activation of the pro-inflammatory transcription factors; nuclear factor-kappa B (NF-kB) resulting in TNF-α, IL-1β, IL-6, IL-8 and NO production [29–31]. LPS activated macrophages also well documented inducer of ROS production which are capable of eliciting a variety of pathological changes including peroxidation of lipids, proteins and DNA and an elevated level of ROS activates mitogen activated protein kinase (MAPKs) and inflammatory transcription factors [32–34]. Recent data described beneficial effects of RVS limited on inflammatory processes and kinase signaling pathways.

Therefore, in this study we investigated the scavenging effect of ROS and anti-inflammatory activities of enzyme-treated and urushiol free RVS extract via immune-redox system in murine macrophage RAW 264.7 cells. We further explored the effect of our experimental enzyme-treatment RVS extract with positive control (commercially available) RVS extract to clarify the effectiveness of our compound. In addition, we also investigated the protective effect of RVS extract on H_2O_2 and LPS-induced oxidative stress and activity of RVS in regulating intracellular molecules associated with cell survival and to confirm biosafety activities of the phenolic and flavonoid components of RVS extract on cell death and immune function in RAW 264.7 cells.

II. MATERIALS AND METHODS

2.1. Preparation of Rhus Verniciflua Stokes (RVS) Extract

The RVS extract used in this study, urushiol-removed RVS extract was provided by Dawoom Food Ltd., Korea and stored in a refrigerator until use. To remove debris from the extract was filtrated by the extract filtration method (Corning bottle top filter, Polystyrene, pore Size 0.22 μm, USA).

2.2. Characterization of Rhus Verniciflua Stokes Extract

The enzyme-treated extract was standardized and allergenic urushiol-removed RVS detoxification and flavonoids compounds were verified by high-performance liquid chromatography (HPLC) analysis.

2.3.1. Cell Culture

The murine macrophage RAW 264.7 cells were cultured and routinely maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone Laboratories, Inc. South Logan, USA,) containing 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc. South Logan, USA) and 1% antibiotic-antimycotic (Gibco, Invitrogen Corporation, Auckland, NZ) in a humidified incubator with 5% CO_2 at 37˚C. The RAW 264.7 cells is adherent in nature to culture flasks within 24 hrs and when the cells reach 80% confluence, the cell growth media was aspirate, made cell suspensions, centrifuged and the pellet were resuspended in culture medium and proceed further steps.

2.3.2. Cytotoxicity Measurement of the RVS extract on RAW 264.7 Cells Using Cell Viability

Live RAW 264.7 cells with RVS treatment were determined with MUSE cell viability and count Kit (Merck Millipore, KGaA, Darmstadt, Germany). The cells ($1x10^5$ cells per well) were seeded in 6-well plate and allow to adhere overnight. After 80% cell confluence, the medium was removed and then cells were treated with different concentrations (0, 12.5, 25, 50 and 100%) of RVS for 24 hrs and cells which were not treated with the RVS indicated as normal control (NC) group in this experiment. The cells were harvested by trypsinization and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended with cell culture medium and made it ready to analyze further activities.

The cell viability also determined using the cellular chemosensitivity reagent cell counting kit-8 (CCK-8, Dojindo molecular Technologies, MD, USA) assay according to manufacturer's instructions. The CCK-8 is highly water-soluble tetrazolium salt (WST-8) allows sensitive colorimetric assays for the determination of viable cells in cell proliferation and cytotoxicity assay following treatment with RVS extract. The WST-8 produces a water-soluble formazan dye upon reduction in the presence of an electron mediator by dehydrogenase in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenase in cells is directly proportional to the number of living cells.

In brief, RAW 264.7 cells ($1x10^4$ cells per well) in 100μL culture medium were seeded in 96-well microplate and incubated at 37˚C for 24 hrs. After 80% cell confluence, the cells were treated with different concentrations (0, 12.5, 25, 50 and 100%) of RVS for 24 hrs and cells which were not treated with the RVS indicated as NC cells group in this experiment. Following

24 hrs of incubation, commercially available CCK-8 solutions and 10μL was added to 100μL cell culture medium and the plates were incubated for a further 1-2 hrs to evaluate the cytotoxic effect of RVS extract. The optical density was determined at 340nm by using DTX-880 multimode microplate reader (Beckman Coulter, Inc., Brea, CA, USA). All experiments were performed in triplicate.

2.3.3. Cell Death Assay

Cell death following RVS treatment was determined using MUSE Annexin-V and cell death reagent. To find apoptosis, cells were treated with 100μM H_2O_2 for 2 hrs and after that cells treated with RVS extract for 24 hrs and harvested cells were mix with the same amount of Muse Annexin-V and cell death reagent and incubated at room temperature for 20 min and then analysis were performed in triplicate data values.

2.4. Induction of Oxidative Stress

The induction of ROS was made with the trial at different time and dose depended manner with H_2O_2 and finally we have decided to expose the cells to 100μM H_2O_2 for 2 hrs and ROS and inflammatory response also induced as well as by LPS treatment in macrophage RAW 264.7 cells. Cells were treated with 5μg/mL LPS concentration for 2 hrs and then treated with different concentration (0, 12.5, 25, 50 and 100%) of RVS for 24 hrs.

2.4.1. Determination of Oxidative Stress

The level of total ROS generation in treated and untreated RAW 264.7 cells groups were measured by the oxidation of 2,4-dichlorodihydrofluorescein diacetate (DCFH-DA) using DTX-880 Multimode microplate reader (Beckman Coulter Inc., Fullerton, CA, USA). Oxidative damage of cells was carried out using H_2O_2 and LPS in RAW 264.7 cells. The molecule DCHF-

DA is freely permeable in cells and after incorporation into cells is converted into the fluorescent 2, 7-dichlorofluorescein (DCF) by oxidative substances. Therefore, DCHF-DA reveals the intracellular production of redox-active substances and has been widely used to investigate oxidative damage in intact cells.

The RAW 264.7 cells ($1x10^4$ cells per well) were seeded in 96-well plate and allowed to adhere overnight. After 80% cell confluence, the old medium was aspirate and then ROS was induced by using 100μM H_2O_2 and 5μg/mL LPS for 2 hrs after that cells treated with different concentrations (0, 12.5, 25, 50 and 100%) of RVS for 24 hrs indicate as experimental group and cells treated with only H_2O_2 and LPS indicate as negative control group and no treatment with RVS indicated as NC cells in this experiment. At the end of the RVS treatment, cells were incubated with 10μM of fluorescent probe DCHF-DA at 37 ℃ for 30 min. The degree of fluorescence, corresponding to intracellular ROS was determined. A dose-dependent response of RVS extract on ROS level and cytotoxicity in H_2O_2 and LPS-treated cells is shown in (Figure 4 and 5).

2.4.2. Determination of Nitric Oxide

The level of NO production in treated and untreated RAW 264.7 cells were determined with using the Griess reagent (Promega Corp., Madison, USA). Nitrite concentration in the medium was measured as an indicator of NO production according to the Griess reaction instruction manual. The assay relies on measuring nitrite (NO_2-), one of the primary, stable and non-volatile breakdown products of NO. Briefly, 50μL of the cell culture medium was mixed with an equal volume of Griess reagent in a 96-well microtiter plate and incubated at room temperature for 15 min. The absorbance was read at 540nm using a DTX-880 multimode microplate reader (Beckman Counter Inc., Fullerton, CA, USA). The NO_2- concentration was calculated by

comparison with the representative NO_2- standard curve generated by serial two-fold serial dilutions of nitrate). Each nitrite standard and sample was assayed in triplicate. A freshly prepared standard curve was used each time when assay was performed.

In brief, RAW 264.7 cells (1×10^4 cells) were seeded in 96-well plates, incubated for 24 hrs and treated with 100μM H_2O_2 and 5μg/mL LPS for 2 hrs and then treated with the indicated concentrations (0, 12.5, 25, 50 and 100%) of RVS for another 24 hrs, then 50μL of cultured medium and Griess reagent were mixed and incubate the plate at room temperature for 30 min, the absorbance at 540 nm was determined with a microplate reader.

2.5. Antioxidant Enzyme Study: Determination of Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and Catalase (CAT) Activity

Effect of purified RVS extract on SOD, GPx and CAT in H_2O_2 and LPS induced RAW 264.7 cells. The cells ($1x10^6$ cells per well) were seeded in 6-well plate and incubated for 24 hrs. The cells were incubated with H_2O_2 (100μM) and LPS (5μg/ml) for 2 hrs to generate oxidative stress by the production of ROS and NO. Then the cells were treated with RVS extract (0, 12.5, 25, 50 & 100%) for 24 hrs. The RAW 264.7 cells were harvested with ice-cold PBS, subjected to freeze-thaw process twice, prepared cell lysate with lysis buffer and then centrifuged at 15000 g for 10 min at 4˚C. The pellets (cellular debris) were rejected and the supernatant was collected and assayed for SOD, GPx and CAT activity.

2. 5.1. Superoxide Dismutase Activity

After RVS treatment of varying concentrations, SOD production from treated RAW 264.7 cells were detected by incubating the samples in the presence of SOD (1U/μL) for subtraction of the SOD-inhibitable signal using SOD detection kit (Biovision, Mountain View, CA, USA).

RAW 264.7 cells ($1x10^6$) were seeded in 6-well plates and incubated for 24 hrs and cells were incubated with H_2O_2 (100μM/ml) for 2 hrs to generate oxidative stress by the production of ROS and then treated with RVS (0, 12.5, 25, 50 & 100%) for 24 hrs. The cells were washed twice with phosphate-buffered saline (PBS) and cells were harvested with chilled PBS, subjected to lysis and then centrifuged at 15000 g for 10 min at 4˚C. The pellets (cellular debris) were rejected and the supernatants, i.e. the cell lysates supernatant were collected and assayed for SOD activity. Then, the post-incubation supernatant in 96-well plate was read with a fluorescent plate reader at 540nm and the SOD activities of different samples were thereby calculated according to the instructions provided with the kit.

2.5.2. Glutathione Peroxidase Activity

RAW 264.7 cells were seeded in 6-well plates for 24 hrs and then cells were incubated with H_2O_2 and LPS at 37˚C for 2 hrs. GPx levels in the RAW 264.7 cells was evaluated by measuring the H_2O_2 scavenging capacity using modified GPx assay kit (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx and was recycled in its reduced state by glutathione reductase and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to $NADP^+$ was accompanied by a decrease in absorbance at 340 nm.

2.5.3. Catalase Assay

RAW 264.7 cells were seeded in 6-well plates for 24 hrs and cells were incubated with H_2O_2 and LPS at 37˚C for 2 hrs. Then, it was treated with various concentrations of RVS extract for another 24 hrs. Catalase activity was detected using catalase detection kit (Biovision, Mountain

View, CA, USA). RAW 264.7 Then, the post-incubation supernatant in 96-well plate was read with a fluorescent plate reader at 540nm.

2.6. Preparation of Cell Lysate

RAW 264.7 cells ($1x10^6$) were cultured in a 6-well plate, cultured overnight then treated with 100μM H_2O_2 and 5μg/ml LPS separately and then the cells were treated with RVS extract (0, 12.5, 25, 50 & 100%) for 1 hrs. Briefly, upon washing cells with ice-cold PBS and were harvested and cell lysis with buffer containing RIPA buffer (Pierce Biotechnology) for 30 min on ice with the addition of protease inhibitor cocktails (Sigma Aldrich) The protein concentration was determined photometrically by a BCA assay (Pierce Biotechnology).

2.6.1. Cell Lysate Inflammatory Cytokines Assay

The concentrations of cell lysate cytokines (IL-1β, IL-6, IL-10, IL-12, INF-γ & TNF-α) were analyzed by using Bio-Plex cytokine assay according to the manufacturer's instructions. For analysis of cytokines, anti-cytokine conjugated beads were diluted 1:25 per well using assay buffer, transferred into each plate well and washed on the wash platform 2 times. Samples, standards and controls respectively were mixed with anti-cytokine conjugated beads and incubated for 2 hrs. After washing, specific biotinylated detection anti-cytokines were added and incubated for 30 min. Streptavidin-PE solution diluted with assay buffer A was added to each well, incubated for 15 min, and washed 3 times with wash buffer A. One hundred microliters assay buffer A was added into each well containing the beads and was read in multi-plex bead suspension array system (Bio-Plex 200, BIO-RAD® , USA).

2.6.2. Western Blot Analysis

The protein expressions of phospho-p38, ERK, JNK, Nrf2 and β-actin in cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins 30μg were separated on 10% by SDS-PAGE gels and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) at 100V for 2 hrs using the wet transfer system. The PVDF membranes was blocked with 5% nonfat milk at room temperature for 2 hrs and incubated with the following primary antibodies: phospho-p38, ERK, JNK, Nrf2 and β-actin (dilution: 1:2000; Cell Signaling Technology, Massachusetts) in Tris buffered saline/tween 20 (1X TBST) containing 5% bovine serum albumin overnight in 4˚C. The secondary antibody used anti-rabbit (dilution: 1:2000; Cell Signaling Technologies) then it was incubated at room temperature for 2 hrs. Specific protein bands were visualized by the enhanced chemiluminescence (ECL Pierce Biotechnology) using UVP Biospectrum 600 Imaging System (UVP, LLC, Upland, CA, USA). β-actin (Cell Signaling Technology) was used as loading controls for the total protein content and showed no differences between groups.

2.7. Statistical Analysis

Each experiment was repeated at least three times. Statistical analyzed performed by analysis of variance (ANOVA) followed subsequent multiple comparison test (Tukey) with GraphPad prism version 5.0 software package (GraphPad l Jolla, CA, USA). Data are mean ± S.E.M. * denotes significant values, *$p<0.05$**,** **$p<0.01$ and ***$p<0.001$ indicate significant differences when tested with ANOVA. Tukey's test was used for post-hoc tests.

III. RESULTS

3.1. Cell Cytotoxicity and Viability Study on RAW 264.7 Cells

3.1.1. Effects of RVS on Cell Viability

To investigate the effects of RVS on cell viability of RAW 264.7 cells, we evaluated cell viability through MUSE cell analyzer. We found that cells viability of experimental RVS treatment (enzyme-treatment RVS extract treatment) and positive control group was increased up to 50% concentration with significance difference. This higher (50%) concentration of RVS extract was shown in both experimental and positive control group with less cell cytotoxicity and higher cell viability (Figure1A and B). In contrast, 100% concentration in combination treatment exhibited higher cell viability effects than other groups (Figure 1C). This result showed that the RVS treatment increased cell viability in a dose-dependent manner in RAW 264.7 cells (Figure 1C).

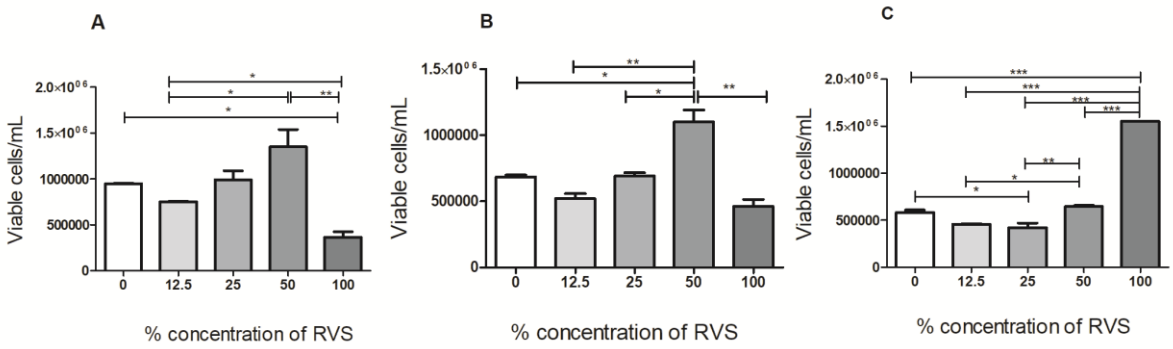

Figure 1. Cell viability effect of RVS on RAW 264.7 macrophage cells.

The cells were treated with RVS for 24 hrs and after 24 hrs treated cells were compared with untreated cells through MUSE cell analyzer. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Number of cells was observed in a concentration dependant manner. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

3.1.2. Effects of RVS on Cell Viability in H_2O_2-induced RAW 264.7 Cells

Further investigation, the protective effect of RVS extracts on cell viability was assessed by CCK-8 assay after 24 hrs incubation on RAW264.7 cells under the oxidative stress induced by H_2O_2. Our result revealed that cell viability was shown higher in NC group whereas least in H_2O_2 induced group (Figure 2A, B and C). Treatment with the RVS extract after H_2O_2 exposure increased the cell viability of RAW cells in a dose-dependent manner in all three groups with same significance difference (Figure 2A, B and C). These results indicate that treating RAW 264.7 cells with the RVS extract attenuated the oxidative damage caused by H_2O_2. In line, positive control RVS extracts significantly increased cell viability most in 50% concentration (Figure 2A) whereas experimental and combination treatment showed increased cell viability in 100% concentration (Figure 2B and C) . These results showed that all the RVS treatment increased the viability of RAW 264.7 cells most in 50 and 100% concentration with least cytotoxic effect.

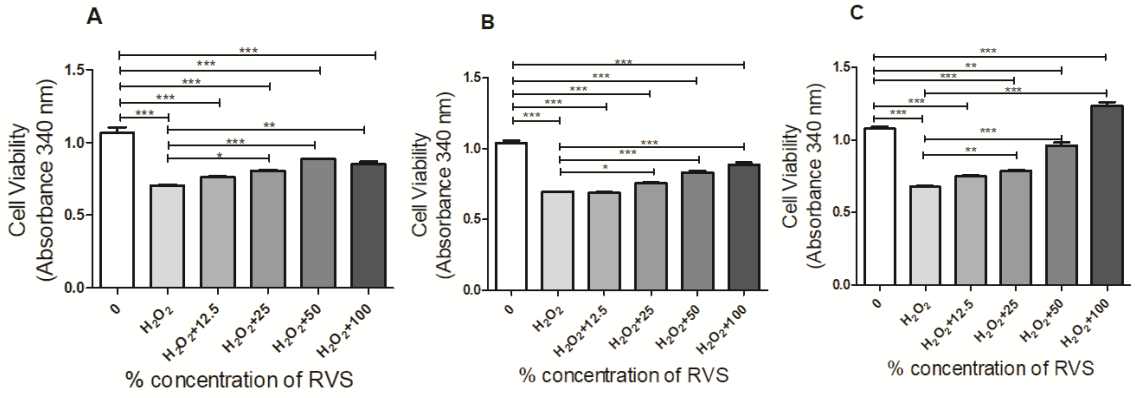

Figure 2. Cell viability effect of RVS against H_2O_2-induced oxidative stress in RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 100μM H_2O_2 for 2 hrs. After 2 hrs, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compare with untreated cells through CCK-8 assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Number of cells was observed in a concentration dependant manner. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.1.3. Effects of RVS on Cell Viability in LPS-induced RAW 264.7 Cells

In the macrophages RAW 264.7 cells, the protective effect of RVS extracts on cell viability was assessed after 24 hrs incubation. Cell viability of LPS-induced RAW 264.7 cells after treatment with various concentrations of RVS extract showed LPS alone did not show any proliferative activity in RAW 264.7 cells. Cell viability of LPS-induced RAW 264.7 cells after treatment with various concentrations of RVS increased cell viability up to a concentration of 50-100% of RVS, suggesting that RVS bioactive compound are able to increase growth of RAW 264.7 cells (Figure 3).

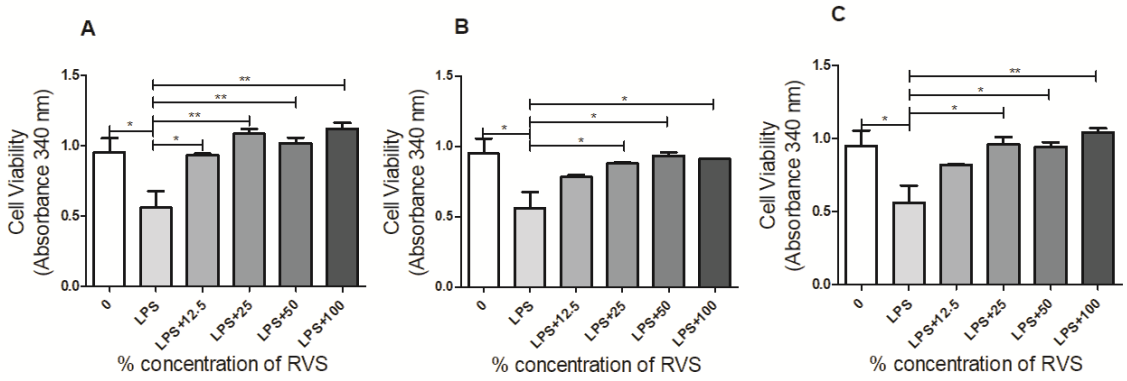

Figure 3. Cell viability effect of RVS against LPS-induced oxidative stress in RAW 264.7 cells.

The cells pre-treated with 5μg/mL LPS for 2 hrs before the cells were treated with different concentration of RVS extracts according to groups and after 24 hrs treated groups were compare with untreated cells through CCK-8 assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Number of cells was observed in a concentration dependant manner. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

3.1.4. Effect of RVS on H_2O_2-induced Intracellular ROS on RAW 264.7 Cells

The radical scavenging effect of enzyme treatment RVS extract with various concentrations on ROS production and cytotoxicity in the presence of H_2O_2 treatment was examined using DCFH-DA assay. A dose-dependent reaction of enzyme-treated RVS extract on ROS level and cytotoxicity in H_2O_2 treated cells is shown in (Figure 4A, B and C). Experimental enzyme treated RVS and combination treatment group showed the reduction in the H_2O_2-induced ROS level with 50 and 100% concentration treatment (Figure 4 B and C) whereas H_2O_2-induced ROS level is increased in 12.5% concentration in all 3 treated groups (Figure 4A, B and C).

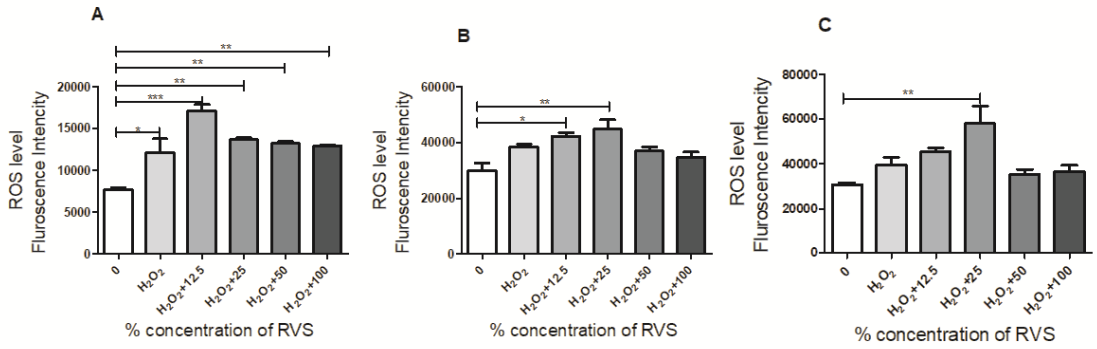

Figure 4. Effect of RVS extracts on H_2O_2-induced intracellular ROS level in RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 100μM H_2O_2 for 2 hrs. After 2 hours, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compared with untreated cells. Total ROS levels in cells were assessed through fluorescence probe DCFH-DA assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.1.5. Effect of RVS on LPS-induced Intracellular ROS on RAW 264.7 Cells

The preventive effect of enzyme treated RVS extract on ROS production in the presence of LPS treatment was examined using DCFH-DA assay. A dose-dependent reaction of enzyme treated RVS extract on ROS level and cytotoxicity in LPS treated cells is shown in (Figure 5A, B and C). Experimental and combination RVS treatment group showed the reduction in the LPS-induced ROS level with 50 and 100% concentration treatment (Figure 5B and C) whereas LPS-induced ROS level is increased in 12.5% concentration in all 3 treated groups (Figure5A, B and C). These result suggested that experimental and combination RVS treatment alleviated the oxidative stress caused by LPS and it may act as an effective antioxidants that could lower the ROS production in RAW 264.7 cells.

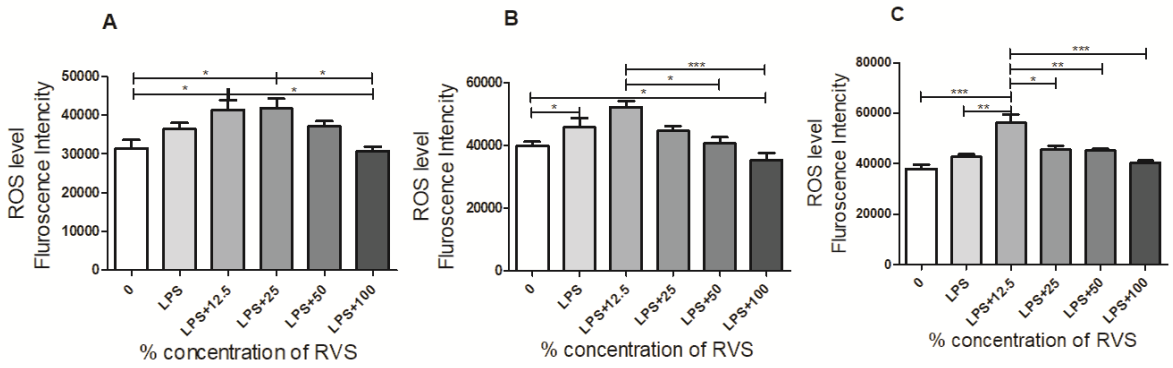

Figure 5. Effect of RVS extracts on LPS-induced intracellular ROS level on RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 5µg/ml LPS for 2 hrs. After 2 hrs, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compared with untreated cells. Total ROS levels in cells were assessed through DCFH-DA assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Number of cells was observed in a concentration dependant manner. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

3.1.6. Effect of RVS on H_2O_2-induced NO Production in RAW 264.7 Cells

Although NO is a pivotal role in many body functions, it plays an important role in pathology of many inflammatory diseases [26]. Hence, inhibition of NO production is essential for prevention of inflammatory diseases. Our result showed significant reduction in the NO level with experimental and combination RVS treatment with 50 and 100% concentration whereas NO production was significantly increased with H_2O_2 induction in experimental group (Figure 6B and C). However in positive control and combination treated group H_2O_2 induction with 12.5% showed higher NO production in RAW 264.7 cells (Figure 6A and C). These results suggested that H_2O_2-induction increased the inflammatory responses in cells, whereas experimental and combination RVS extract treatment markedly reduced NO production.

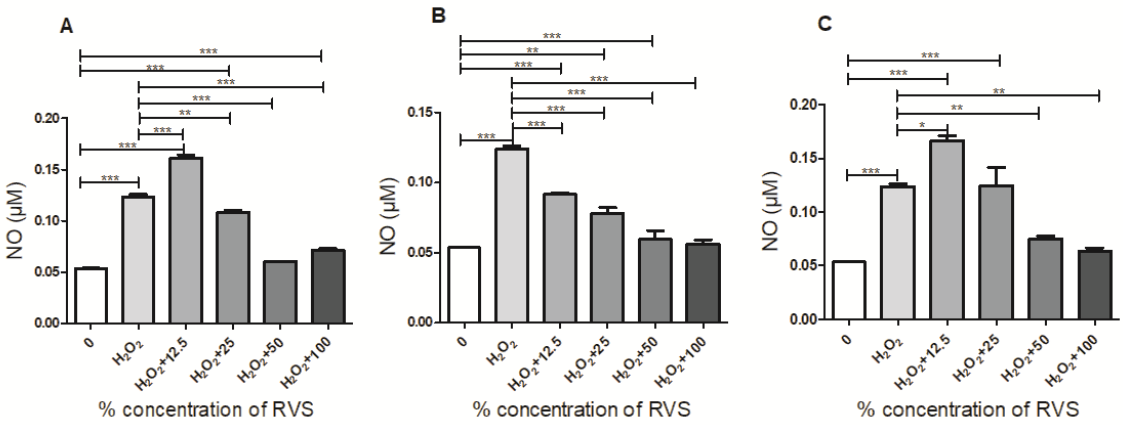

Figure 6. Anti-inflammatory effects of RVS extract in H_2O_2-induced NO production on RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 100μM H_2O_2 for 2 hrs. After 2 hrs, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compared with untreated cells. Total NO levels in cells were assessed through Griess reagent assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.1.7. Effects of RVS in LPS-stimulated NO Production on RAW 264.7 Cells

To confirm the effect of RVS extract on the suppression of inflammatory responses simulated with LPS for 2 hrs in RAW 264.7 cells, the levels of NO production were measured with Griess reagent assay. As shown in figure 7, in positive control, experimental and combination treated group LPS stimulated cells showed higher NO production in RAW 264.7 cells (Figure 7A, B and C), whereas treating cells with RVS extract significantly reduced NO level with experimental and combination RVS treatment with 50 and 100% concentration (Figure 7B and C).

In this study, to confirm which concentration of RVS extract suppressed inflammation on RAW 264.7 cells, where the higher concentration of experimental RVS showed it significantly suppressed LPS-induced NO production (Figure 7A, B and C). This result suggested that 100% RVS extract showed the strongest NO reducing effect in LPS-activated RAW 264.7 cells.

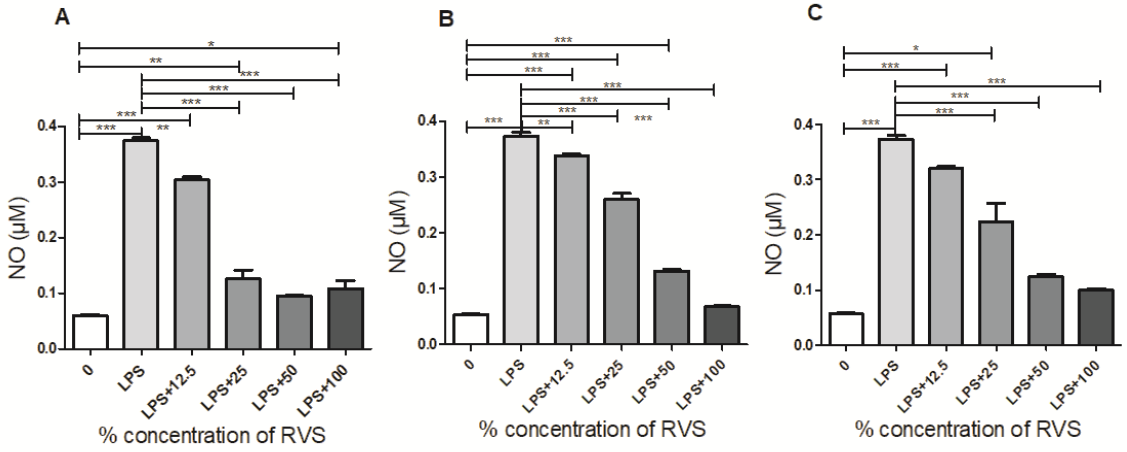

Figure 7. Anti-inflammatory effects of RVS extract in LPS-induced NO production on RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 5μg/mL LPS for 2 hrs. After 2 hrs, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compared with untreated cells. Total NO levels in cells were assessed through Griess reagent assay. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

3.1.8. Effects RVS Extract on Cell Death

Determination whether RVS could induce cytotoxic cell death was performed in RAW 264.7 cells after 24 hrs treatment of RVS extract using MUSE cell death and count kit. The result represented as in approximately % of viable cells in figure 8A, B and C. As shown in figure 8, all RVS treatment with their different concentration showed, RVS extract with H_2O_2 showed significant protection of H_2O_2-induced cytotoxic cells death in RAW 264.7 (Figure 8A, B and C). RVS treated cells viability reached to normal control level where H_2O_2 alone induced cytotoxic cells death and decrease number of viable cells. These results confirmed that RVS extract protected oxidative stress-induced cell death and induced anti-apoptotic effects in RAW 264.7 cells. To further understand the molecular mechanism of RVS effects on RAW 264.7 cells, we examined the expression of proteins which associated with RVS effects.

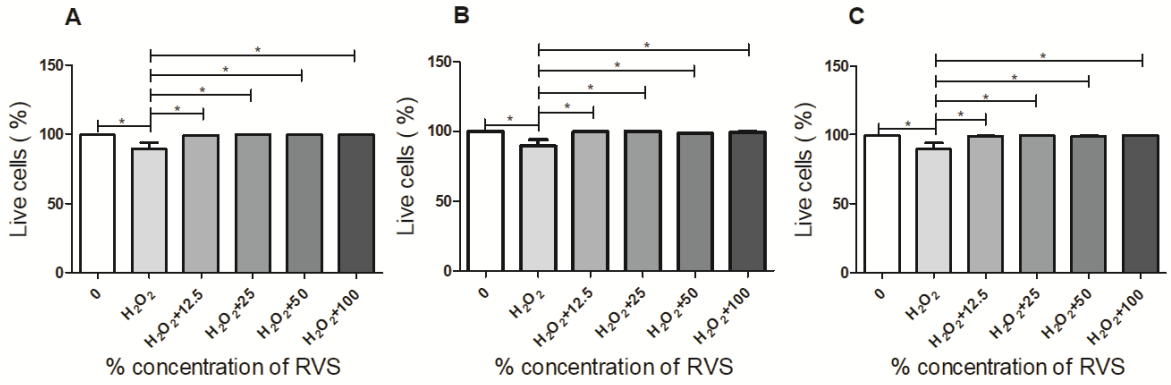

Figure 8. Effect of RVS on proliferation and death of RAW 264.7 cells.

Cells were treated with different concentration of RVS extract after pre-treated with 100μM H_2O_2 for 2 hrs. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. The percentage of live cells count and compared between untreated control cells and RVS treated cells. Reduction in no. of cells is observed in a concentration dependant manner using Muse cell death and count kit. Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.2. Endogenous Antioxidant Enzyme Activities in H_2O_2-induced Oxidative Stress

This study investigated the levels of anti-oxidant activity to confirm the ROS mechanism after H_2O_2 induction. The effects of RVS change the antioxidant status in RAW 264.7 cells. Comparative study of % SOD Activity of RVS with respect to control cells plotted in figure 9. The higher concentration of experimental RVS extract significant increased SOD activity compared to H_2O_2-induced control cells (Figure 9A and B), where the low concentration of combination RVS significantly enhanced SOD enzyme activity in RAW 264.7 cells (Figure 9C). The results in figure 9A suggesting a higher dose have a higher SOD activity in positive control RVS extract.

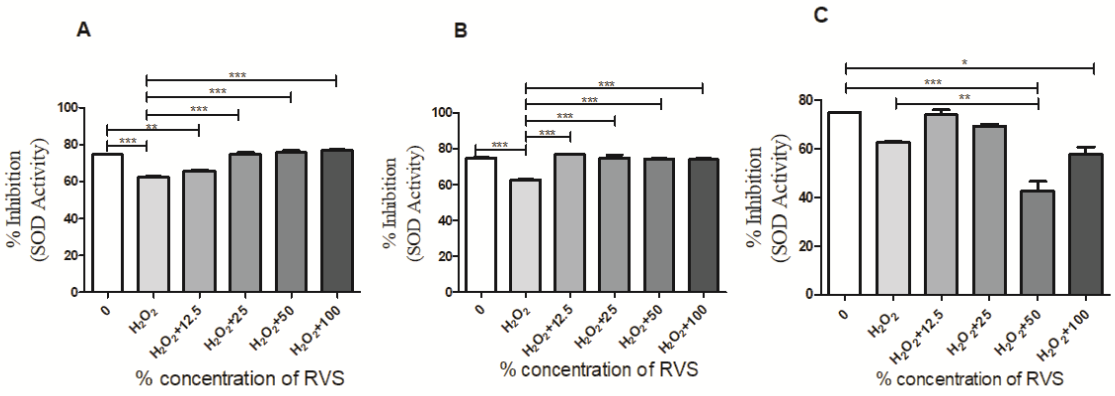

Figure 9. Antioxidant enzyme SOD activity in RVS treated RAW 264.7 cells.

The RAW 264.7 cells were pre-treated with 100μM H_2O_2 for 2 hrs. After 2 hrs, the cells were treated with different RVS extracts according to groups and after 24 hrs treated groups were compared with untreated cells. SOD activity was assessed through SOD activity assay kit (Biovision). (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. Data values were expressed as mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.2.1. Effect of RVS on Antioxidant Enzyme GPx Activity in RAW 264.7 Cells

Here, we examined whether antioxidant enzyme restore in H_2O_2-induced macrophage RAW 264.7 cells. Oxidative stress-induced ROS will initially deplete intracellular antioxidant activity. Although the experimental RVS extract were not able to fully recover GPx activity to the normal control level but 12.5 and 50% RVS extract seem enhanced the GPx activity compared to H_2O_2-induced control RAW 264.7 cells (Figure 10B) as well as this concentration also recovered slightly higher GPx activity compared to LPS treatment only in figure 11B. However in combination treated group with H_2O_2 induction did not recover GPx activity compared to H_2O_2 only but LPS induction with 12.5 and 100% showed slightly higher GPx activity compared to LPS control group respectively (Figure 10C and 11C).

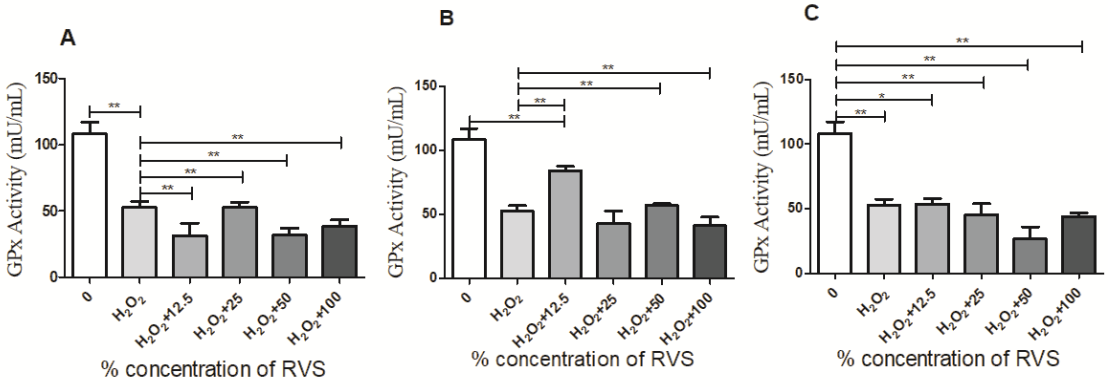

Figure 10. Antioxidant effect of RVS extract in H_2O_2-stimulated on RAW 264.7 cells.

The cell treated with different concentration of RVS for 24 hrs after pre-treated with H_2O_2 for 2 hrs. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. GPx activity was assessed according to GPx activity assay kit (Biovision). Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

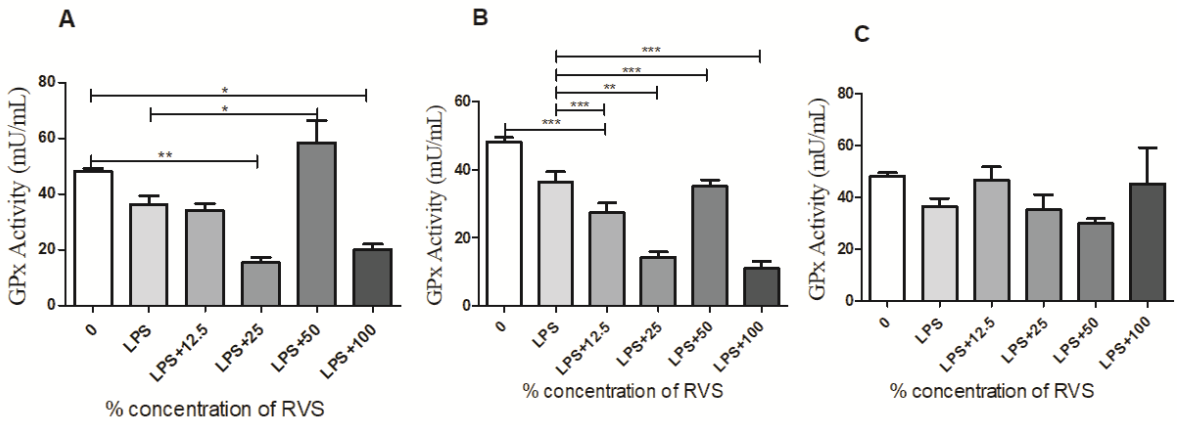

Figure 11. Antioxidant effect of RVS extract in LPS-stimulated on RAW 264.7 cells.

The cell treated with different concentration of RVS for 24 hrs after pre-treated with LPS for 2 hrs. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. GPx activity was assessed according to GPx activity assay kit (Biovision). Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.2.2. Effect of RVS on Antioxidant Enzyme Catalase Activity in RAW 264.7 Cells

We examined antioxidant related CAT enzyme activity in H_2O_2 and LPS-induced macrophage RAW 264.7 cells. The higher concentration of experimental RVS extract was not able to fully recover to the control level but 12.5-25% RVS seem recovered these activities to an extent in normal control RAW 264.7 cells (Figure 12B & 13B).

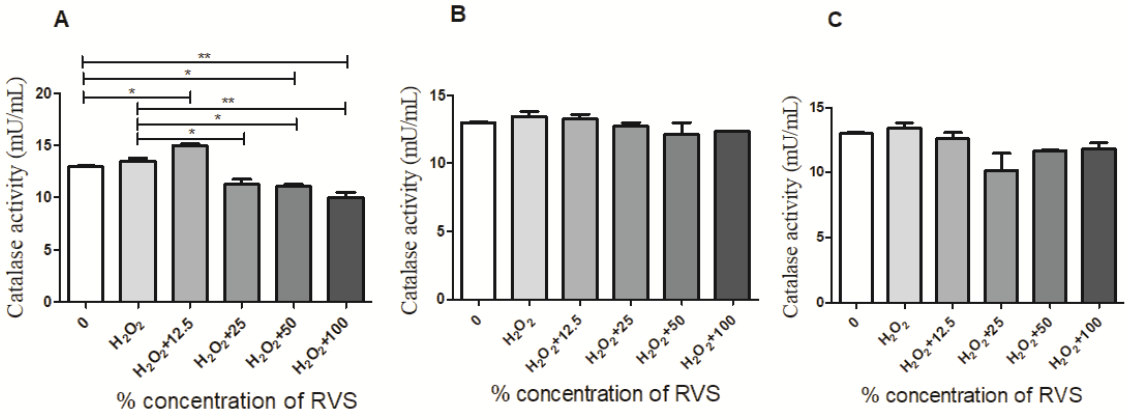

Figure 12. Antioxidant enzyme catalase activity in H_2O_2-stimulated on RAW 264.7 cells.

The cell treated with different concentration of RVS for 24 hrs after pre-treated with H_2O_2 for 2 hrs. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. CAT activity was assessed according to CAT activity assay kit (Biovision). Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, **$p<0.01$ and ***$p<0.001$.

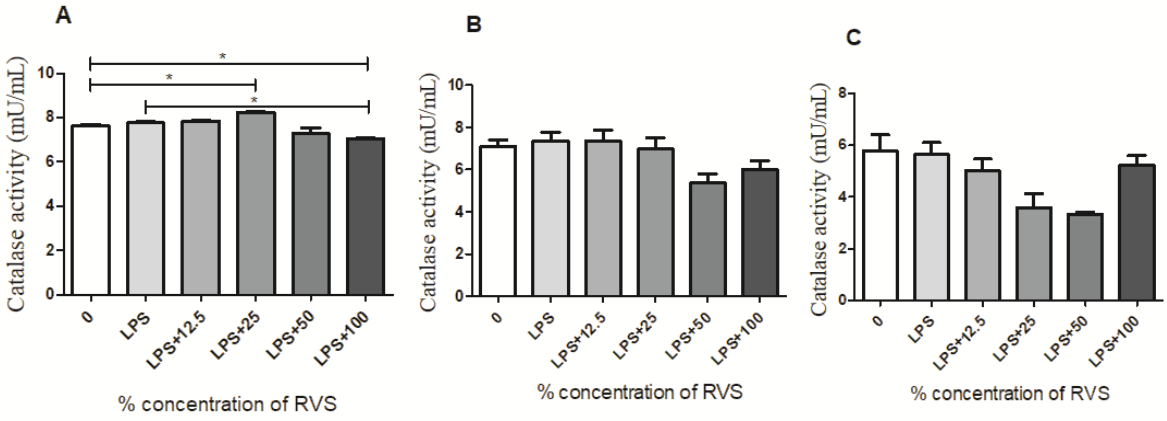

Figure 13. Antioxidant enzyme catalase activity in LPS-stimulated on RAW 264.7 cells.

The cell treated with different concentration of RVS for 24 hrs after pre-treated with LPS for 2 hrs. (A) Positive control RVS, (B) Experimental RVS and (C) Combination RVS extract. CAT activity was assessed according to CAT activity assay kit (Biovision). Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.3. Effect of RVS Extract on Inflammatory Cytokines in RAW 264.7 Cells

Using the RVS extracts, we assayed the effects of RVS on inflammatory response in RAW 264.7 cells in dose-dependent manner. INF-γ is an important activator of macrophage cell and aberrant levels of INF-γ are associated with a number of autoinflammatory diseases. High concentration of experimental RVS somehow showed decreasing concentration of INF-γ level, whereas combination RVS treatment increased this level with their increasing concentration (Figure 14E). On the other hand, without stimulation of TNF-α level was low, wherein cells were treated with different concentration of experimental RVS did not show inflammatory response where positive control 12.5-25% RVS treatment induced inflammatory response in RAW 264.7 cells (Figure 14F). IL-1β and IL-6 showed decrease level in all experimental RVS treatment group but only high concentration treatment have effect on IL-10 and IL-12 (Figure 14A, B, C and D).

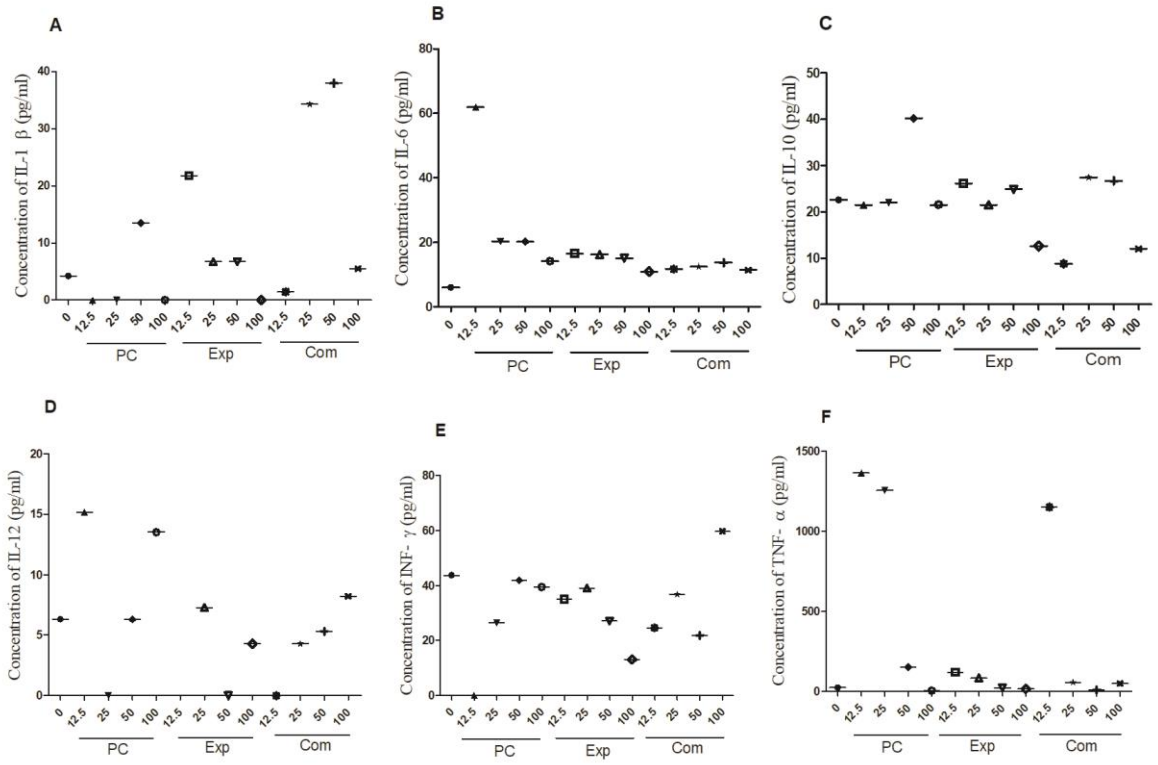

Figure 14. Inhibitory effects of RVS extract on cytokine production in RAW 264.7 cells.

RAW264.7 cells were treated in the absence or presence of indicated concentrations of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS for 1 hr. The levels of (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-12, (E) INF-γ and (F) TNF-α concentration were determined in the cell lysate using the using Bio-Plex Cytokine Assay. All values are represented as mean ± SD.

3.3.1. Inflammatory Cytokines Mediate H_2O_2-induced Oxidative Stress on RAW 264.7 Cells

Investigating the anti-inflammatory effect of RVS extract on suppression of inflammatory responses simulated with H_2O_2 and LPS for 2 hrs in RAW 264.7 cells. Overall trend of IL-1β, IL-6, INF-γ and TNF-α concentration in H_2O_2-treated cells were higher than non-treatment (normal control group) except IL-10 and IL-12 level. The levels of TNF-α did not increase in experimental RVS treatment but positive control and combination 12.5-50% RVS treatment elevated of TNF-α level (Figure 15F). Pro-inflammatory cytokines IL-1β levels decreased in all RVS treatment group wherein PC and Exp. 100% RVS decreased INF-γ level (Figure 15A). Experimental RVS showed decreasing levels of IL-6 with their increasing concentration while PC and combination RVS treatment showed the opposite trend and all 100% RVS treatment downregulated IL-10 level and IL-12 levels were elevated in all RVS treatment but were not in PC 100% concentration compared to normal control level (Figure 15B, C and D).

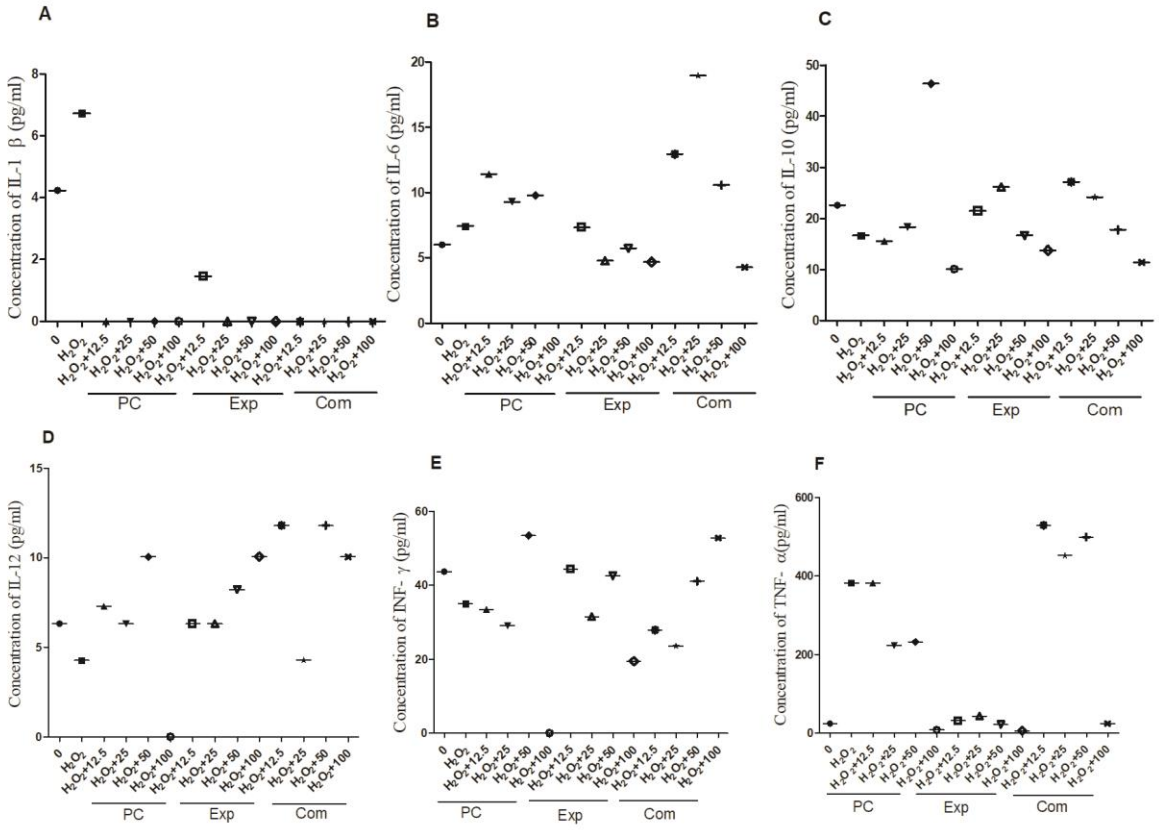

Figure 15. Inflammatory cytokines mediate H_2O_2-induced oxidative stress in Raw 264.7 Cells.

RAW264.7 cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after stimulated with H_2O_2 (100μM/mL) for 2hrs. The levels of (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-12, (E) INF-γ and (F) TNF-α concentration were determined in the cell lysate using the using Bio-Plex Cytokine Assay. All values are represented as mean ± SD.

3.3.2. Inflammatory Cytokines in LPS-induced RAW 264.7 cells

To confirm the effect of RVS extract on the suppression of inflammatory responses on LPS-stimulated pro-inflammatory mediators such as IL-1β, IL-6, IL-10, IL-12, INF-γ and TNF-α in RAW 264.7 cells. Changes in the cellular inflammation under LPS and RVS treatment were also observed a reversal of LPS-induced alteration in cellular cytokines following treatment with various concentrations of RVS extract. The cell treated with LPS showed increase level of inflammatory reactions but whereas Exp. RVS treatment decreased the level of IL-1β and IL-6 dose-dependent (Figure 16A and B). Similar trend was observed in INF-γ and TNF-α concentration in experimental RVS-treated RAW 264.7 cells compare to positive control and combination RVS treatment groups. Comparing cytokine levels with PC, experimental and combination RVS treatment induced a dose-dependent inhibition of proinflammatory cytokines production. On the other hand, RVS slightly stimulated IL-10 and IL-12 expression 12.5-50 μg/mL in RAW 264.7 cells (Figure 16C and D).

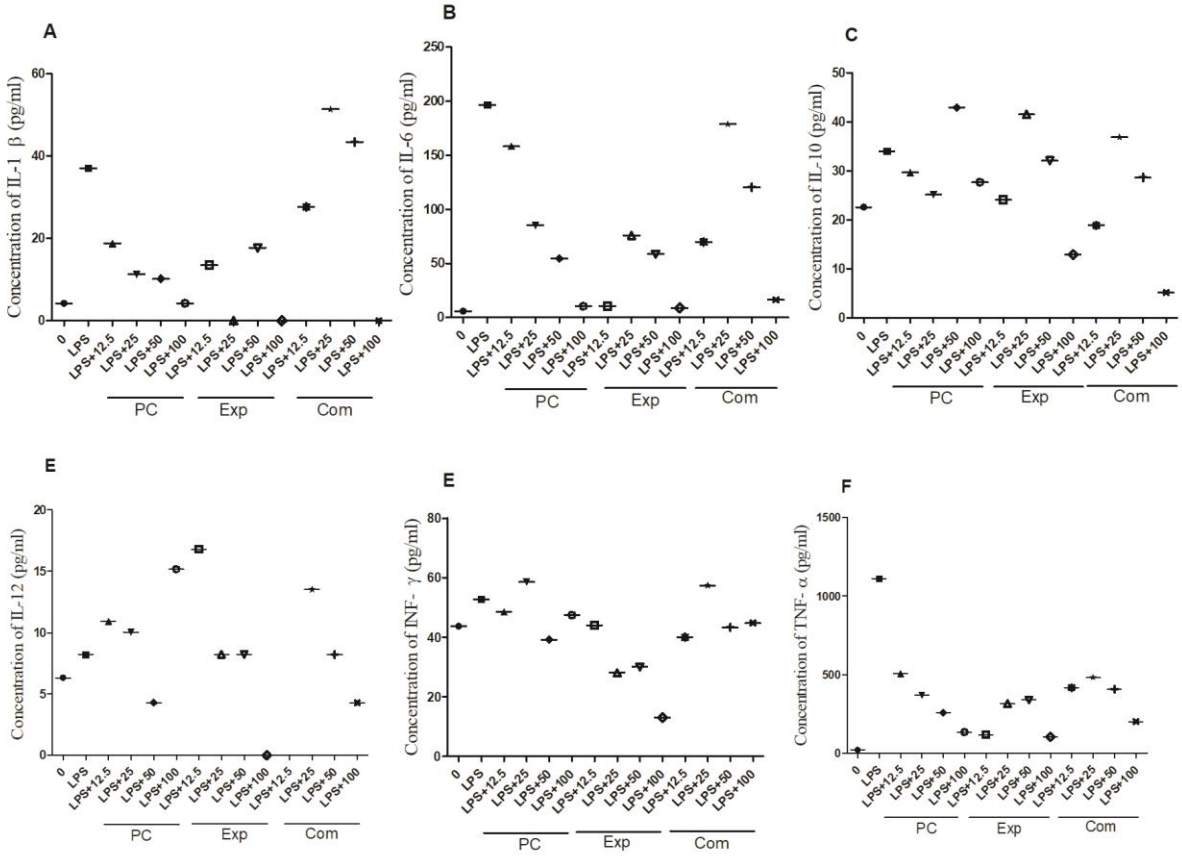

Figure 16. Inflammatory cytokines mediate LPS-induced Oxidative Stress in Raw 264.7 Cells.

RAW264.7 cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after stimulated with 5μg/mL LPS for 2 hrs. The levels of (A) IL-1β, (B) IL-6, (C) IL-10, (D) IL-12, (E) INF-γ and (F) TNF-α concentration were determined in the cell lysate using the using Bio-Plex Cytokine Assay. All values are represented as mean ± SD.

3.4. Effects of RVS on MAPK against H_2O_2 and LPS-induced Oxidative Stress in RAW 264.7 Cells

To fully elucidate the underlying molecular mechanism of antioxidant and anti-inflammatory effect mediated by RVS extract, we hypothesized those different pathways that can lead to inflammatory immune response. LPS interacts with cellular receptor and activates several intracellular signaling pathways involved in the macrophage RAW 264.7 cells immune response including the MAPK pathway [33]. Therefore, we investigated the influence of RVS on both H_2O_2 and LPS-induced activation of ERK, JNK and p38-MAPK. To prove our hypothesis, we determined the SDS-PAGE analysis utilizing phosphorylation specific primary antibodies from whole cell homogenates. Phosphorylation and thereby activation of the studied MAPKs were increased by H_2O_2 and LPS, which was attenuated by RVS treatment. This effect of RVS showed that there was a marked dose dependent strongest down regulation of p38 phosphorylation (Figure 17 & 18), increased phosphorylation of p-ERK (Figure 19 & 20) and did not show any effect on c-Jun terminal kinase (JNK).

3.4.1. RVS Decreases p38 Expression in RAW 264.7 Cells

Since p38 and ROS are mediators in inflammatory reactions, the expression of p-p38 in RAW 264.7 cells was assessed. RVS suppressed phosphorylation of p38 expression induced by H_2O_2 and LPS in RAW 264.7 cells. The PC and experimental 50-100% concentration of RVS showed higher suppressive effect on p38 phosphorylation and also in combination treatment suppressed dose-dependently except 100% (Figure 17). RVS suppressed phosphorylation of p38 expression induced by LPS in RAW 264.7 cells, where PC and experimental RVS increased suppression of p38 with their increasing concentration but combination treatment show opposite trend (Figure 18). These results suggested that experimental RVS possessed ability to suppress inflammatory

response wherein combination treatment showed inflammatory cell signaling response at 100% concentration (Figure 17 & 18).

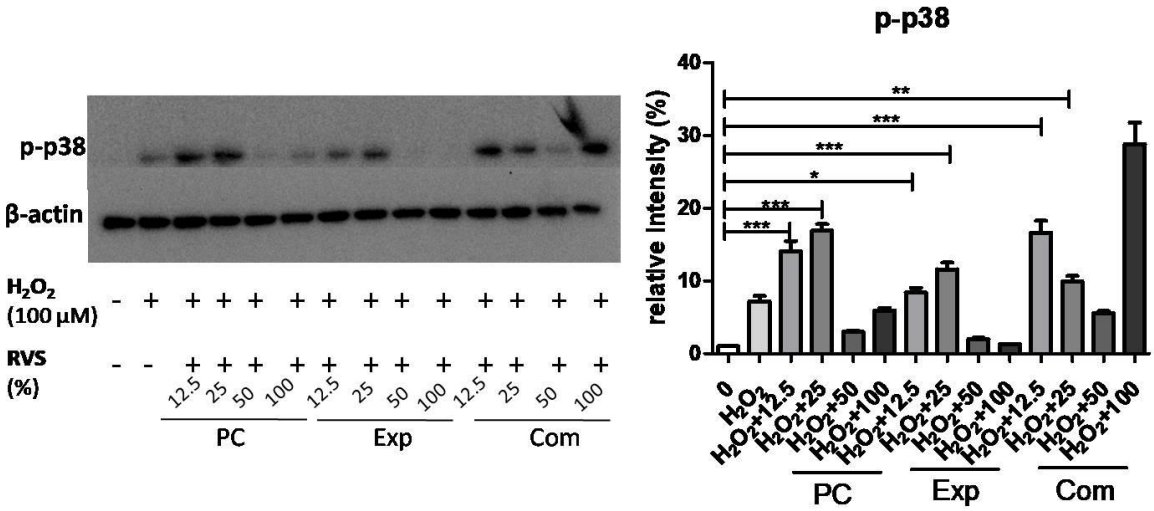

Figure 17. MAPK protein: p-p38 expression in H_2O_2-induced RAW 264.7 cells.

Cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after stimulation with 100μM H_2O_2 for 2 hrs. Cell lysates were subjected to western blot analysis with specific antibodies. Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$ **$p<0.01$ and ***$p<0.001$.

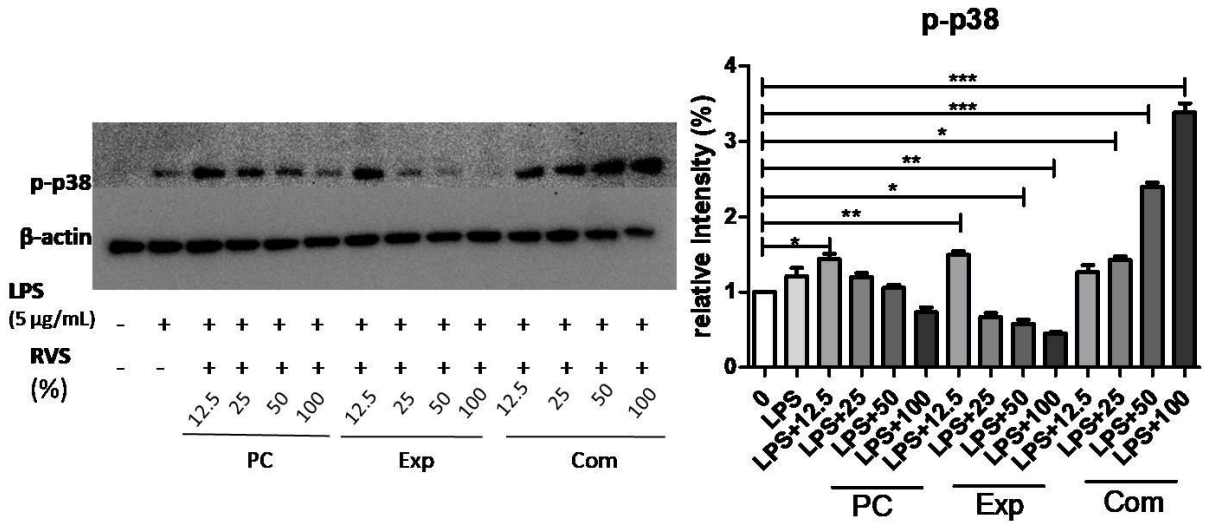

Figure 18. MAPK protein: p-p38 expression in LPS-induced RAW 264.7 cells.

Cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after stimulation with 5μg/mL LPS for 2 hrs. Cell lysates were subjected to western blot analysis with specific antibodies. Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.4.2. Effect of RVS on p-ERK Expression in RAW 264.7 Cells

We investigated the influence of RVS on H_2O_2 and LPS-induced activation of ERK in RAW 264.7 cells. RVS increased the extracellular signal-regulated kinase (p-ERK) expression in a specific dose-dependent manner. All RVS treatment 100% concentration showed higher expression of ERK in both H_2O_2 and LPS-induced RAW 264.7 cells (Figure 19 & 20). These results suggest that RVS have effect on MAPK–induced cell proliferation through phos-phorylation of ERK signaling pathway.

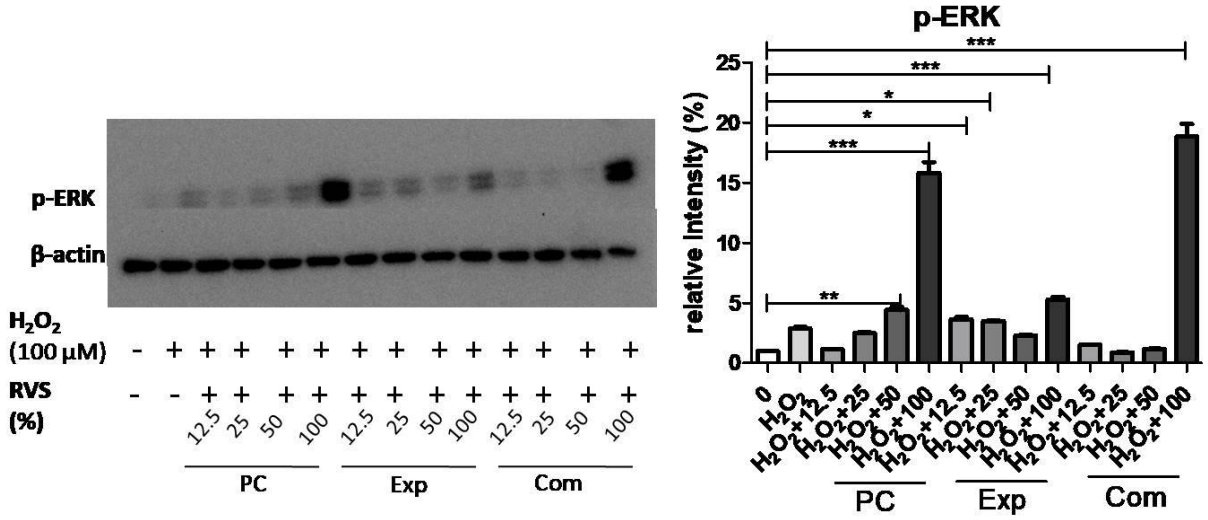

Figure 19. MAPK protein: p-ERK expression in H_2O_2-induced RAW 264.7 cells.

Cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after pretreated with 100μM H_2O_2 for 2 hrs. Cell lysates were subjected to western blot analysis with specific antibodies. Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

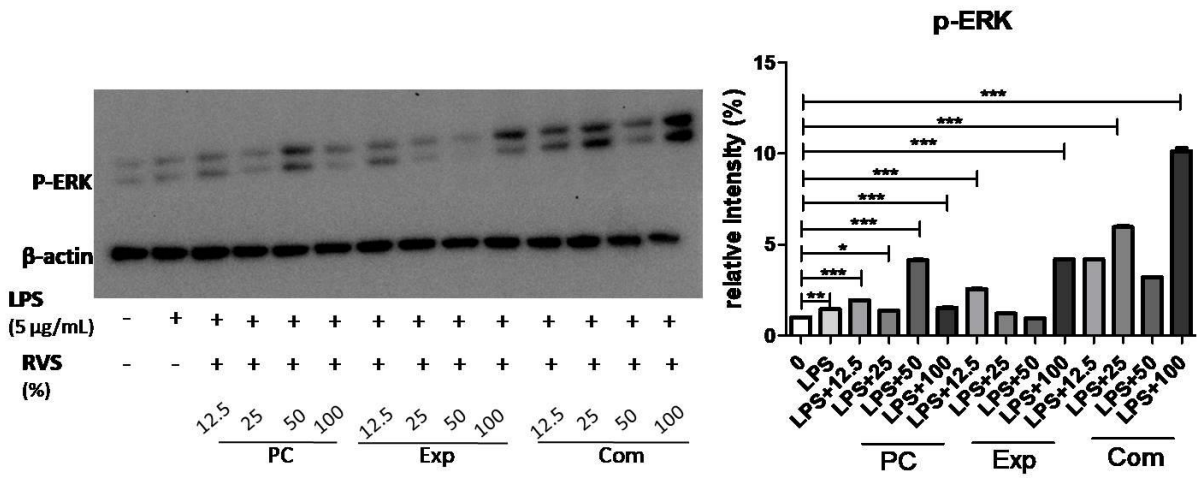

Figure 20. MAPK protein: p-ERK expression in LPS-induced RAW 264.7 cells.

Cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after pretreated with 5μg/mL LPS for the indicated times course. Cell lysates were analyzed by western blot with antibodies specific for phosphorylated ERK (p-ERK). Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.4.3. Effects of RVS on Nrf2 expression through nuclear translocation in H_2O_2-induced RAW 264.7 Cells

Furthermore, we examined the effects of RVS on nuclear factor-E2-related factor 2 (Nrf2) expressions and antioxidant activity in RAW 264.7 macrophages cells. In this study, we investigated the potential involvement of antioxidant activities through nuclear translocation of Nrf2 in the anti-oxidant activity elicited by flavonoids isolated from RVS extract. The activation of p38 and ERK proteins known to be involved in the regulation of Nrf2 expression were also investigated. We found that treatment with experimental and combination RVS led to Nrf2 phosphorylation may induce Nrf2 activation and antioxidant enzyme expression. The result showed that there was a marked dose dependent increase in phosphorylation of phosphor NRf2 in RAW 264.7 macrophages (Figure 21).

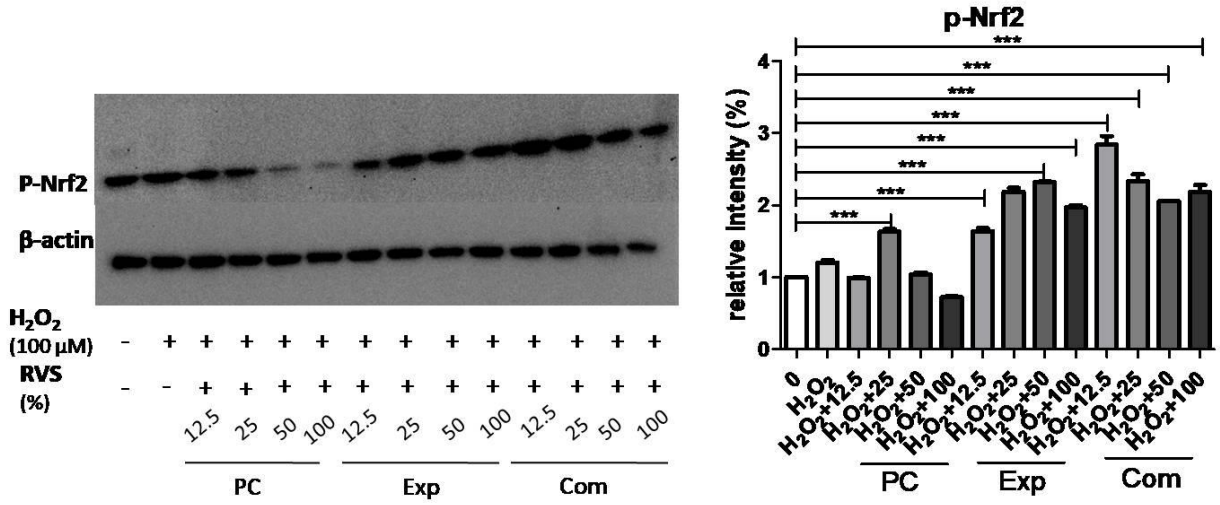

Figure 21. Effect of RVS on Nrf2 antioxidant signalling against H_2O_2-induced RAW 264.7 cells.

Cells were treated with different concentration of Positive control (PC) RVS, Experimental (Exp.) RVS and Combination (Com.) RVS after pretreated with 100μM H_2O_2 for the indicated time course. Cell extracts were analyzed by western blot with antibodies specific for phosphorylated Nrf2. Western blot analyses for Nrf2 expression was performed as described in the materials and methods. Data are mean ± S.E.M. Significant difference was analyzed with ANOVA Tukey's test, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

IV. DISCUSSION

Rhus verniciflua Stokes has been a traditional plant medicine in East Asia and it is well known that RVS has been found to have various biological activities [1- 5, 14-18]. Recently, much attention has been focused on the protective function, especially antioxidative effect of naturally occurring compounds and on the mechanisms of their action. Phenolic compounds have been considered to play an important role as dietary antioxidants for the prevention of oxidative damage in living systems [21, 22]. The antioxidant capability of these phenolic compounds is widely reported in the literature. Previous studies have shown that flavonoids compound isolated from RVS has various pharmacological effects, especially antioxidant and anti-inflammatory activities.[27, 35, 36]. In particular, it has been shown that these natural compounds showed scavenging activity against superoxide anion radical, ·OH and H_2O_2 which are often generated by biological oxidation reactions of exogenous factors [36, 37]. We mentioned in this study, these ROS are known to cause human diseases such as cancer, heart disease, inflammation and many other negative effects on the human body [20, 38, 39]. Previous studies have shown that ethanol extract from RVS posses various bioactive properties [14]. In this study, the effects of enzyme-treatment RVS extract on radical scavenging activities and inhibition of inflammatory response in macrophage RAW264.7 cells were determined. The enzyme-treatment RVS extract exhibited significantly reduced the level of intracellular oxidative stress with their concentration to compare with commercially available positive control RVS extract. More efficient antioxidant activities in the RVS extract originated from the presence of higher concentrations of potent antioxidant compounds.

Furthermore, we have shown differential effects of enzyme-treatment RVS extracts on macrophage RAW 264.7 cell viability. The potent cytotoxic activities of RVS have been

supported by the observations in cell viability study. Urushiol-removed enzyme-treatment RVS extract did not affect cell cytotoxicity. This RVS extract increased cell viability in a concentration-dependent manner and also effectively enhanced the cell growth stimulation in H_2O_2 and LPS-induced RAW 264.7 cells. These results suggested that enzyme-treatment RVS extract correlated with non-cytotoxic effect and also prevent inflammatory response in H_2O_2 and LPS-induced macrophage RAW264.7 cells. We effectively obtained non-cytotoxic RVS extract without cells damage by enzyme-treatment detoxifying method.

We examined the protective effect of RVS extract under oxidative stress (H_2O_2 and LPS)-induced intracellular ROS level in RAW 264.7 cells. Here, we demonstrate that enzyme-treatment RVS extract significantly reduced intracellular ROS level in both H_2O_2 and LPS-stimulated RAW 264.7 cells. Free radical scavenging protects cells from oxidative damage and their consequence pathogenesis involving ROS and attenuated the oxidative damage showed the radical scavenging activity of the RVS extract antioxidant effect (Figure 4 & 5). In addition, NO has a pivotal role in many body functions and it has also been implicated in the pathological processes, especially in inflammatory diseases, including arthritis, myocarditis and nephritis [26, 40-42]. Therefore, inhibition of NO is essential for prevention of inflammatory diseases. To confirm the effect of RVS extract on the suppression of inflammatory responses in RAW 264.7 cells, the levels of NO were measured. In this study, we showed that enzyme-treatment flavonoids contained RVS extract dose-dependent significantly reduced NO production in RAW 264.7 cells (Figure 6 & 7). As shown in figure 6 and 7, H_2O_2 and LPS increased inflammatory responses, whereas treating cells with RVS extract markedly reduced H_2O_2 and LPS-induced NO levels in macrophage RAW 264.7 cells. These results indicated that RVS extract may be

potentially beneficial in the treatment of inflammatory diseases through the inhibition of NO production.

There is evidence that ROS induce apoptosis, from studies in which antioxidants have been used to inhibit apoptotic mediators induced by H_2O_2 and their behavior [43, 44]. In addition, cell death can be induced when a cell becomes less effective at scavenging or detoxifying ROS. Therefore, alleviating oxidative stress can reduce oxidative damage to and apoptosis of cells. We examined the protective activity of cytotoxic cell death of RVS extract in macrophage RAW 264.7 cells. In further studies on the RVS flavonoids protect against oxidative cell death by scavenging ROS and protect cytotoxic cell death in macrophage RAW 264.7 cells (figure 8). However, there are no data that clearly indicate the mechanisms by which the RVS extract scavenges radicals or acts as an antioxidant, nor have the active compounds of the extract been identified.

It was well known that antioxidative defense enzymes play pivotal roles in preventing cellular damage. This study showed that RVS had antioxidant properties evidenced by different oxidative targets including the reduction of intracellular ROS production and enhancement of the activities of antioxidant enzymes (SOD, GPx and CAT). In antioxidant enzyme study, oxidative stress has been generated by adding both H_2O_2 and LPS to RAW 264.7 cells. By inducing this oxidative stress the antioxidant property of RVS have been studied by analyzing the SOD activity. It was well known that antioxidative defense enzymes play pivotal roles in preventing cellular damage. The result of the study goes like this in SOD activity. Our results shown that purified experimental RVS extract enhanced % of SOD activity in H_2O_2-induced RAW 264.7 cells compared to no treatment stress group. Oxidative stress is believed to play an important role in H_2O_2-induced cell damage [45] and ROS initially depleted intracellular antioxidant activity [46].

H_2O_2 is usually removed by GPx and it is one of the body's most potent antioxidant defenses, as it protects cells from damage by free radicals. We noted that GPx activities decreased in H_2O_2-induced RAW 264.7 cells where RVS treatment showed slightly higher GPx activity compared to an extent in without H_2O_2 and LPS-induced RAW 264.7 cells, although the higher concentration of RVS did not show this effect. These results could imply a positive effect in RVS-treated cells that could lower the ROS content in RAW 264.7 cells wherein higher concentration of experimental RVS also did not recover CAT activity in both H_2O_2 and LPS-induced RAW 264.7 cells.

To further confirm immunomodulation, we measured cytokines level in H_2O_2 and LPS-induced in RAW 264.7 macrophage cells. Cytokine profiling clearly showed a significant reduction of pro-inflammatory cytokines such as IL-1β, IL-6, INF-γ and TNF-α in RVS treated RAW 264.7 cells compared to non-treatment cells (Figure 14, 15 & 16). H_2O_2 and LPS increased inflammatory responses, whereas treating cells with RVS extract showed dose-dependent markedly reduced IL-1β, IL-6, IL-6, INF-γ and TNF-α in figure 15 & 16. It revealed that RVS flavonoids suppressed cytoxicity-induced inflammatory response in RAW 264.7 cells compared to untreated H_2O_2 and LPS-induced cells. Meanwhile, we also investigated the modulation of RVS in inflammatory signal transduction mediated through extracellular signal-regulated protein kinase (ERK) pathway. MAPK pathways are also associated with inflammation and the ERK pathway is activated by inflammation [47]. Therefore, our findings suggest that RVS extracts can have a positive influence on regulating the proinflammatory factors and relative genes in LPS-stimulated macrophages through ERK1/2-phosphorylation. Since it has been suggested that apoptosis plays a critical role in tissue homeostasis and so we determined whether the cytotoxicity of RVS is due to apoptosis, apoptosis analysis was performed in RAW 264.7 cells

after RVS treatment and result shows the expression of intracellular molecules associated with cell proliferation, assessed using western blot analysis. To further understand the molecular mechanism of RVS-induced cell proliferation in RAW 264.7 cells, we examined the expression of proteins associated with cell growth after RVS treatment. RVS extract up regulated MAPK-induced ERK express in a dose-dependent manner. MAPKs are a group of serine/threonine protein kinases that are activated in response to diverse extracellular stimuli such as ROS and mediate signal transduction from the cell surface to the nucleus. This study also found out that Nrf2 and MAPK protein are involved in the antioxidant effect of RVS against H_2O_2-induced oxidative stress. Under unstressed conditions, Nrf2 is constitutively degraded through binding to Keap1 (Kelch-like ECH-associated protein 1), an adapter protein of E3 ubiquitin ligase. Nrf2 is a transcription factor that regulates expression of many detoxification or antioxidant enzymes. It is plausible that H_2O_2 and/or LPS transiently increases the intracellular level of ROS and/or activates p38 MAPK signaling pathway, which may possibly lead to facilitating the dissociation of Nrf2 from Keap. Upon oxidative stress, Nrf2 is induced at protein levels through redox-sensitive modifications on cysteine residues of Keap1, a component of the E3 ubiquitin ligase that targets Nrf2 for ubiquitin-dependent degradation. The MAPKs have previously been proposed to regulate Nrf2 in response to oxidative stress. However, the exact role of MAPKs and the underlying molecular mechanism remain unclear [48, 49]. Furthermore, RVS flavonoids compound treatment caused nuclear accumulation of Nrf2 and increased the promoter activity of antioxidant response elements (AREs) [50, 51]. Since Nrf2 upregulates numerous antioxidant genes, elimination of ROS has been considered to be the molecular basis of Nrf2-mediated anti-inflammatory capacity of flavonoids [52]. Nrf2 phosphorylation which may induced Nrf2 activation and antioxidant enzyme expression which may partially mediated by MAPK. The

resultant Nrf2/ARE activation induced phase II detoxification or antioxidant enzyme, thereby potentiating cellular defence capacity against H_2O_2 and LPS-induced cell death.

Chromatograms results obtained from HPLC showed RVS extract derivatives and flavonoids as major compounds are the pharmacological active constituents in many medicinal plants [21,22, 53]. According to HPLC results, flavonoids were suggested as fustin, fisetin, quercetin, butein, kaempferol, sulfuretin, protocatechuic acid, ethyl gallate and catechol [4, 6-9]. These flavonoids such as kaempferol and butein exhibit several pharmacological properties, acting as antioxidant and cell protection agent [54, 55]. Fustin is one of the major flavonoids in Toxicodendron vernicifluum reported to have anti-inflammatory effects, while sulfuretin showed the strongest NO suppressive effect in LPS-activated BV-2 cells [56]. Therefore, based on the previous reports as well as our findings, although sulfuretin was the most active flavonoid in RVS extract, fisetin, fustin, or another flavonoid might contribute to suppressing inflammatory components and ROS. These phenolic compounds in enzyme-treatment RVS extract may be contribute to the antioxidant activities and protective effect against oxidative stress-induced cells death. However, it is need to identify exactly compounds from RVS extract by using specific single use of these compounds *in vitro* and *in vivo* model.

In summary, the present study provides data that suggest that RVS extract effectively increased cell viability and provides a protective effect against cytotoxicity induced by H_2O_2 and LPS in macrophage RAW 264.7 cells in a dose-dependent manner. The RVS extract significantly reduced the level of intracellular oxidative stress in RAW 264.7 cells induced by H_2O_2 and LPS. Therefore, based on our findings, probably that phenolic and flavonoid compounds in RVS extract might function as antioxidants, thereby attenuated intracellular ROS and possibly slowing suppressed inflammatory components NO production and inflammatory

cytokines level. The antioxidant and cell protection action of enzyme-treatment RVS extracts, therefore, may be useful in developing new herbal medicine against oxidative stress.

V. CONCLUSION

Collectively, the RVS extract and its active flavonoids compound showed non-toxic effect and enhanced the cell viability and reduced apoptosis in dose-depended manner in murine macrophage RAW 264.7 cells. The enzyme-treatment RVS extract significantly prevented oxidative stress (H_2O_2)-induced cell death and reduced intracellular oxidative stress in RAW 264.7 cells induced by H_2O_2 and as well as LPS that supported the hypothesis of purified enzyme-treatment RVS and its phenolic compounds might function as antioxidants, thereby reduced the intracellular ROS production and enhanced the cellular protection effects. Most importantly, RVS demonstrated a counter balanced ROS/Antioxidant paradigm as shown by the reduction of ROS/RNS levels and consistently, the activities of ROS scavenging enzymes such as SOD and GPx level were increased. In relation to that, the inflammatory markers such as IL-1β, IL-6, IL-10, Il-12, INF-γ and TNF-α in RVS treatment cells with presence of H_2O_2 and LPS induction were lower which implies mediation in the inflammatory response. Lastly, it was found out that Nrf2 was involved in the antioxidant effect of RVS against H_2O_2-induced oxidative stress and nuclear accumulation of Nrf2 and increased the promoter activity of antioxidant response elements (AREs) contribute to the anti-inflammation. This study shows that antioxidant and cell protection action of RVS extracts, therefore, may be useful in developing new herbal medicine against oxidative stress diseases and could improve immune cells function. Further studies are needed to find out exact cellular and molecular mechanisms to confirm various pharmacological actions of RVS extract whether may be used as therapeutic agents to ameliorating oxidative stress *in vivo.*

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VII. KOREAN ABSTRACT

초록

RAW264.7 세포주를 이용하여 옻나무 진액이 면역 레독스기전에 미치는 효과

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옻나무에는 fustin, fisetin, quercetin, butein, kaempferol, sulfuretin, protocatechuic acid, catechol, ethyl gallate와 같은 폴로페놀이나 플라보노이드 생리활성성분이 있어 항산화와 항염증효과를 나타내는 것으로 알려져 있다. 본 연구에서는 효소처리를 통해 옻나무수액으로부터 우루시올을 제거한 옻나무진액을 사용하여 효과를 확인하고자 하였다. 옻나무 진액추출물의 항산화 효과와 immune-redox balance 를 평가하기 위하여 과산화수소(H_2O_2)와 Lipopolysaccharide (LPS)로 RAW 264.7 세포에 세포독성을 유발한 후 세포생존능력과 활성산소 제거능력을 측정하였다. 옻나무진액의 세포독성에 대한 효과는 RAW 264.7 세포의성장률과 CCK-8 assay를 통하여 검증하였다. 옻나무진액의 잠재적 항염증효과를 확인하기위하여 과산화수소와 LPS로 RAW 264.7 세포에 산화스트레스와 염증반응을 유도하였다. 세포독성실험을 위하여 과산화수소와

LPS로 염증반응을 유도한 RAW 264.7 세포에 옻나무 진액 0, 12.5, 25, 50, 100% 를 각각 24시간 동안 처리한 후 세포생존능력을 측정하였다. 그 결과 옻나무진액의 처리는 대식세포 RAW 264.7 세포에서 세포 내 활성산소와 산화질소 생성을 유의하게 감소시켰으며 세포의 생존율의 감소를 억제시키는 결과를 보여주었다. 결론적으로 우루시올이 제거된 옻나무진액을 세포에 처리하였을 때 세포내의 활성산소의 생성이 억제됨으로써 항산화효과가 있음을 확인하였으며 옻나무진액의 적절한 사용은 산화적스트레스나 염증성 질환에 도움을 줄 수 있을 것으로 판단된다.

키워드: 옻나무 진액, 플라보노이드, 산화적 스트레스, 염증, 항산화능