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Protective mechanism of carbon tetrachloride-induced acute hepatic injury in Sprague-Dawley (SD) rats by Lycium ruthenicum

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Running Head: HepatoprotectionofLycium ruthenicum

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ABSTRACT
Lycium ruthenicum known as black Goji (wolfberry) belongs to Solanaceae family. It has rich Anthocyanin, the free radical scavenger, and other active components. Since liver injury in animals can be protectedbyAnthocyanin,we were interested to investigate the effect of L.ruthenicumextract (LRE) on the carbon tetrachloride (CCl_4)-induced acute liver injury.Sprague-Dawley (SD) rats were given intragastrically for 7 days,with and without LRE (0, 10, 30 mg/kg) and positive controls of silymarin (30 mg/kg) or anthocyanin (1.2 mg/each rat). After pretreatment, the animals were injected intraperitioneally with 1000μl/kg of 40% CCl_4 and sacrificed three hours later. The results showed thathistopathology and serum levels of ALT, AST, nitric oxide (NO), reactive oxygen species (ROS), interleukin-1β (IL1β), IL6,andprostaglandin E2 (PGE2)were significantly reduced by LRE in the pretreated rats. The protective mechanism of LRE was further examined for signaling pathways from the liver tissue with western blot assay. Consistently, the CCl_4-induced protein expressionsof MAP kinases, COX-2, iNOS, Bax, and caspase-3 were significantly reduced by LRE. In summary, the hepatic protection mechanism of LRE wasattributed to its antioxidative and anti-inflammatory effect through several signaling pathways.

Key words: Anthocyanin; acute liver injury; signaling pathways.

I. INTRODUCTION

Goji berry of Lycium barbarum is a popular Chinese traditional medicinal food that has been reported for its anti-inflammatory, anti-angiogenic effects and enhancing immune response (Wuet al., 2012; Ren et al., 2012). It is also considered to be one of the richest natural sources of zeaxanthin, a non-provitamin a carotenoid shown to protect the eyes (Zhou et al., 1999). Active components of L. barbarum fruits such as polysaccharides, flavonoids and carotenoids have been investigatedvigorously for these effects (Zhou et al, 1999; Fraser and Bramley, 2004; Luo et al., 2004; Kocyigit and Sanlier, 2017). However, a black Goji berry,Lyciumruthenicum of the Solanaceae family is less known, and only a few studies of biological effects for L.ruthenicum have been reported(Duan et al., 2015; Gong et al., 2015; Lin et al, 2015).It is distributed mainly in Qinghai and Xinjiang, China and used as traditional Tibetan medicine (Lv et al., 2013).Fruits of L. ruthenicumcontainmainly polysaccharides and anthocyaninbut devoid of zeaxanthin(Zheng et al., 2011;Islam et al., 2017).Anthocyanin are natural compounds found in plant-based foods (Williamson, 2017; Leonget al, 2016; Wang et al, 2018) and powerful antioxidants for scavenging reactive oxygen species (ROS) as well as anti-inflammatory effects (Choiet al., 2016; Kimet al., 2017; Lin et al, 2012).

Liver diseaseassociated with toxicological damageis often induced by chemicals, drugs and viruses that cause inflammation, oxidative stress, immunologicaland apoptotic reactions in the liver. CCl4 is a hepatotoxin widely used in animal modelsfor induction of oxidative stress in liver and other organs (Noa et al., 2003; Yang et al, 2010; Zhuet al., 2012). Since L.ruthenicum extract (LRE) contains polysaccharides and OPC, it should be able toprotect hepatic injury by reducing the CCl_4-induced oxidative stress. Therefore, we

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investigated the effect of LRE on CCl_4-induced acute liver injury rat model.

II. MATERIALS AND METHODS

Chemicals and Reagents

L.ruthenicum was obtained from the local market (Qaidam, Qinghai, China).LRE was prepared by blending with 95% ethanol (1 ml/g) for 5 min and then distilled water (8 ml/g) for additional 5 min and stored at 4°C until use. The preparation was stirred for 3 min before administering to the animals.ELISA kits for the measurement ofalanine aminotransferase (ALT)and aspartate aminotransferase (AST) were obtainedfrom Sigma- Aldrich (Steinheim, Germany).CytokinesELISA kits, interleukin-1β (IL1β), IL6, and PGE2 were obtainedFromR&D (Minneapolis, MN, USA).

Assays of Anthocyanin content in the LRE

The dried LRE was homogenized with deionized water (1:10; weight/volume) and centrifuged at 5000×g for 10 min at 4 °C. Anthocyanin concentration in the LR extract was determined by using the Nakata method(Nakata and Ohme-Takagi, 2014). The anthocyanin content (Abs_{530}/g F.W.) was measured using a microplate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA, USA) and calculated by [Abs_{530} - (0.25 x Abs_{657})] × 10 (dilution). To correct contribution of chlorophyll to the absorbance at 530 nm, the formula Abs_{530} - (0.25 x Abs_{657}) was used.

Animals and Experimental Protocol

Male Sprague-Dawley (SD) rats, aged 6 months (400 ± 20g), were purchased from National Laboratory Animal Center (Taipei, Taiwan). The study was carried out followed the guideline of the Institutional Animal Care and Use. Animals were kept in the housing facilities, at least three days to adapt to the environment before the experiment and maintained at 23 ± 2 °C, light and dark of the 12 h cycle with food and water provided ad libitum. Forty-eightrats were randomly assigned to eachgroup of six for the experiment. Rats were given intragastrically with or without LRE (0, 10, 30 mg/kg) or positive control of anthocyanin (1.2 mg/rat) andsilymarin (mg/kg) for 7 days. All rats except for the normal control were injected intraperitoneally (i. p.) with 40% CCL_4 (1000 μg/kg) on days 6 and 7 to induce acute hepatic injury. Blood was collected from these animals 3 h later. Animals were sacrificed witha method of chloral hydrate euthanasia(400 mg/kg, i.p.) and livers were collected for western blot assay.

Histological Assessment

Liver samples taken from the central part of the right large lobe were fixed with 10%PBS-buffered formalin for 24 h, then washed, dehydrated and embedded in paraffin.Sections of 5 μm thick were prepared and stained with hematoxylin and eosin (H-E), andthen a histological assessment was performed.

Assay for Serum Enzymes and Components

Sera were collected from each animal 24 and 48 h after CCl4 treatment and measured the concentration of nitrate by the Griess reagent method.Nitric oxide (NO) was determined by Griess reagent assay. The absorbance at 450 nm was determined using a microplate reader (spectraMAX 340, Molecular Devices, Sunnyvale, CA, USA).ELISA assay.Cytokines, interleukin-1β (IL1β), IL6, and PGE2 were measured by ELISA kits (R&D, Minneapolis, MN, USA).

Measurement of reactive oxygen species

The serum accumulation of ROS was determined usingH_2DCF-DA, which is a nonfluorescent compound thataccumulates in serum following deacetylation. H_2DCFthen reacts with ROS to form fluorescent dichlorofluorescein(DCF).Serum fluorescence was monitored on a FluoroskanAscent fluorometer (Labsystems Oy, Helsinki, Finland)using an excitation wavelength of 485 nm and emissionwavelength of 538 nm.

Western blot assay

Tissue (0.3 g) from liver was adding 9 times (V/W) of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol), homogenized for 1 min, The homogenate solution was centrifuged at $12000_{\times}$g, 4 °C, for 10 min and the supernatant was store at -80°C until assay.Extracted protein samples from each treatment group (containing50μgofprotein)were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to immobile polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated for 1 h with 5% dry skim milk in TBST buffer (0.1M Tris-HCl, pH 7.4, 0.9%NaCl, 0.1% Tween-20) to block non-specific binding. Then, they were incubated with rabbit

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antibodies against p-p38, p-JNK, p-ERK, COX-2, iNOS, Bax, caspase-3, and anti-β-actin (Abcam, Cambridge, UK). Subsequently, the membranes were incubated with the conjugated affinity goat anti-rabbit IgG (Jackson, West Grove, PA, USA).Expression of these proteins was detected by a chemilumine scence detection system according to the manufacturer's instructions (ECL, Amersham, Berkshire, UK).The band intensity was quantified with a densitometric scanner (PDI, Huntington Station, NY, USA).

III. RESULTS

Determination of anthocyanin content in the LRE

TheLycium ruthenicumwas known as rich in Anthocyanincontent.The quantity of Anthocyanin in extracts of The Lycium ruthenicum wasdetermined using the method described by Nakata method. Anthocyanin concentration in the LRE(dry) was 24.1 ± 7.7 mg/g.

Effects of LRE on Histological Changes

Thehistopathology showed that LRE protected rat from acute hepatic injury significantly (Figure 1, P< 0.05). The pathological changes of tissue disruption, submassive centrizonal necrosis, fatty changes, and inflammatory cell infiltration were significantly reduced by LRE as compare with the CCl_4 only.

Effects of LRE on the Levels of Serum Enzymes and Components

Rat pretreated with LSE (10, and 30 mg/kg), or silymarin (30 mg/kg), or anthocyanin (1.2 mg/each rat), had a reduced CCl_4-induced hepatic injury as compared with the vehicle control. The levels of hepatic enzymes AST and ALT weresignificantly elevated in the CCl_4 treated group.The results showed that the serum levels of ALT and AST were significantly reduced by LRE in the pretreated rats(P<0.05; Figure 2).Similarly, LRE reduced CCl_4-induced NO and ROS production in the serum of CCl_4treated SD rats, dose dependently (Figures 3 and 4, P< 0.05). Consistently, LRE could reduce IL1β, IL6, and PGE2 productions in CCl_4 stimulated SD rats significantly (P< 0.05;Figure 5 and 6).

After pretreatment, the SD rats were injected intraperitioneally with 1000μl/kg of 40% CCl_4stress. The results showed that histopathology and CCl_4-elevatedserum levels of ALT, AST, nitric oxide (NO), reactive oxygen species (ROS), interleukin-1β (IL1β), IL6,andprostaglandin E2 (PGE2) were significantly reduced by LRE in the pretreated rats.

Western Blot of MAPKs, COX-2, iNOS, Caspase-3, and Bax

Since several signaling pathways are involved with CCl_4-induced hepatic injury, effects of LRE on these pathways were further examined by western blot assay. The protein expression of the hepatic apoptosis and inflammatory proteinwere all significantly increased in the CCl_4 treated groupcompared with that in control group (P <0.05). However, as shown in Fig. 7, we found that the LREwith high concentration reduced the expression of proteins in signaling pathways significantly: JNK (28%),ERK (19%),p38 (29%)MAPKs,Bax (52%), COX-2 (33%),iNOS (53%),andCaspase-3 (48%), respectively to the CCl_4-induced non-pretreated rats (P < 0.01). (P< 0.05; Fig. 7).

IV. DISCUSSION

Oxidative stress is a key factor in the development of liver disease (Yang et al., 2000). Studies have shown that antioxidants ameliorate acute hepatic injury in various animal models (Hsu et al., 2009; Deng et al., 2012). In the present study, pretreatment with LRE improved the CCl_4-induced hepatic injury.The CCl_4-induced pathological change was significantly reduced by LRE that distorted tissue architecture; submassive centrizonal necrosis, fatty changes, and inflammatory cell infiltration were similar to the normal controls. Antioxidants are able to protect against hepatic injury by scavenging oxidative free radicals (Kim et al., 2010). Our results were consistent with other reports that antioxidants improved the serum AST and serum ALT levels and histopathology against CCl_4-acute or chronic injuries (Kim et al., 2010; Bak et al., 2016). Consistently, serum levels of ROS and NOwere significantlysuppressed by LRE. These resultssupported its hepatoprotective effect. In the present study, anti-inflammatory effect of LRE was tested in animal models. The results showed that LRE pretreatment reduced CCl_4-inducedhepatic injury and inflammatory factors in the liver tissue and serum, dose dependently. TheCCl_4 induced serum levels of ROS and NO weresignificantly reduced by LRE pretreated and dose dependently. And,CCl_4 induced serum levels of IL1β, IL6, and PGE2 inflammatory factors in the serum, weresignificantly reduced by LRE pretreated animals.The hepatoprotection and anti-

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inflammatory mechanism of LRE might be related to the inhibition of CCl_4 induced MAPKs, COX2, iNOS, and caspase-3 expressions.

Although all goji berries are rich in phenolic, L.ruthenicum contains the highest phenolic, condensed tannin and anthocyanin content (Islam et al., 2017). Anthocyaninhas displayed antioxidative effectson aging, diabetic,ischemic heart,and liver steatosis models (Schmitzeret al., 2010;Hsuet al., 2016;Haoet al., 2016;Wang et al., 2017). Therefore, the reduced serum ROS and NOlevelsmightbe attributed to the antioxidative effects of anthocyanin. Anthocyanin is powerful antioxidant for scavenging reactive oxygen species (ROS) and anti-inflammatory (Choiet al., 2016; Kimet al., 2017). We found that CCl_4-induced COX-2 and iNOS protein levels were also reduced by LRE pretreatment in a dose-dependent manner similar to effects of other antioxidants (Kim et al., 2010; Bak et al., 2016). These results suggest that LRE exerts effects in suppressing inflammatory responses. In addition, polysaccharides from LRE could also protect against CCl_4-induced-inflammation (Peng et al., 2012).Thus, LRE could be used as potential drug for reducing acute hepatic toxicity in CCl_4-treated animal.

LRE significantly reduced NO, IL1β, IL6, and PGE_2 productions in the serum of CCl_4-stimulated animals. Furthermore, LRE was able to suppressCCl_4induced JNK, ERK, and p38 MAPK signal pathways.MAPKs signaling pathways are involved in a relay in transmitting signals to the downstream transcription factors like NF-κB by regulating transcriptional activation of a variety of genes encoding COX-2, iNOS, TNF-α, and IL-1β. (Kim and Choi, 2010).It has been reported that CCl4-induced liver injury is involved a combination of oxidative stress, inflammation, and apoptosis from the NF-κB, JNK, ERK, p38 MAPK, COX-2, and Bcl-2/Bax signaling pathways (Xie et al., 2015; Kim et al., 2011; Baket al., 2016; Chen et al., 2017). Our results showed that LRE reduced the CCl_4-induced JNK, ERK, p38 MAPKs, and COX-2 signaling pathways.Therefore, mechanism of LRE suppression of ROS and NOmight due to theinhibition of theseCCl_4-induced signal pathways. Our results of LRE also agreed with reports on hepatoprotectionthat showed the reduced Bax and Caspase-3apoptotic pathway (Lu et al, 2012; Chen et al., 2017; Zeng et al., 2017).

V. CONCLUSION

In conclusion, LRE effectively ameliorates CCL4-induced acute hepatic injury by inhibition of ROS,NOand PGE-2. Thisprotectmechanism was mainly through the suppression of apoptoticand inflammatorysignaling pathways such as JNK, ERK, and p38 MAPKs, COX-2, Bax, and Caspases.

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Figure Legends

Fig. 1.Protective effect of LRE on the CCl_4-induced hepatic injury. Histopathology (H&E stain) of hepatic slices from rats from (A) no treatment control group; (B) the CCl_4 stress group; (C) the silymarin (30 mg/kg) group; (D) the anthocyanin (1.2 mg/each rat) group, and the CCl_4 treatment supplemented with 10mg/kg(E) and 30 mg/kg(F) the LRE group. Photographs show the hepatic section with 200× magnification. Tissue disruption and inflammatory cell infiltration was more severe in CCl_4-stressonlythan in control or LRE groups.

Rat pretreated with LSE (10, and 30 mg/kg), or silymarin (30 mg/kg), or anthocyanin (1.2 mg/each rat), had a reduced CCl_4-induced hepatic injury as compared with the vehicle control.

Fig. 2.Effect of LRE on serum AST and ALT levels in rats with and without CCl_4 stress.The results showed that CCl_4-elevated serum levels of ALT and ASTwere significantly reduced by silymarin (30 mg/kg, S30),anthocyanin (1.2 mg/each rat, A1.2),and LRE in the pretreated rats.Data are expressed as the mean ± SD (n = 8). *P< 0.05 as compared to the CCl_4 stress group.

International Journal of Pharmaceutical Research and Applications
Volume 7, Issue 6 Nov-Dec 2022, pp: 1928-1936 www.ijprajournal.com ISSN: 2456-4494

Fig. 3. The effect of LRE on serum nitric oxide level of rats under CCl_4treated. LRE reduced CCl_4-induced NO production in the serum of CCl_4treated SD rats, dose dependently. Data are expressed as the mean ± SD (n = 8). *P< 0.05 as compared to the CCl_4 treated group.

Fig. 4. The effect of LRE on serum ROS level of rats under CCl_4 treated.The silymarin (30 mg/kg, S30) andanthocyanin (1.2 mg/each rat, A1.2)significantlydiminishedCCl_4-induced ROS in serum of rats. LRE scavengedCCl_4-induced ROS production in the serum of CCl_4 treated SD rats, dose dependently. Data are expressed as the mean ± SD (n = 8). *P< 0.05 as compared to the CCl_4 group.

Fig. 5.Effects of LRE on serum IL-1βand IL-6 levels of rats under CCl_4-stress. Serum IL-1 and IL-6 were determined by ELISA method after CCl_4 treatment. Data are presented as the mean ± SD (n = 8). *P< 0.05 as compared to theCCl_4 group.

Fig. 6. The effect of LRE on serum PGE2 production in CCl_4 stimulatedSD rats. LRE could reduce PGE2 generation in CCl_4 stimulated SD rats significantly.Data are expressed as the mean ± SD (n = 6). *P< 0.05 as compared to the CCl_4 group.

Figure 7.Effect of LRE on CCl_4-activatedsignaling pathway.The p-JNK, p-ERK, p-P38 MAP kinases, Bax,COX-2, iNOS and Caspase-3 proteins expression in hepatic cell of CCl_4 stimulated SD rats were determined by Westernblot assay. The protein expression of the hepatic apoptosis and inflammatory proteinwere all significantly increased in the CCl_4 treated groupcompared with that in control group.Values represent the mean from three independentexperiments. *P< 0.05 as compared to the CCl_4 control.

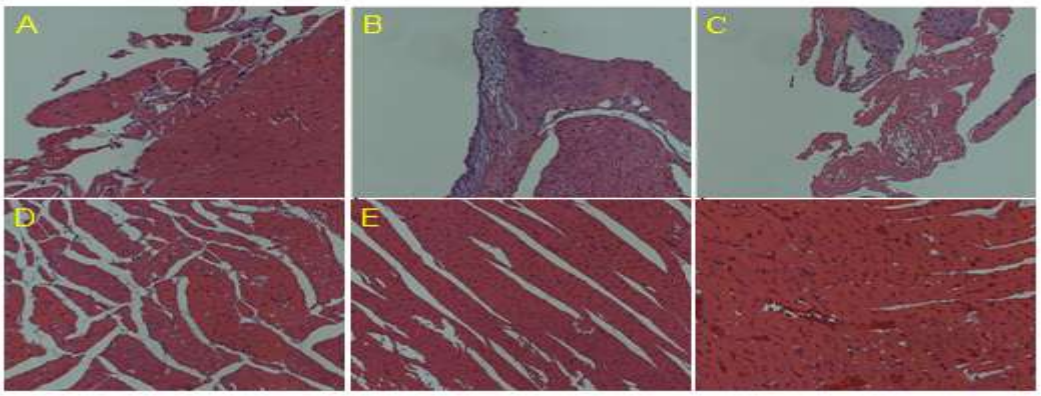

Figure 1.

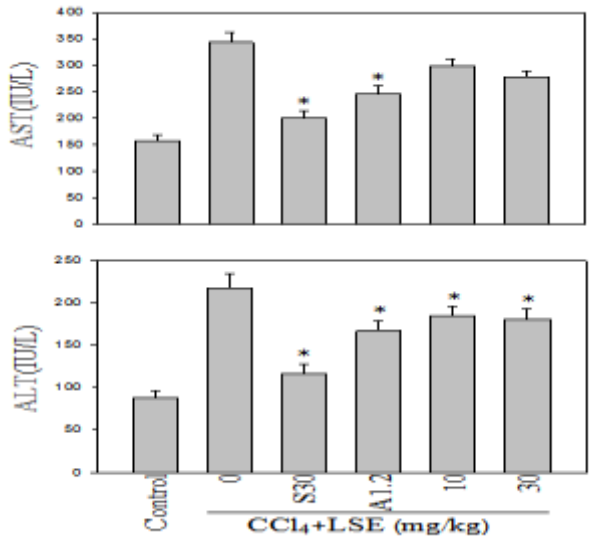

Figure 2

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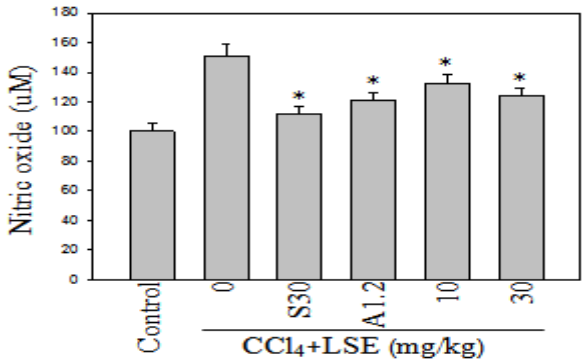
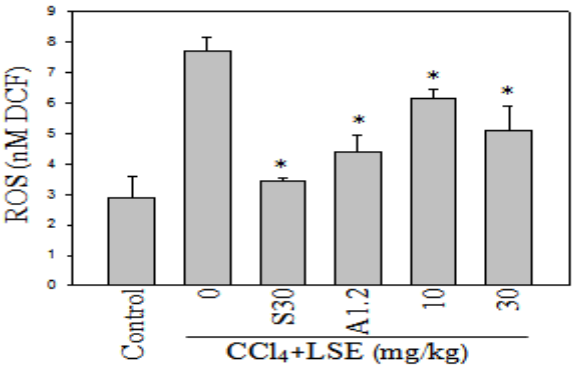


Figures 3



Figures 4

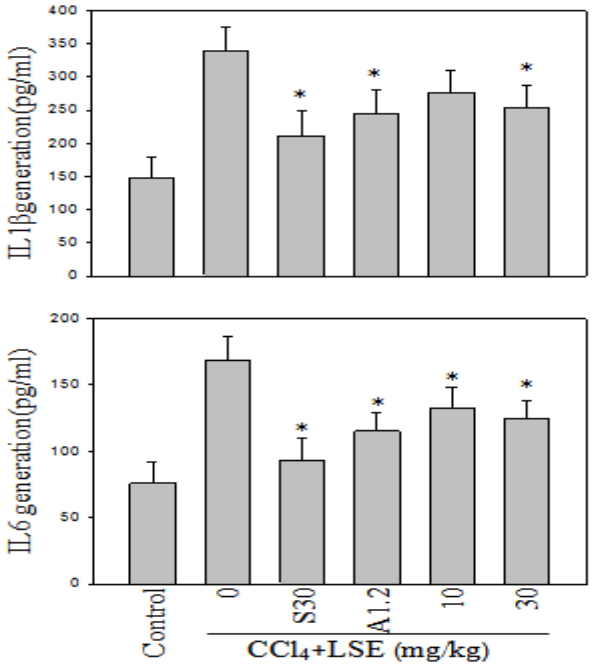


Figure 5

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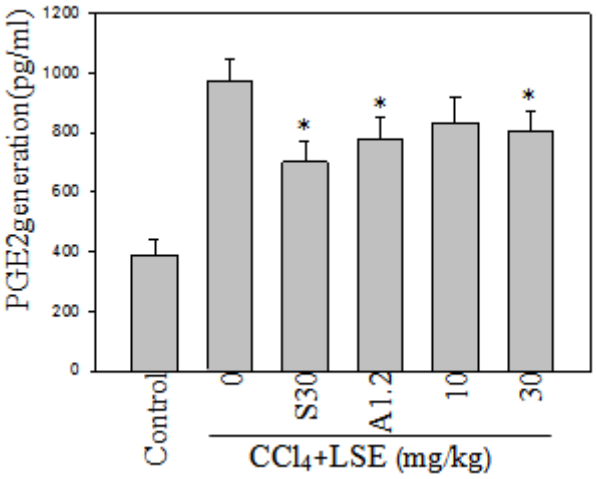

Figure 6.

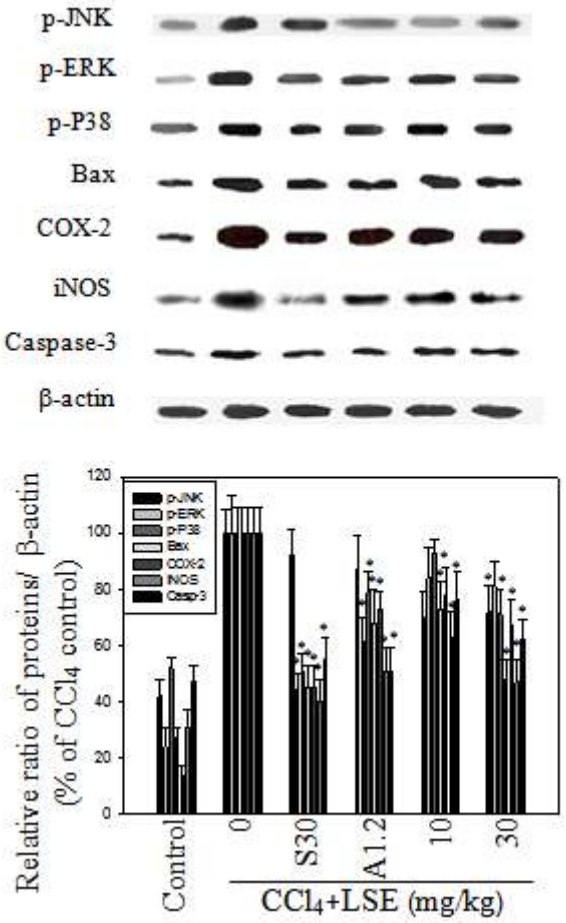

Fig. 7