PROTECTIVE EFFECTS OF MOUTAN CORTEX RADICIS AGAINST ACUTE HEPATOTOXICITY

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Abstract

This study evaluated the potential beneficial effect of Moutan Cortex Radicis (MCR) in a murine model of carbon tetrachloride (CCl_4)-, D-galactosamine (GalN)- and α-naphthylisothiocyanate (ANIT)-induced liver injury. Acute hepatotoxicity was induced by intraperitoneal injection of CCl_4 (10 μL/kg), GalN (700 mg/kg), and ANIT (40 mg/kg). Animals received MCR (30, 100, and 300 mg/kg) orally at 48, 24, and 2 h before and 6 h after administration of CCl_4, GalN, and ANIT. Serum activities of aminotransferase were significantly higher at 24 h after CCl_4 or GalN treatment. These changes were attenuated by MCR. Histopathological analysis revealed multiple and extensive areas of portal inflammation, hepatocellular necrosis, and an increase in inflammatory cell infiltration. These changes were inhibited by MCR. Serum total bilirubin concentration increased and bile flow decreased significantly 48 h after ANIT treatment, which was attenuated by MCR. Our results suggest that MCR has a protective effect on acute liver injury.

Keywords: carbon tetrachloride, D-galactosamine, inflammation, Moutan Cortex Radicis, α-naphthylisothiocyanate

Abbreviations: ALT: alanine aminotransferase; ANIT: α-naphthylisothiocyanate; AST: aspartate aminotransferase; CCl_4: carbon tetrachloride; GalN: D-galactosamine; MCR: Moutan Cortex Radicis; TBIL: total bilirubin

Introduction

Liver diseases constitute a major medical problem of worldwide significance and affect high proportions of the population (Farghali et al., 2009). Many environmental toxins cause liver injury to humans; despite new advances in hepatology, treatment of liver disease does not resolve the problems caused by these toxins. Furthermore, despite considerable progress in treatment of liver disease by oral hepatoprotective agents, the search for newer drugs continues due to the limitations of existing synthetic drugs (Pushpavalli et al., 2009). Therefore, there has been considerable interest in the role of complementary and alternative medicines for treatment of liver diseases.

Carbon tetrachloride (CCl_4)-induced liver injury in a range of laboratory animals is regarded as an analogue of the liver damage caused by various hepatotoxins in humans (Muriel, 1998). Involvement of bioactivation by a microsomal cytochrome P450-dependent monooxygenase system, resulting in formation of trichloromethyl free radicals, leading to hepatocellular damage in hepatic necrosis caused by CCl_4 has been suggested (Morio et al., 2001). D-Galactosamine (GalN) is a well-established hepatotoxicant that induces a diffuse type of liver injury closely resembling human viral hepatitis (Aristatile et al., 2009). GalN-induced liver injury reduces the intracellular pool of uracil nucleotides in hepatocytes, thus inhibiting synthesis of RNA and proteins (Siendones et al., 2004). α-Naphthylisothiocyanate (ANIT) is a hepatotoxicant that damages biliary cells and hepatocytes; therefore, it is used experimentally in rodents as a model of intrahepatic cholestasis (Xu et al., 2004). ANIT is detoxified by glutathione conjugation within hepatocytes and the complex of ANIT-glutathione is secreted into bile. However, the ANIT-glutathione conjugate dissociates rapidly in bile, leading to release of the parent compound, and exposes biliary epithelial cells to toxic concentrations of ANIT, thus causing hepatobiliary toxicity (Ramaiah and Jaeschke, 2007).

Moutan Cortex Radicis (MCR), the root cortex of *Paeonia suffruticosa* Andrews (Paeoniaceae), is a well-known traditional herbal medicine used in treatment of cardiovascular and female genital diseases (Hirai et al., 1983; Sakamoto et al., 1992). MCR removes heat from the blood, promotes blood circulation, and removes blood stasis (Chun et al., 2007). MCR has potent analgesic, sedative, anti-inflammatory, and anti-microbial properties (Jiang et al., 2007). Furthermore, MCR has a protective effect on acetaminophen-induced hepatotoxicity in mice (Shon and Nam, 2004). Paeonol, a major phenolic component of MCR, has recently been shown to alleviate alcoholic liver injury via inhibition of hepatic steatosis and inflammation (Hu et al., 2010).

Therefore, this study examined the hepatoprotective effect of MCR against acute hepatotoxicity induced by CCl_4, GalN, and ANIT.

Materials and Methods

Preparation of an ethanol extract from MCR

MCR (voucher No. 20100107) was purchased from the Kyung-Dong market (Seoul, Korea) and a dried voucher specimen was authenticated. 500 g of MCR was extracted in 70% ethanol at room temperature for 24 h, and three times at 55

doi: 10.4314/ajtcam.v8i5S.2

- 60˚C for 4 h in 10 times volume of 70% ethanol. Each extract was filtered through a 150 mesh and concentrated using a rotary evaporator (Model: Cosmos 660, KyungSeo Machine Co., Incheon, Korea). The dry powder of extracts was obtained by freeze-drying and the yield was 18.0%. MCR was dissolved in 10% Tween 80 (vehicle) for the experiments.

Treatment of animals

Male ICR mice (25-30 g) and Sprague-Dawley rats (190 – 210 g) were obtained from Daehan Biolink Co., Ltd., (Eumseong, Korea) and acclimatized to the laboratory conditions at Sungkyunkwan University for at least one week. Animals were maintained in a room with controlled temperature and humidity (25 ± 1˚C and 55 ± 5%, respectively) with a 12 h alternating light–dark cycle. Prior to experimentation, the animals were fasted for 18 h, but given tap water *ad libitum*. All animals were treated humanely under the Sungkyunkwan University Animal Care Committee Guidelines.

CCl_4–induced hepatotoxicity in mice

CCl_4 was dissolved in olive oil and administered intraperitoneally (10 µL/kg). Mice were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated CCl_4; iii) MCR 30 (30 mg/kg)-treated CCl_4; iv) MCR 100 (100 mg/kg)-treated CCl_4; v) MCR 300 (300 mg/kg)-treated CCl_4. MCR was administered orally at 48 h, 24 h, and 2 h before and 6 h after CCl_4 injection. Dose and timing of MCR were selected based on our preliminary studies. Blood samples were taken from the abdominal aorta and the liver was removed 24 h after CCl_4 treatment.

GalN–induced hepatotoxicity in rats

GalN was dissolved in phosphate buffered saline (PBS) and administered intraperitoneally (700 mg/kg). Rats were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated GalN; iii) MCR 30 (30 mg/kg)-treated GalN; iv) MCR 100 (100 mg/kg)-treated GalN; v) MCR 300 (300 mg/kg)-treated GalN. MCR was administered orally at 48 h, 24 h, and 2 h before and 6 h after GalN injection. Blood samples were taken from the abdominal aorta and the liver was removed 24 h after GalN treatment.

ANIT–induced hepatotoxicity in rats

ANIT was dissolved in olive oil and injected intraperitoneally (40 mg/kg). Rats were randomly assigned to five groups: i) vehicle-treated control; ii) vehicle-treated ANIT; iii) MCR 30 (30 mg/kg)-treated ANIT; iv) MCR 100 (100 mg/kg)-treated ANIT; v) MCR 300 (300 mg/kg)-treated ANIT. MCR was administered orally 48 h, 24 h, and 2 h before and 6 h after ANIT injection. Blood samples were taken from the abdominal aorta and the liver was removed 48 h after ANIT treatment.

Serum aminotransferase activities and total bilirubin concentration

Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity and total bilirubin (TBIL) concentration were determined by standard spectrophotometric procedures using the ChemiLab ALT, AST, and TBIL assay kits (IVDLab Co., Ltd., Uiwang, Korea), respectively.

Histological analysis

The anterior portion of the left lateral lobe of the liver was sectioned and used for histological analysis. Tissues were fixed by immersion in 10% neutral-buffered formalin. Samples were then embedded in paraffin, sliced into 5 µm sections, stained with hematoxylin-eosin, followed by blinded histological assessment. The degree of portal inflammation, hepatocellular necrosis, and inflammatory cell infiltration was evaluated (Frei et al., 1984). Histological changes were evaluated in non-consecutive, randomly chosen x 400 histological fields.

Bile flow

At 48 h after ANIT treatment, rats were anesthetized intraperitoneally with ketamine (60 mg/kg body weight) and xylazine (10 mg/kg body weight) and placed on a heating pad in order to maintain body temperature. A midline incision was made in the rat, and the common bile duct was isolated and cannulated with PE 10 tubing. Bile was collected for a 60 min period. Bile and liver samples were weighed, and bile flow was calculated, assuming a density of 1 g/mL.

Statistical analysis

The overall significance of the results was examined using one-way analysis of variance (ANOVA). Differences between the groups were considered statistically significant at $P < 0.05$ with the appropriate Bonferroni correction made for multiple comparisons. Results are presented as mean ± S.E.M.

Results and Discussion

In this study, the protective effect of MCR was examined using a model of CCl_4-, GalN, and ANIT-induced

doi: 10.4314/ajtcam.v8i5S.2

hepatotoxicity. CCl_4, a classic hepatotoxin, caused acute, reversible liver injury characterized by centrilobular necrosis (Son et al., 2007). Hepatotoxicity of CCl_4 is thought to involve two phases. The first phase consists of activation by cytochrome P450 2E1, 2B1, or 2B2 in the liver to form a highly reactive trichloromethyl radical and then a trichloromethyl peroxy radical (Yamaji et al., 2008). These radicals can attack membrane phospholipid stimulating lipid peroxidation and cell lysis. This damage to the structural integrity of the liver was observed from elevated serum levels of hepatospecific enzymes, i.e. AST and ALT (Aktay et al., 2000). The second phase is caused by the inflammatory response, which involves activation of Kupffer cells, probably by free radicals. Activation of Kupffer cells is accompanied by production of proinflammatory mediators (Chen et al., 2004).

As shown in Table 1, serum ALT activity in the control group averaged 25.5 ± 1.2 U/L. However, the value in the vehicle-treated CCl_4 group showed a dramatic increase (1983.0 ± 28.6 U/L, $P < 0.01$), indicating severe hepatocellular damage. Treatment with MCR, at doses of 30, 100, and 300 mg/kg resulted in a markedly attenuated increase in ALT activity to approximately 66.3%, 54.3%, and 37.3% of that in the vehicle-treated CCl_4 group, respectively. Consistent with the ALT data, the serum level of AST increased significantly from 46.4 ± 6.7 U/L to 1764.0 ± 158.1 U/L, and this increase was reduced by 30, 100, and 300 mg/kg of MCR. Histological observation of liver samples strongly supported the release of aminotransferases by damaged hepatocytes, as well as the protective effect of MCR. The histological features shown in Figure 1A demonstrated normal liver lobular architecture and cell structure in the control group. In contrast, the vehicle-treated CCl_4 group exhibited various histological changes to the liver, including cell necrosis, fatty metamorphosis in adjacent hepatocytes, ballooning degeneration, cell inflammation, and infiltration of lymphocytes and Kupffer cells (Figure 1B). These alterations were significantly attenuated by 300 mg/kg of MCR, showing mild hepatocellular necrosis and inflammation (Figure 1C). These results suggest that MCR may have potential clinical applications for treatment of liver disorders.

In terms of both morphological and functional aspects, GalN-induced acute injury in rat livers is well-established and recognized as resembling viral hepatitis in humans (Decker and Keppler, 1972). The toxic effect of GalN is connected with an insufficiency of UDP-glucose and UDP-galactose, as well as loss of intracellular calcium homeostasis. These changes affect cell membranes and organelles, as well as synthesis of proteins and nucleic acids (Devaki et al., 2009). Recent studies have demonstrated that GalN can induce hepatic hypoxia/hypoperfusion and trigger production of reactive oxygen species from affected hepatocytes, infiltrated leukocytes, and activated Kupffer cells and enhance mitochondrial apoptosis- and the proinflammatory cytokine-signaling pathway, contributing to oxidative stress and inflammation in the liver (Lin et al., 2009).

Table 1: Effect of MCR on serum aminotransferase activities in mice after CCl_4 administration

Group	Dose (mg/kg)	ALT (U/L)	AST (U/L)
Control		25.5 ± 1.2	46.4 ± 6.7
CCl_4			
Vehicle		1983.0 ± 28.6**	1764.0 ± 158.1**
MCR	30	1316.0 ± 196.2$^{**, \#\#}$	1181.0 ± 128.0$^{**, \#\#}$
	100	1076.0 ± 120.1$^{**, \#\#}$	1239.0 ± 94.5$^{**, \#}$
	300	739.4 ± 174.2$^{**, \#\#}$	960.3 ± 86.7$^{**, \#\#}$

The results are presented as mean ± S.E.M. of 8-10 mice per group. ** Denotes significant differences ($P < 0.01$) compared with the control group; $^{\#}$, $^{\#\#}$ denote significant differences ($P < 0.05$, $P < 0.01$) compared with the vehicle-treated CCl_4 group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; MCR: Moutan Cortex Radicis.

Table 2: Effect of MCR on serum aminotransferase activities in rats after GalN administration

Group	Dose (mg/kg)	ALT (U/L)	AST (U/L)
Control		44.4 ± 1.0	81.5 ± 0.8
GalN			
Vehicle		235.7 ± 28.6**	664.6 ± 41.7**
MCR	30	168.8 ± 9.2$^{**, \#}$	539.4 ± 27.5$^{**, \#}$
	100	155.9 ± 13.3$^{**, \#}$	468.8 ± 18.8$^{**, \#\#}$
	300	148.3 ± 19.2$^{**, \#}$	530.1 ± 28.3$^{**, \#}$

The results are presented as mean ± S.E.M. of 8-10 rats per group. ** Denotes significant differences ($P < 0.01$) compared with the control group; $^{\#}$, $^{\#\#}$ denote significant differences ($P < 0.05$, $P < 0.01$) compared with the vehicle-treated GalN group. ALT: alanine aminotransferase; AST: aspartate aminotransferase; MCR: Moutan Cortex Radicis.

doi: 10.4314/ajtcam.v8i5S.2

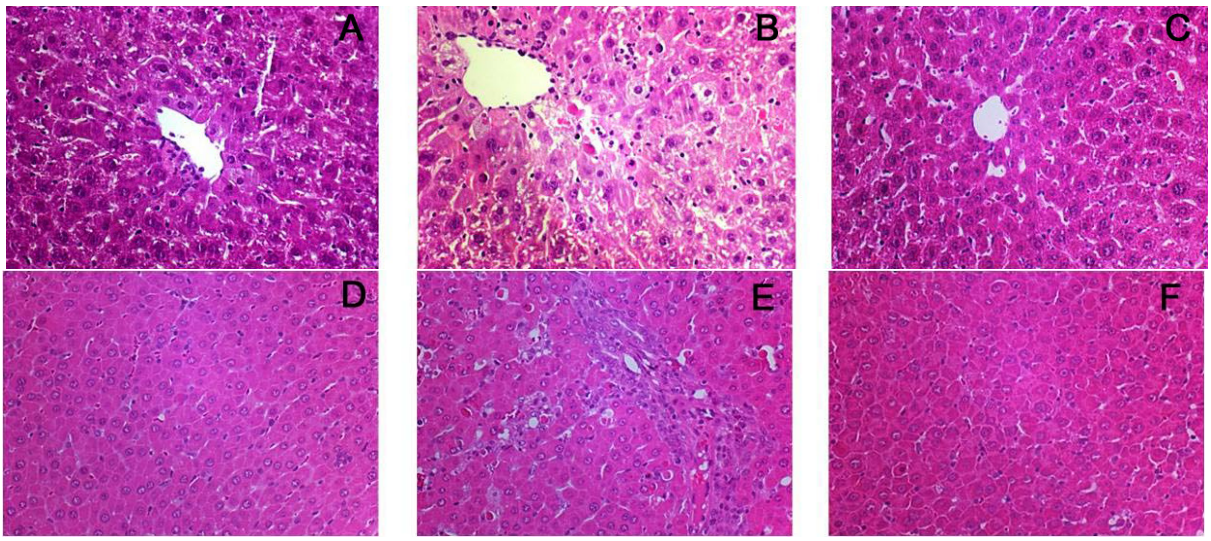


Figure 1: Histological features of liver sections stained with hematoxylin and eosin at 24 h after CCl_4 (A-C) or GalN (D-F) exposure. Typical images were chosen from each experimental group (original magnification ×400). (A): The control group, showing normal lobular architecture and cell structure; (B): The vehicle-treated CCl_4 group, showing multiple and extensive areas of portal inflammation and hepatocellular necrosis, and a moderate increase in inflammatory cell infiltration; (C): CCl_4 + MCR (300 mg/kg) group, showing minimal hepatocellular necrosis and inflammatory cell infiltration, and mild portal inflammation; (D): The control group, showing normal hepatic architecture; (E): The vehicle-treated GalN group, showing hepatocellular necrosis with extensive inflammation and inflammatory cell infiltration; (F): GalN + MCR (300 mg/kg) group, showing mild hepatocellular necrosis and inflammatory infiltration.

In the present study, serum ALT activity in the control group was 44.4 ± 1.0 U/L. The vehicle-treated GalN group showed a significant increase in serum ALT activity at 24 h after GalN injection (235.7 ± 28.6 U/L, $P < 0.01$). In contrast, treatment with MCR at doses of 30, 100, and 300 mg/kg attenuated the increase in ALT activity to approximately 71.6%, 66.1%, and 62.9% of that in the vehicle-treated GalN group, respectively. The serum level of AST also showed a significant increase from 81.5 ± 0.8 U/L to 664.6 ± 41.7 U/L, and this increase was reduced by 30, 100, and 300 mg/kg of MCR (Table 2). The histological features shown in Figure 1D demonstrate normal liver lobular architecture and cell structure in the control group. However, livers exposed to GalN showed multiple and extensive areas of portal inflammation and hepatocellular necrosis, randomly distributed throughout the parenchyma, as well as a moderate increase in inflammatory cell infiltration (Figure 1E). These pathological changes were attenuated by 300 mg/kg of MCR (Figure 1F). Taken together, our results suggest that MCR has a potential for use as a therapeutic agent for treatment of acute hepatitis.

The key function of the liver is to synthesize, concentrate, and secrete bile acids and to excrete other toxicants, such as bilirubin. ANIT induces bile duct epithelial cell necrosis, followed by cessation of bile flow, and consequent hyperbilirubinemia (Hasegawa et al., 2008). Due to its dose-dependent effects and high reproducibility between studies, ANIT is a typical hepatotoxin used in the study of intrahepatic cholestasis (Hasegawa et al., 2008). This drug provokes an acute cholestatic hepatitis, due in part to its recycling through repeated rounds of glutathione conjugation and biliary excretion (Kodali et al., 2006). The drug is initially detoxified in hepatocytes by conjugation with glutathione (Xu et al., 2004). Instability of the ANIT-glutathione conjugate and recycling rounds of ANIT metabolism result in high ANIT concentration in bile (Luyendyk et al., 2009). This causes bile duct epithelial cell damage and formation of foci of hepatic necrosis characterized by dead hepatocytes (Luyendyk et al., 2009).

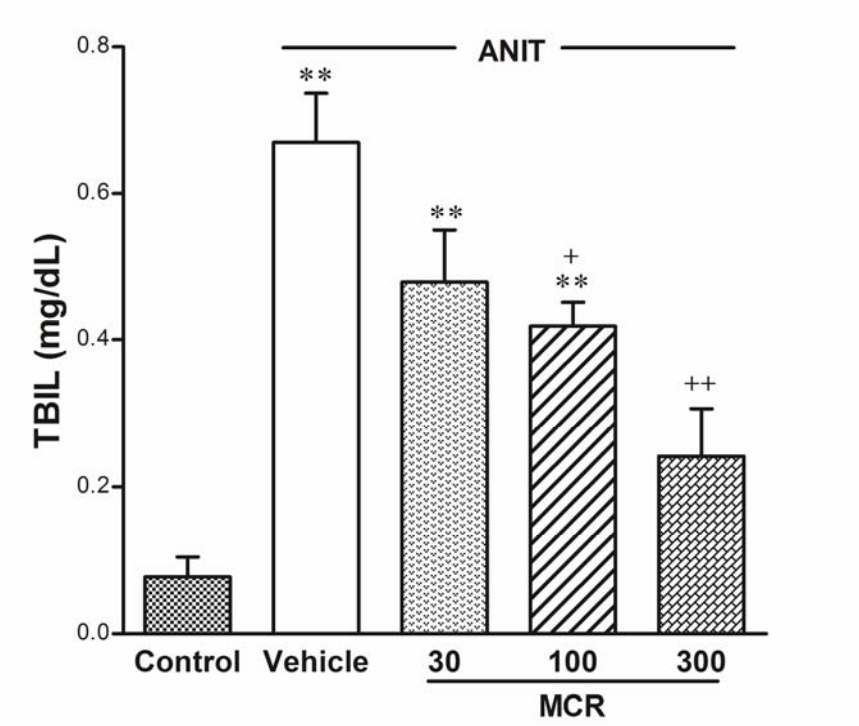
According to our results, serum TBIL concentration was 0.077 ± 0.027 mg/dL in the control group. However, the vehicle-treated ANIT group showed significantly increased serum TBIL concentration by approximately 8.6 times that of the control group. Treatment with MCR at doses of 100 and 300 mg/kg attenuated an increase in TBIL concentration to approximately 62.5% and 36.1% of that in the vehicle-treated ANIT group, respectively (Figure 2A). Bile flow in the control group was 2.249 ± 0.252 mL/min/g liver. In the vehicle-treated ANIT group, bile flow showed a significant decrease (0.379 ± 0.023 mL/min/g liver, $P < 0.01$); however, treatment with MCR, at doses of 30, 100 and 300 mg/kg attenuated this decrease in bile flow (Figure 2B). These results indicate that MCR may offer protection to hepatocytes and bile duct epithelial cells against acute liver cholestasis.

Conclusion

MCR may prevent CCl_4-, GalN-, and ANIT-induced acute hepatic injury. This study provides evidence of the potential of MCR as an alternative treatment for liver diseases. Further studies will be needed in order to achieve a full understanding of the underlying mechanism in the hepatoprotective effect of MCR against acute hepatotoxicity.

doi: 10.4314/ajtcam.v8i5S.2

A



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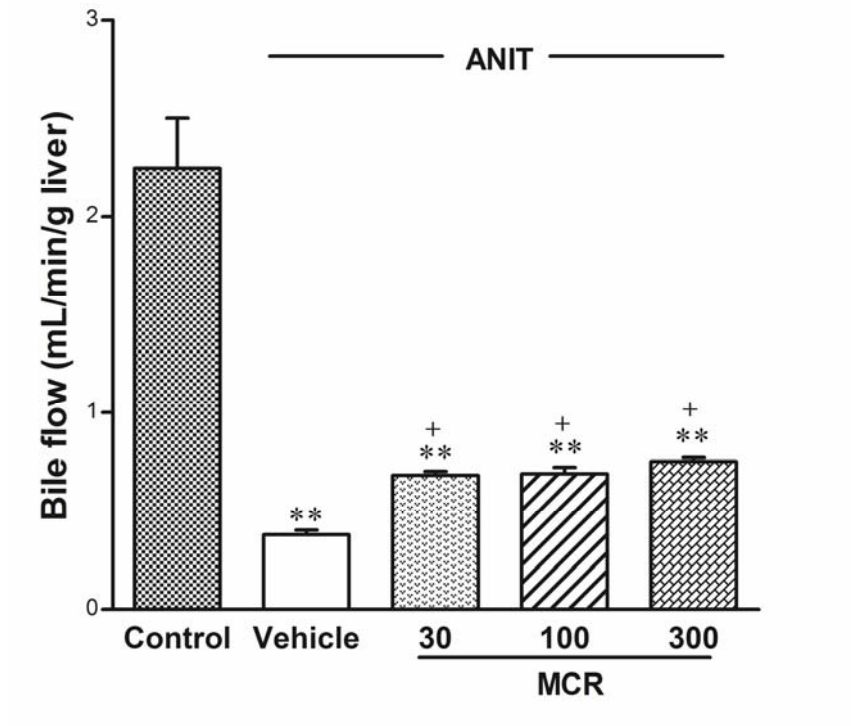

Figure 2: Effect of MCR on serum total bilirubin (A) and bile flow (B) in ANIT-induced acute hepatotoxicity. The values are represented as mean ± S.E.M. for 8-10 rats per group. ** Denotes significant differences ($P < 0.01$) compared with the control group; +, ++ denote significant differences ($P < 0.05$, $P < 0.01$) compared with the vehicle-treated ANIT group. ANIT: α-naphthylisothiocyanate; TBIL: total bilirubin; MCR: Moutan Cortex Radicis.

Acknowledgments

This work is financially supported by the Ministry of Education, Science and Technology (MEST), the Ministry of

doi: 10.4314/ajtcam.v8i5S.2

Knowledge Economy (MKE) through the fostering project of HUNIC.

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doi: 10.4314/ajtcam.v8i5S.2