PEANUT ROOT EXTRACTS AND RESVERATROL INHIBIT LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION BY SUPPRESSION OF MAPKS SIGNALING PATHWAYS IN BV-2 CELLS

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ABSTRACT

Peanut Root (PR), in the field of agricultural waste, contains resveratrol and bear potent antioxidative activity. The effect of the PR extract and resveratrol on lipopolysaccharide (LPS) induced inflammation of BV-2 microglial cells and acute liver injury in Sprague-Dawley (SD) rats was investigated. The results showed that the PR extract and resveratrol reduced LPS-induced nitric oxide (NO), interleukin (IL)-1, IL-6, reactive oxygen species (ROS), and prostaglandin E_2 (PGE_2) production in BV-2 cells. The PR extract and resveratrol significantly decreased serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in LPS-treated rats. Furthermore, the water extract, with exception of the ethanol extract, of the PR extract dose-dependently inhibit LPS-induced mitogen-activated protein kinases (MAPK), and slightly inhibit cyclooxygenase-2 (COX-2) expression in BV-2 cells. Taken together, these results indicated that the protective mechanism of PR extract involved an antioxidant effect and inhibition of MAP kinases and COX-2 expressions in LPS-induced inflammation of BV-2 cells.

Key words: ALT, AST, COX-2, MAPK, Resveratrol, ROS.

INTRODUCTION

The peanut plant (*Arachis hypogaea*) has been extensively studied in recent years as the search for their antioxidative compounds have intensified. Compounds reported in peanut root include resveratrol, arachidin-1, and arachidin-3 (Abbott *et al.* 2010). Resveratrol and its derivatives are natural stilbenes associated with many health benefits which include those conferred by their antioxidant, neuroprotection, and anticancer properties (Shingai *et al.* 2011; Madhyastha *et al.* 2013; Temraz *et al.* 2013). Hepatitis is commonly caused by pathogenic infection (including hepatitis viruses and Gram-negative bacteria), and alcohol- or drug-induced liver toxicity. Its pathology is initiated by a cascade of inflammatory events from viral-, alcohol-, or endotoxin-stimulated inflammatory cells and hepatic Kupffer cells to produce various pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin(IL)-1, IL-6, IL-12, and interferon(IFN)-λ (Malhi

and Gore 2008; Chao *et al.* 2009; Mandrekar and Szabo 2009). Lipopolysaccharides (LPS) can induce the Kupffer cells to produce reactive oxygen species (ROS) (Kono *et al.* 2000). Inflammatory response to stimuli or injury is often exacerbated by the resultant swelling or edema of tissue, pain (due to increased pressure in tissues or by inflammatory mediators), or even cell damage (Sosa *et al.* 2002; Yam *et al.* 2008). Therefore, chronic hepatitis leads to cirrhosis and eventually hepatocellular carcinoma. LPS-stimulated microglia, macrophages, and Kupffer cells activate phosphorylation and kinase activities of ERK1/2, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and subsequently, cytokine production (Geppert et al. 1994; Jiang et al.2002). Evidence indicates that inducible COX may have both pro- and anti-inflammatory properties through the generation of different types of prostaglandins (Poligone and Baldwin 2001). Prostaglandin E_2 (PGE_2) strongly

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synergizes with the inflammatory cytokine. Thus, the employment of anti-inflammatory agents may be helpful in the treatment of various inflammatory conditions including hepatitis. There are no reports on the extract of peanut root used as anti-inflammation. It had been reported that resveratrol suppresses serum tumor necrosis factor alpha (TNF-α) and hepatic injury by carbon tetrachloride-induced liver toxicity (Roy et al. 2011). However, the mechanism of protective liver affected by PR extract and resveratrol on LPS-induced liver toxicity had not been reported. Therefore, the aim of this present study is to investigate the mechanism of anti-inflammatory effects of water extract from PR and resveratrol using *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Reagents: LPS from Escherichia coli serotype 0111:B4 was obtained from Sigma (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (H$_2$DCF-DA) was obtained from Molecular Probe (Eugene, Oregon, USA). Peanut Root powder (100 mg) was added to 1 ml of RO water (as hydrophilic solution) or 99.5 % ethanol (as hydrophobic solution), mixed well by vortex for 5 min, and centrifuged at 2000 g for 10 min. Finally, the PR extract supernatants were filtered by sterile membranes respectively.

High-Performance liquid chromatography (HPLC) analysis of resveratrol content of peanut root: The resveratrol contents of peanut root were characterized by HPLC using a Shimadzu SCL-LC 10A HPLC fitted with a SIL 10AD autosampler. Chromatography was performed with an ODS HYPERSIL (Thermo Scientific) reverse phase column (25 cm × 0.46 cm i.d., 5 μ) and a UV''vis detector (Shimazu Systems Co., Foster City, CA). The mobile phase of catechins assay contained 5 % formic acid (solvent A) and methanol (solvent B), with a linear gradient from A/B (92:8) to A/B (73:27) over a period of 40 min with a flow rate of 1 mL/min. The detector was set at 306 nm.

Animals: Male Sprague-Dawley rats (300-400 g) obtained from the National Laboratory Animal Center (Taipei, Taiwan) were maintained in the Animal Center of the Chinese Medical University (Taichung, Taiwan). The animal studies were performed following the procedures from the Guidebook for the Care and Use of Laboratory Animals (2002), published by the Chinese Society of Animal Science in Taiwan. The rats were divided into six groups and starved for 12 h before the intraperitoneal (IP) drug administration. One control group was given saline (blank), while the experimental group (L) was given 50 μg/kg of LPS. The PR extract and resveratrol groups were given 10 or 30 mg/kg of *Gardenia jasminoides* extract and 1 or 10 mg/kg of gallic acid by gastric gavage after an injection of LPS. Liver tissues were fixed with 10 % formaldehyde solution over-night and H&E stained. However, hepatic function was assessed by measuring serum alanine aminotranferease (ALT) and aspartate aminotransferase (AST) with an automatic blood analyzer (Hitachi High-Technologies, Tokyo, Japan).

Cell Culture: Murine BV-2 cell line was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator under 5% CO$_2$. Confluent cultures were passed by trypsinization. For experiments, cells were washed twice with warm DMEM (without phenol red), and then treated in a serum-free medium. In all experiments, cells were treated with the PR extract and resveratrol for the indicated times after the addition of activating agent, 2 μg/ml LPS. The PR extract and resveratrol were dissolved in phosphate-buffered saline (PBS) and filtered through a 0.2 μM filter.

Nitric oxide assay: Nitrite was measured as NO in a Greiss test. Briefly, a serum sample reacted with equal volume of Griess reagent (0.1% naphthylethylene diamine and 1% sulfanilamide (1:1) in H$_3$PO$_4$) in 96-well plates for 10 min. Therefore, the absorbance at 540 nm was measured in a microplate reader.

Cytokine assay: Cytokines (IL-1β, IL-6) and PGE$_2$ were measured by ELISA kits (R&D, Minneapolis, MN, USA). Also, the absorbance at 450 nm was determined using a microplate reader (spectraMAX 340). The TNF-α, IL-1β and IL-6 levels in the tissue supernatant were determined by ELISA kits (R&D). Protein concentration (pg/μg) in liver tissue was determined using a dye-based protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

ROS generation: ROS was measured with 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCF-DA). H$_2$DCF-DA was dissolved in methanol and de-

acetylated in serum mixed with 10 μM H$_2$DCF for 10 min in the dark. The reaction solution was plated in 96-well plates and monitored on a Fluoroskan Ascent Fluorometer (Labsystems Oy, Helsinki, Finland) using an excitation wavelength of 485 nm and emission wavelength of 538 nm.

Serum AST/ALT: Concentration of blood samples were taken from tail veins by venipuncture during the experiment to measure the serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations from rats of each group. On this note, AST and ALT were detected by the automatic blood analyzer.

Western blotting: BV-2 cells were homogenized in ice-cold lysis buffer (1:10, wt/vol) containing 20 mmol/L 4-(2-hydroxyethyl)-1- piperazineethane sulfonic acid (pH 7.2), 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. This solution was centrifuged at 10,000 $\times$ g for 20 min at 4 °C. 50 μg of protein was run on an 8% or 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto nitrocellulose membranes (NEN Life Science, Boston, MA, USA) at 1.2 A for 3 h. The membranes were blocked in 5% milk in Tris-buffered saline with Tween-20. The membrane was thereafter incubated with polyclonal rabbit iNOS antibody (BD Biosciences, San Diego, CA, USA), diluted 1:1000 in blocking buffer. In addition, membranes were incubated with secondary anti-rabbit IgG conjugated to alkaline phosphatase (1:3000; Jackson ImmunoResearch, Philadelphia, PA, USA) and detected with a alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl-phosphate/ nitroblue tetrazolium) (Kirkegaard & Perry, Baltimore, MD, USA).

Statistical analysis: All data were expressed as mean ± S.D. For single variable comparisons, a Student's t-test was used. For multiple variable comparisons, data were analyzed by one-way ANOVA using the Dunnett's test. P-values of less than 0.05 or 0.01 were considered significant.

RESULTS AND DISCUSSION

Effect of the peanut root extract and resveratrol on nitrite production in LPS-stimulated BV-2 cells: It was first tested which fraction (hydrophobic or hydrophilic) of the PR extract could inhibit inflammation and nitrogen free radicals. Murine BV-2 cell line was used as an inflammation model. The

BV-2 cells were stimulated with LPS and then treated with various concentrations of the PR extract and resveratrol for 24 h. Nitrite content was measured using the Griess reaction. The results showed that nitrite production inhibit dose-dependently by the hydrophobic fraction of the PR but was not affected by the hydrophilic fraction (Fig. 1). Thus, the hydrophobic solution of the PR extract was used in further experiments.

Effect of the PR extract and resveratrol on inflammatory cytokines: BV-2 cells were treated with 2 μg/ml LPS in combination with 1, 5, and 10 mg/ml of the PR extract and 1 μM resveratrol. Cultured supernatants were used to measure IL-1β and IL-6 by ELISA assay. The results showed that the PR dose-dependently decreased IL-1β and IL-6 productions in BV-2 cells (Fig. 2), although the effect of PR on IL-6 production was more prominent than IL-1β.

Effect of the PR extract and resveratrol on LPS-induced PGE$_2$ release: To investigate the PR extract and resveratrol effect on inflammation, PGE$_2$ release was measured in the LPS-induced BV-2 cell culture. The results showed that the PR extract decreased the PGE$_2$ released from BV-2 cells dose-dependently (p < 0.05) (Fig. 3).
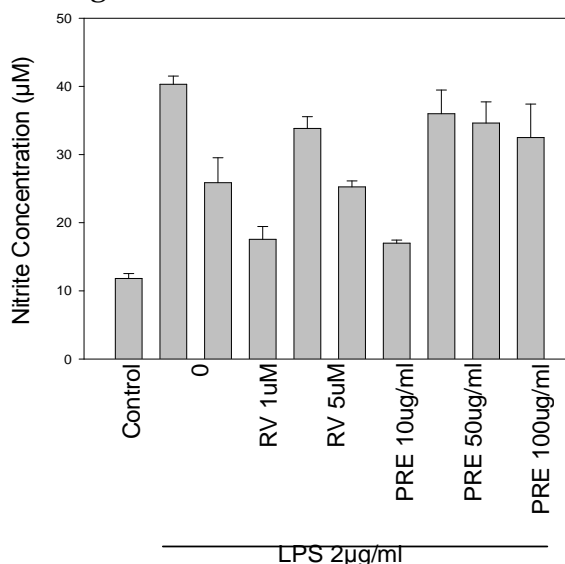

FIG. 1: PR extract tested the inhibition of nitrogen free radicals. BV-2 cells were stimulated with 2 mg/ml LPS and then treated with various concentrations of PR extract and resveratrol (RV) for 24 h. Nitrite production inhibited dose-dependently by the PR extract and resveratrol. The results showed that hydrophobic fraction of PR extract significantly decreased nitrite production, but was not affected by the hydrophilic fraction (PRE-W).
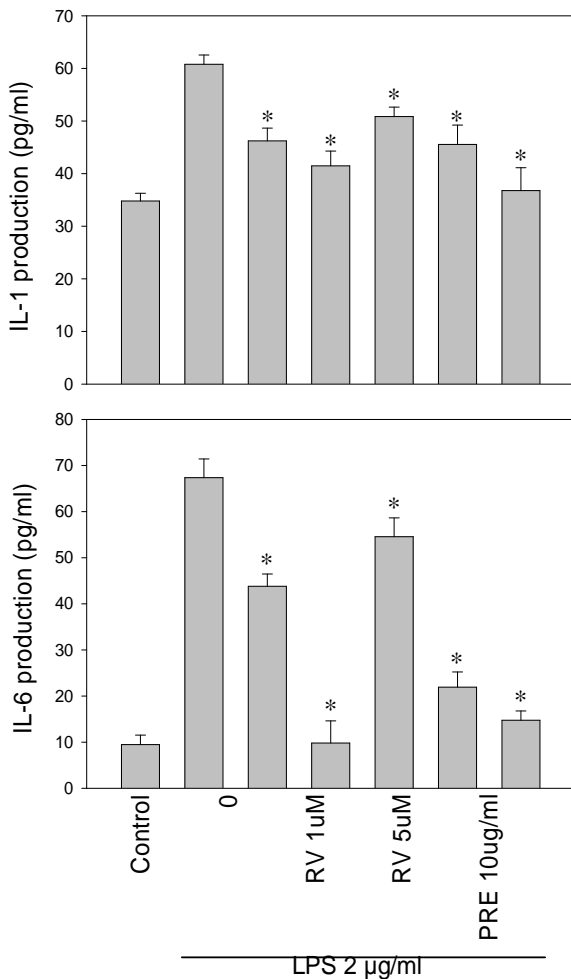
FIG. 2: BV-2 cells were treated with 2 mg/ml LPS in combination with 1, 5, and 10 mg/ml of PR extract and 1, 5 mM resveratrol. PR extract and resveratrol dose-dependently decreased IL-1â and IL-6 productions in BV-2 cells (p < 0.05).

Effect of the PR extract and resveratrol on ROS generation: To investigate the antioxidant activity of the PR extract and resveratrol on ROS generation, BV-2 cells were treated with H_2O_2 (1 mM) alone or with the PR extract and resveratrol. The ROS generation was increased by H_2O_2, but ROS generation was dose-dependently scavenged by the PR extract (Fig. 4).

Effect of the PR extract and resveratrol on hepatic enzyme activity of SD rats: The anti-inflammatory effect of the PR extract and resveratrol were further studied in an acute hepatic injury model of SD rats. These rats were then pretreated with resveratrol (1 or 10 mg/kg) or PR extract (10 or 30 mg/kg) for three days, and then injected with 50 µg/
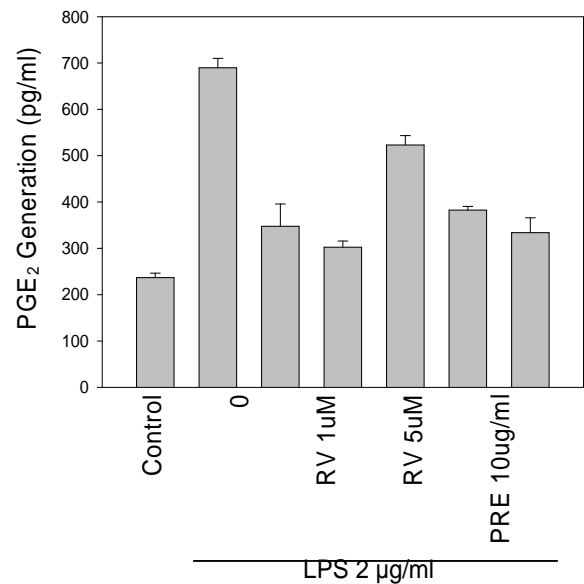

FIG. 3: To investigate the PR extract and resveratrol effect of inflammation, PGE_2 release was measured in the LPS-induced BV-2 cell culture. PR extract and resveratrol decreased PGE_2 release from BV-2 cells dose-dependently (p < 0.05).

kg LPS to induce liver injury. Both the PR extract and resveratrol supplementation decreased serum AST and ALT levels when compared to the LPS control (Fig. 5).
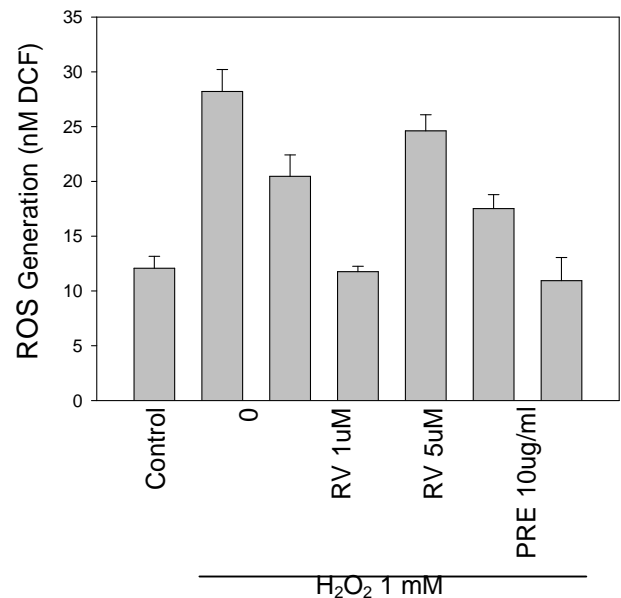

FIG. 4: To investigate the antioxidant activity of PR extract and resveratrol on ROS generation, BV-2 cells were treated with H_2O_2 (1 mM) alone or with PR extract and resveratrol for one hour. The ROS generation was increased by H_2O_2, but ROS generation was dose-dependently scavenged by PR extract and resveratrol.
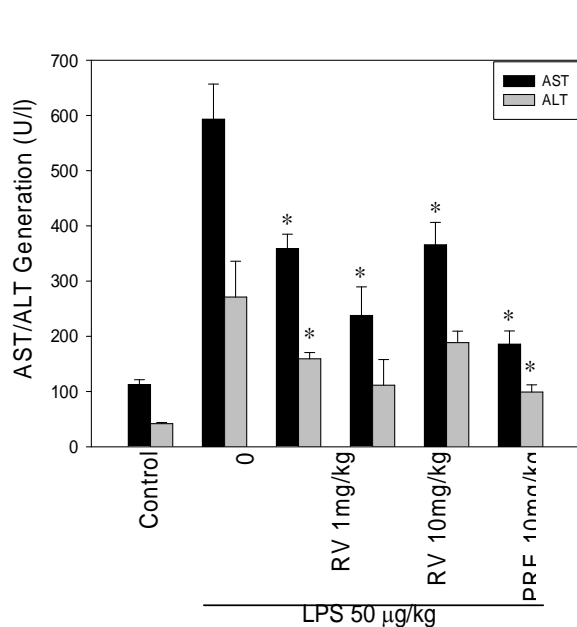
FIG. 5: The anti-inflammatory effect of PR extract and resveratrol were further studied in an acute hepatic injury model of SD rats. Rats were pretreated with resveratrol (1 or 10 mg/kg) or PR extract (10 or 30 mg/kg) for three days, and then injected with 50 mg/kg LPS to induce liver injury. Both PR extract and resveratrol supplementation decreased serum AST and ALT levels compared to the LPS control.

Effect of the PR extract and resveratrol on LPS-Induced MAPKs and COX-2 expression: The effect of the PR extract and resveratrol on cell signaling was investigated both in vitro and in vivo. ES dosedependently inhibited LPS-induced JNK, p38 MAPK, and partially inhibited COX-2 in BV-2 cells. Similarly, the PR extract inhibited LPS-induced p38 MAPK and partially inhibited COX-2 in the livers of SD rats (Fig. 6).

Effect of the PR extract and resveratrol on LPS-induced hepatic injury of rats: To analyze the PR extract and resveratrol effects on LPS-induced hepatic injury of rats, we also examined the liver pathology. Lesions and leukocyte infiltration were prominently induced in LPS-treated rats. However, this pathology was greatly reduced in the PR extract and resveratrol-treated rats (Fig. 7).

The results of this study showed that the PR extract and resveratrol protected rats from LPS-induced injury. Hepatic lesions and leukocyte infiltration were prominently reduced by the PR extract and resveratrol treatment. In addition, the levels of AST and ALT were decreased by the PR extract and resveratrol significantly. Thus, this
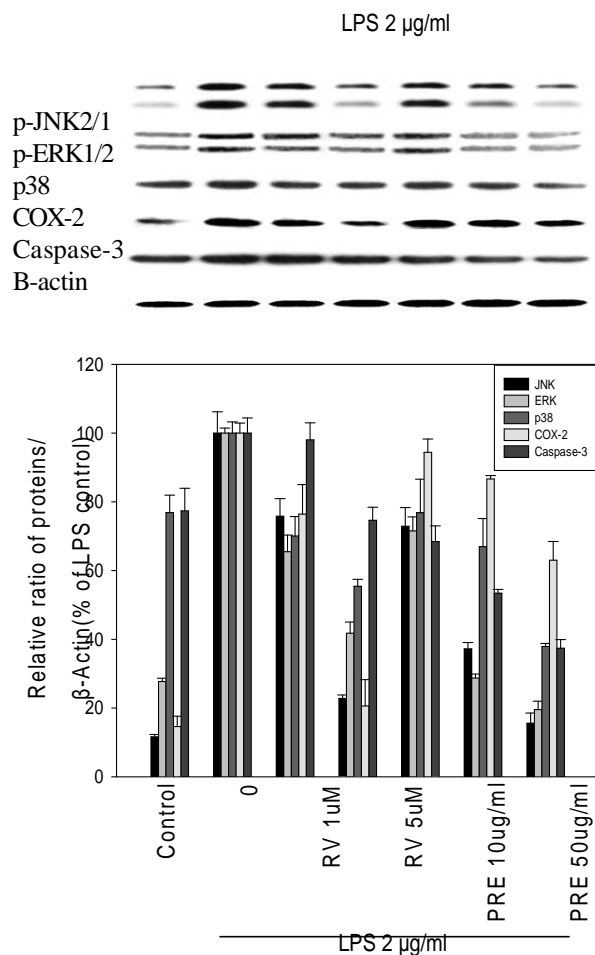

FIG. 6: The effect of PR extract and resveratrol on cell signaling was investigated in BV-2 cells. PRE and resveratrol dose-dependently inhibited LPS-induced MAPKs, and inhibited COX-2 and caspase-3 in BV-2 cells.

hepatoprotective effect is consistent with the resveratrol effects on other animal models such as $CC1_4$, or LPS, D-GalN, and dimethylnitrosamine (Fan *et al.* 2009; Farghali *et al.* 2009; Lee and others 2010). Hence, the results of the present study clearly showed that the mechanism of the hepatoprotective effect by the PR was mainly due to the suppression of p38 MAPK and, to a lesser degree, of COX-2. During liver fibrosis formation, JNK and p38 might have had opposite effects (Schnabl and others 2001). This showed that chronic alcohol prompts LPS-induced p38 and ERK1/2 activation but decreases JNK in murine hepatic macrophages (Kishore *et al.*). Therefore, our result confirmed that LPS-induced MAPKs such as ERK and p38 may also contribute to acute liver injury. Nevertheless, other studies had demonstrated
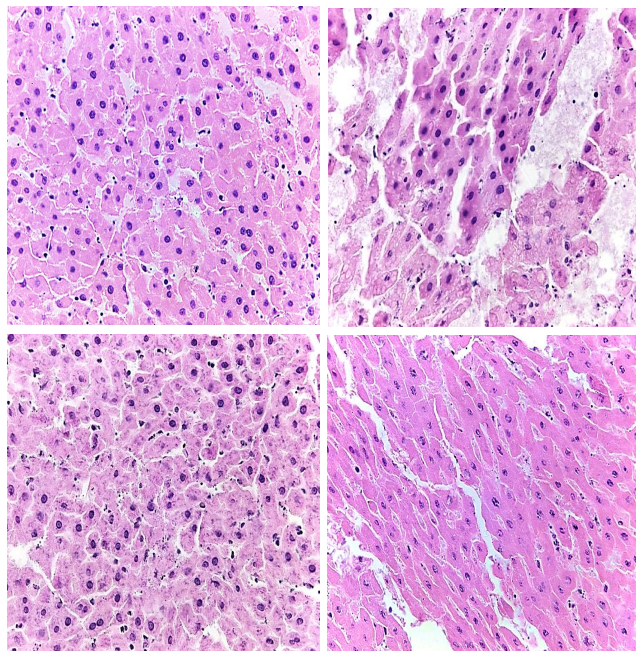
FIG. 7: Protective effect of PR extract and resveratrol on LPS-induced hepatic injury in SD rats. Liver slices from rats with no treatment (A, control) or treated with 50 mg/kg LPS i.p. (B), and supplemented with 30 mg/kg resveratrol; (C) and 30 mg/kg PRE (D) showed the differences in liver pathology. Photographs show the liver slices from rats with 400 x magnification. Partial to extensive vacuolar degeneration of liver cells in LPS stimulated rats were significantly higher than the others, but, this pathology was reduced in the PR extract and resveratrol-treated rats.

that JNK and p38 MAPKs activation following liver ischemia and reperfusion are associated with the induction of apoptosis and necrosis while inhibitors of p38 and JNK can reduce the liver damage (Mandrekar and Szabo 2009; King *et al.* 2009; Toledo-Pereyra, *et al.* 2008). The mechanism of the hepatitis virus which causes liver injury was also involved in the MAPKs pathway. Hence, studies had shown that hepatitis B, C, and E viruses (HBV, HCV and HEV) modulated the MAPK signaling pathway (Panteva and others 2003). Moreover, studies have also shown that deregulated MAPKs were often found

to contribute to the development of many cancers, including hepatocarcinoma (HCC). The early studies on the ERK pathway have led to the development of the multikinase inhibitor Sorafenib, the first effective systemic drug for the targeted treatment of human HCC. In addition, the functions and molecular mechanisms of JNK and p38 in HCC development have also been addressed using mouse models (Min et al. 2011). The results of the PR extract and resveratrol suppression of MAPKs were consistent with these hepatoprotective mechanisms. Furthermore, the ethanolic extracts of the PR had been identified as an anti-inflammatory effect in the present research. However, the data of the present study showed that the alcoholic extract of the PR, with exception of the water extract, reduced LPS-induced NO, IL-1α, IL-6, ROS, and PEG2 production in BV-2 cells. In addition, the PR extract and resveratrol reduced JNK2/1, ERK1/2, and p38 MAPKs phosphorylation, and slightly reduced COX-2 expression in BV-2 cells in a dose-dependent manner. Thus, the antioxidant effects were also shown in the LPS-induced liver injury in SD rats, since the MAPKs phosphorylation and COX-2 expression were similarly reduced by the PR extract and resveratrol treatment.

CONCLUSION

The data of this study provided sufficient evidence that the anti-inflammatory effect and hepatoprotective mechanism of the PR extract and resveratrol might occur through the modulation of MAPKs and COX-2 signaling pathways. Since folk medicine only used the decoction of the PR extract for anti-inflammatory conditions, therefore it warrants further studies to find out the major active compounds in the ethanolic extract of the PR extract.

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