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Thymoquinone, a bioactive component of *Nigella sativa* Linn seeds or traditional spice, attenuates acute hepatic failure and blocks apoptosis via MAPK signaling pathway in mice

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Abstract:

Thymoquinone (TQ), a bioactive natural product obtained from the black cumin seeds of *Nigella sativa* Linn, is a widely used spice or herb. The present study investigated the hepatoprotective effect of TQ on acute hepatic failure induced by D-galactosamine (D-GalN) and lipopolysaccharide (LPS) in mice. Mice were intragastrically administrated of TQ (5 or 20 mg/kg) for 12 h and 1 h prior to D-GalN (700 mg/kg)/LPS (10 μg/kg) injections and then sacrificed 8 h after treatment with D-GalN/LPS. TQ pretreatment declined the mortality induced by D-GalN/LPS and reversed liver damage. TQ attenuated D-GalN/LPS-induced hepatocyte apoptosis, which confirmed by suppressing caspase activation, PARP cleavage and Bax/Bcl-2 ratio. Importantly, TQ attenuated the D-GalN/LPS-mediated phosphorylation of JNK, ERK and p38. Furthermore, TQ suppressed the production of proinflammatory cytokines. These findings suggested that TQ could modulate D-GalN/LPS-mediated acute hepatic failure by inhibiting caspase activation, consistent with the mitochondrial pathway of apoptosis and MAPK signaling pathway.

Keywords: *Nigella sativa,* thymoquinone, acute hepatic failure, apoptosis, MAPK

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; D-GalN/LPS, D-galactosamine/lipopolysaccharide; ERK, extracellular signal regulated kinase; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; PARP, poly ADP-ribose polymerase; TNF-α, tumor necrosis factor-α.

Introduction

Acute hepatic failure is a clinical syndrome induced by viral hepatitis, alcohol or other hepatotoxic agents, leading to a high morbidity and mortality [1]. Liver transplantation is the specific available therapy, which limited for the rarity of organ. Thus, 80 - 90% of the high mortality is observed in patients with acute hepatic failure. Rodents challenged with D-galactosamine (D-GalN) sensitized significantly to lipopolysaccharide (LPS). It has been recognized as a promising model, D-GalN/LPS induced liver injury is similar to clinical acute hepatic failure [2]. Over-production of several cytokines and inflammatory mediators are caused by the combination of D-GalN and LPS [3]. D-GalN/LPS show a more severe and rapid acute hepatic failure in mice, which also surpasses exclusive use of LPS.

LPS is one of the major factors that regulate the inflammatory response by stimulating various proinflammatory mediator cytokines. In LPS-induced inflammation, LPS complex activates mitogen activated protein kinases (MAPK) signaling pathway [4]. In addition, it is reported that D-GalN/LPS induced MAPK activation in mice [5]. Furthermore, MAPK signaling cascades are activated by a variety of growth factors involved in kinds of biological responses, such as the production of cytokine and cell death [6]. Apoptosis can be induced through extrinsic pathway followed by caspase-8 activation and via the mitochondrial pathway by triggering the Bcl-2 family [7].

Thymoquinone (TQ), a bioactive natural product obtained from the black

cumin seeds of *Nigella sativa* Linn, is a widely used spice or herb throughout India and the Middle East [8]. Black cumin seed oil has been used as a traditional medicine of a range of diseases for a long history, such as diabetes, hypertension, inflammation, gastrointestinal disturbances, and cancer [9, 10]. The anti-tumor activity of TQ has been reported in cells derived from ovarian, breast and colon cancers [11]. For a recent study, they showed the dual effect of TQ in apoptosis in cancer cells. They showed TQ reduced the viability of human colon cancer HCT116 cells. And treatment of cells with TQ induced apoptosis, which was associated with the upregulation of Bax and inhibition of Bcl-2 expression [12]. For instance, TQ was shown to possess anti-inflammatory and antioxidant effects [13]. TQ had a protective effect against liver fibrosis induced by CCl_4, and inhibited the LPS-induced proinflammatory response in LX2 cells [14, 15]. Based on the researches *in vitro* and *in vivo*, it is appropriate that TQ should move from testing on the bench to clinical experiments [11]. In our previous study, TQ represented a potential new source of medicine for treating hepatic injury, targeting at LPS-activated hepatic stellate cells *in vitro* [16], inhibiting TLR4 signaling pathway and activating LKB1-AMPK signaling pathway *in vivo* [17]. In this study, we aimed to investigate the hepatoprotective effect of TQ on acute hepatic failure induced by D-GalN/LPS in mice, and focus on the role of apoptosis and MAPK signaling pathway.

Materials and methods

Animals

Male kunming mice were obtained from Yanbian University Laboratory Animal Centre (SPF, SCXK (J) 2011 - 0007). Animals (6 - 8 weeks old and 18 - 23 g) were housed in cages with bedding of flakes of wood at 22 ± 2°C and relative humidity of 50% - 60% with 12:12 h light-dark cycle. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Yanbian University.

Experimental design

Fifty mice were randomly divided into five groups for survival experiment (ten mice per group): normal, D-GalN/LPS, silymarin + D-GalN/LPS, TQ (20) + D-GalN/LPS and TQ (5) + D-GalN/LPS. In the TQ (Sigma Chemical Co., St Louis, MO, USA) and silymarin (Aldrich Chemical Co., Inc. Milwaukee, WI, USA) treated group, mice were intragastrically administered of TQ at doses of 20 mg/kg and 5 mg/kg and silymarin at dose of 100 mg/kg for 12 h and 1 h prior to the D-GalN/LPS injections. Then the mortality for 48 h after injected intraperitoneally with D-GalN (700 mg/kg; Sigma Chemical Co., St Louis, MO, USA) and LPS (10 μg/kg; Sigma Chemical Co., St Louis, MO, USA) was observed.

Thirty-six mice were randomly divided into the following six groups (six mice per group): normal, D-GalN/LPS, silymarin + D-GalN/LPS, TQ (20) +

D-GalN/LPS, TQ (5) + D-GalN/LPS and TQ (20). TQ or silymarin was intragastrically administrated to mice at 12 and 1 h prior to D-GalN/LPS injections. Then the mice (except for the normal group) were injected intraperitoneally with D-GalN (700 mg/kg)/LPS (10 μg/kg). At 8 h after injections of D-GalN/LPS, the mice were sacrificed and blood from the carotid artery was collected. Liver tissue was removed immediately and then was frozen immediately in liquid nitrogen and kept at -80°C until subsequent analyzed.

Histopathology analysis and serum ALT and AST levels

Liver samples were sliced into 4 μm sections prepared from frozen sections stained with hematoxylin and eosin (H&E) for histological assessment. Serum ALT and AST levels were examined after D-GalN/LPS injections by using assay kits of Nanjing Jiancheng Bioengineering Institute in China according to the manufacturer's instructions.

Western blot analysis

The protein extracts of liver tissue were used to determine protein concentration by the BCA Protein Assay Kit (Beyotime, Jiangsu, China). Fifty micrograms of whole liver tissue extracts were loaded per lane on 10% or 12% SDS-polyacrylamide gels for electrophoresis. The proteins were electroblotted onto a PVDF membrane and blocked with 5% skim milk for 1 h at room

temperature, and then incubated with specific primary antibody. The primary antibodies for caspase-8, caspase-9, p-p38 and Bcl-2 were purchased from Santa Cruz Biotechnology (1:500). Antibodies for Bax, extracellular signal regulated kinases (ERK), c-Jun N-terminal kinases (JNK), PARP, p-ERK, p-JNK and p38 were purchased from Cell Signaling Technology (1:500). Antibody for β-actin was purchased from Abcam (1:5000). After binding of an appropriate secondary antibody for 1 h at room temperature, protein bands were visualized by the BeyoECL plus kit (Beyotime Institute of Biotechnology). Quantitative analysis of bands intensities were performed using Quantity One software (Bio-Rad, USA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from liver tissue by the Trizol kit according to the manufacturer's protocol. cDNA was prepared using 1μg of total RNA. The mRNA expressions of IL-1α, IL-1β, IL-18 and GAPDH were investigated by RT-PCR (Applied Biosystems® Veriti® Thermal Cyclers). The following primer sequences were used for PCR: interleukin-1α (IL-1α), 5'-CTTGAGTCGGCAAAGAAATC-3' and 5'- GAGATGGTCAATGGCAGAAC-3'; IL-1β, 5'- GTACATCAGCACCTCACAAG-3' and 5'-CACAGGCTCTCTTTGAACAG-3'; IL-18, 5'- GATCAAAGTGCCAGTGAACC-3' and 5'-AACTCCATCTTGTTGTGTCC-3'. GAPDH was used as the housekeeping gene control. The reaction conditions were comprised of 2 min

at 95 °C, and then 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 1 min at 72 °C. The final extension was done at 72 °C for 10 min. PCR products were resolved in 2% agarose gel, ethidium bromide stained special bands were visualized under UV light and photographed.

Statistical analysis

Data were expressed as mean ± S.D. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were used in determining the statistical significance between different treatment groups in reference to either normal or D-GalN/LPS mice; statistical significance was set at $p<0.05$. Calculations were performed using the GraphPad Prism program (Graphpad Software, Inc, San Diego, USA).

Results

Lethality in mice

As shown in Fig. 1, mice treated with D-GalN/LPS began to die occurred 6 h after D-GalN/LPS injections, and the lethality rate reach 100% within 14 h. However, mice pretreated with 20 or 5 mg/kg TQ and 100 mg/kg silymarin prior to D-GalN/LPS injections exhibited 60%, 40% and 70% survival rate. 20 and 5 mg/kg of TQ were used as the optimal effective dose for examining the hepatoprotective effect against D-GalN/LPS-induced liver injury.

Histopathology changes and serum biochemical parameters in the liver

At 8 h after D-GalN/LPS treatment, livers showed severe areas of necrosis, apoptosis, inflammatory cell infiltrate. TQ treatment ameliorated the pathological alterations in mice in Fig. 2A. TQ (20) group showed normal liver lobular structure, and histological changes in the liver were not observed in the normal group (Fig. 2A).

Serum ALT and AST activities are the routine tests for liver function. As shown in Fig. 2B and C, the serum levels of ALT and AST at 8 h after the injections of D-GalN/LPS were higher than the normal group, which indicate severe liver injury. However, the mice administration of TQ and silymarin showed decreases in the serum of ALT and AST activities. And TQ (20) group didn't affect serum ALT and AST levels.

Effects of TQ on proinflammatory cytokines levels

To determine whether TQ suppresses inflammation caused by D-GalN/LPS, we examined the levels of proinflammatory cytokines in the liver including IL-1α, IL-1β and IL-18 by RT-PCR. The three cytokines levels in D-GalN/LPS group were higher than the normal group (Fig. 3). In contrast, TQ attenuates these cytokines levels, suggesting that TQ ameliorated the increases of D-GalN/LPS-induced proinflammatory cytokines.

TQ inhabited caspase activation and PARP cleaved

We further examined the anti-apoptotic effect of TQ on D-GalN/LPS-induced liver injury. As shown in Fig. 4, the active form of caspase-8, caspase-9 and cleaved PARP protein expressions were significantly increased than the normal group, while TQ treatment decreased expressions of active caspase-8, caspase-9 and PARP cleaved compared with D-GalN/LPS group. Silymarin also inhibited the caspase activation and PARP cleavage against D-GalN/LPS-induced acute hepatic failure (Fig. 4).

TQ regulated Bcl-2 and Bax protein expressions

Bcl-2 family was critical regulator of the apoptosis pathway, functioning as inhibitor Bcl-2 and promoter Bax of cell death. We therefore investigate Bcl-2 family protein expression by western blot analysis. The results demonstrated that Bcl-2 protein was less expressed but the Bax protein was highly

206 expressed in the D-GalN/LPS group. The expression of Bcl-2 was increased
207 by pretreatment with TQ, while Bax levels were decreased by pretreatment
208 with TQ as the D-GalN/LPS group (Fig. 5). The protein levels were digitized as
209 a percentage of the normal Bax/Bcl-2 ratio. The same to the immunoreactive
210 band, the Bax/Bcl-2 ratio was decreased with TQ pretreatment as the
211 D-GalN/LPS group (Fig. 5).

212

213 **TQ inhibited MAPK phosphorylation induced by D-GalN/LPS**

214 It has been well established that MAPK are redox sensitivity and involved in
215 apoptosis, such as JNK and ERK [18]. So we investigated whether the ERK,
216 JNK and p38 were involved in protection of TQ on D-GalN/LPS-treated mice.
217 There was no markedly change in total levels of ERK, JNK and p38. The
218 phosphorylation of ERK, JNK and p38 protein expressions were significantly
219 increased than the normal group, however, the phosphorylation of ERK, JNK
220 and p38 levels were declined by pretreatment with 20 and 5 mg/kg TQ (Fig. 6).

Discussion

In our study, TQ effectively attenuated acute hepatic failure induced by D-GalN/LPS in mice, including destruction of the structure of the hepatic lobules and inflammation. This was confirmed by the weakened levels of serum ALT and AST, MAPK phosphorylation and caspase activation in the TQ-treated group.

MAPK are major signal transduction molecules involved in regulating a variety of cellular responses, such as proliferation, differentiation, survival, and apoptosis. The MAPK family includes JNK, ERK and p38 well-characterized subfamilies [19, 20]. The three major MAPK proteins present different roles in inflammatory diseases in different capacities. JNK signaling pathway is one of the most important apoptosis-signaling pathways, and activated by various forms of liver injury. P38 is involved in regulating cellular responses to stress and cytokines. It has been reported that cell survival and apoptosis are regulated through the ERK MAPK pathway in various cancer cells [21]. This study focused on JNK, ERK and p38 MAPK, and the results showed that D-GalN/LPS induced MAPK phosphorylation, whereas TQ reduced the elevation of phosphor-JNK, phosphor-ERK, and phosphor-p38 proteins in liver tissues (Fig. 6).

Many molecular components are involved in apoptosis tightly linked to the presence and activation of MAPK family. The JNK-mediated cytochrome release might contribute to caspase-3 activation and the onset of apoptosis [22].

Inhibitors of MAPK, especially p38 MAPK, have been demonstrated to reduce LPS-induced metabolic activity and up-regulate pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-1β [23]. LPS are characteristic components of the cell wall of Gram negative bacteria, LPS treated mice show cytotoxicity and liver injury [24, 25]. In addition, LPS can induce lethal liver failure when simultaneously administered with D-GalN. D-GalN is a typical hepatotoxin and often used in pharmacodynamics research to induce hepatic injury. This model of liver damage provides a useful system for screening and investigating drugs that can be used in the treatment of disease [26]. Under stimulation of D-GalN/LPS, liver macrophages release pro-inflammatory cytokines. In our study, TQ reduced the release of pro-inflammatory cytokines IL-1α, IL-1β and IL-18 (Fig 3). Cytokines sensitized hepatocytes to activate tissue damage and caspase family. Caspase-8 is an initiator caspase, which is activated by a variety of apoptotic signals. Activated initiator caspases could cleave and activate effector caspases, such as caspase-3, which in turn cleave a variety of cellular substrates, most notably PARP through multiple signaling pathways. Our study confirmed that TQ inhibited caspase-8 and caspase-9 activations and PARP cleaved induced by D-GalN/LPS (Fig 4).

Several studies have explained the role of JNK in hepatocyte apoptosis induced by D-GalN/LPS through phosphorylation-dependent control of the anti-apoptosis factor, Bcl-2 [27, 28]. Bcl-2 and Bax are essential for apoptosis and in the eventual activation of caspase in Bcl-2 family [29]. The anti-apoptosis

protein Bcl-2 and the pro-apoptosis protein Bax are known as the regulation of anti-apoptosis [30]. Regardless of how the JNK signaling regulates the Bcl-2 superfamily members, as a whole, Bax/Bcl-2 ratio determine whether the cell survives or apoptosis. The higher this ratio is, the more possibility apoptosis would occur [31]. In this study, we showed that administration of TQ markedly decreased the Bax/Bcl-2 ratio induced by D-GalN/LPS (Fig 5). These data indicated that TQ-induced apoptosis in D-GalN/LPS treated mice was associated with the regulation of the Bcl-2 family.

Considering all of the findings, TQ protected hepatocytes against D-GalN/LPS-induced liver injury through inhibiting apoptotic signaling pathways. In addition, TQ suppressed the phosphorylation of MAPK signaling pathway. Thus, results of this study showed that TQ might be a potential pharmacological agent in preventing acute hepatic failure.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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352 **Figure legend**

353 Fig.1. Lethality in mice. TQ (20 or 5 mg/kg) or silymarin (100 mg/kg) were
354 intragastrically administered at 8 and 1 h prior to D-GalN/LPS injections (n=10).
355 The survival rate of mice was monitored for 48 h after intraperitoneally injected
356 with D-GalN (700 mg/kg)/LPS (10 μg/kg).

357

358 Fig. 2. Histopathological changes and serum biochemical parameters. Hepatic
359 tissue was collected 8 h after D-GalN/LPS injections and all sections were
360 stained with H&E and serum parameters of ALT and AST levels were
361 determined. (A) Histopathologic analysis with black arrows indicating the
362 hepatocyte necrosis or inflammatory infiltration. All slides are 200 ×
363 magnification. (B) Serum ALT. (C) Serum AST. $^{###}p<0.001$, significantly
364 different vs normal group. ***p<0.001, *p<0.05, significantly different vs
365 D-GalN/LPS group. NS, nonsignificant TQ (20) vs normal group.

366

367 Fig. 3. Effects of TQ on proinflammatory cytokines levels. mRNA expressions
368 of IL-1α, IL-1β and IL-18 were detected by RT-PCR. The GAPDH mRNA band
369 was used to confirm equal loading and to normalize the data. Values from
370 densitometric analysis are the mean ± S.D. of three independent experiments.
371 $^{###}p<0.001$, significantly different vs normal group. ***p<0.001, **p<0.01,
372 *p<0.05, significantly different vs D-GalN/LPS group.

373

374 Fig. 4. TQ inhabited caspase activation and PARP cleaved. Caspase-8 and
375 caspase-9 active form were detected as fragments of 18 kDa and 10 kDa.
376 PARP was cleaved to 89 kDa via Western blotting with specific antibodies.
377 β-actin protein band as loading control. Values are means ± S.D. of three
378 independent experiments. ###p<0.001, significantly different vs normal group.
379 ***p<0.001, **p<0.01, significantly different vs D-GalN/LPS group.
380
381 Fig.5. TQ regulated Bcl-2 and Bax protein expressions. β-actin protein band as
382 loading control. Densitometric tracing of Bax and Bcl-2 was expressed as a
383 percentage of the normal Bax/Bcl-2 ratio. Values are means ± S.D. of three
384 independent experiments. ###p<0.001, significantly different vs normal group.
385 ***p<0.001, significantly different vs D-GalN/LPS group.
386
387 Fig.6. Effects of TQ on the expression of MAPK. Phosphorylation (P) and total
388 (T) of ERK, JNK and p38 expressions were detected via Western blotting.
389 β-actin protein band as loading control. Values are means ± S.D. of three
390 independent experiments. ###p<0.001, significantly different vs normal group.
391 ***p<0.001, significantly different vs D-GalN/LPS group.

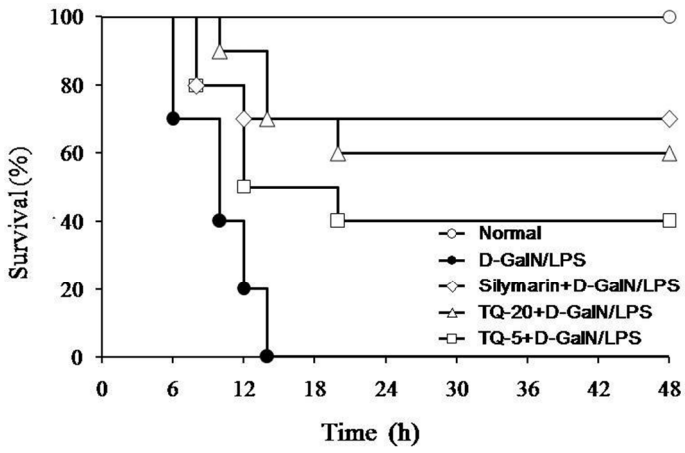

Fig 1

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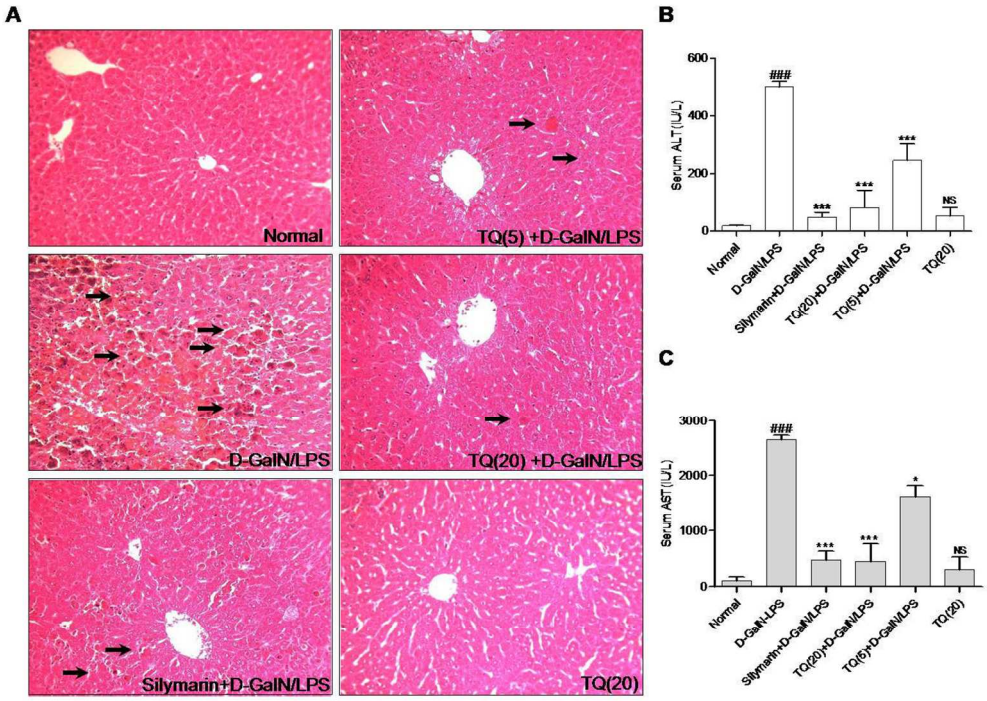

Fig 2

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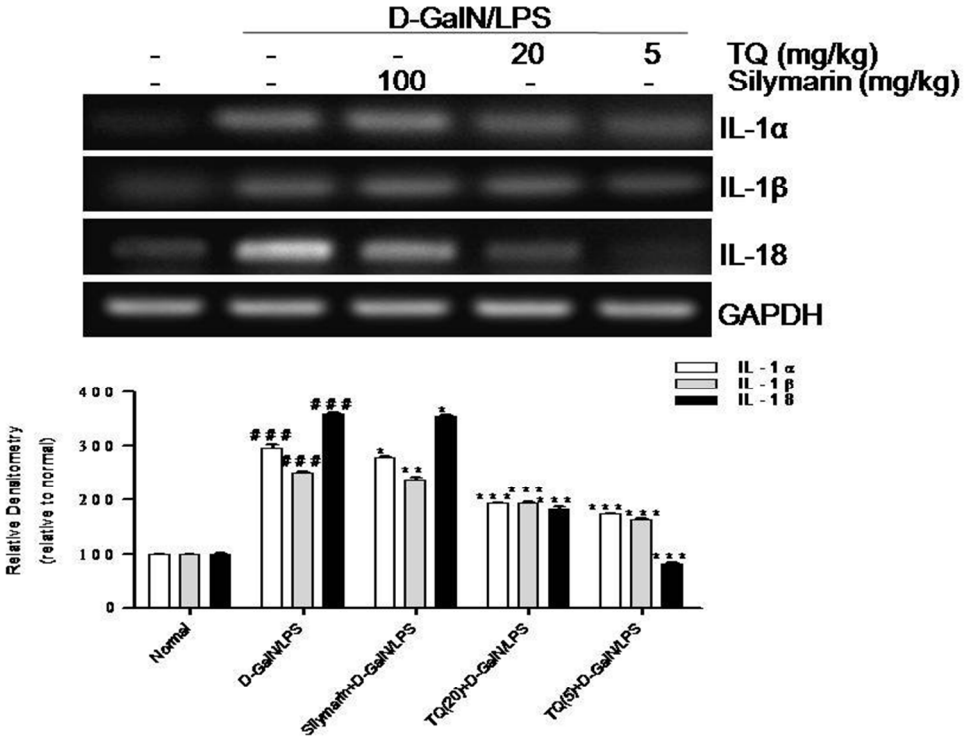

Fig 3

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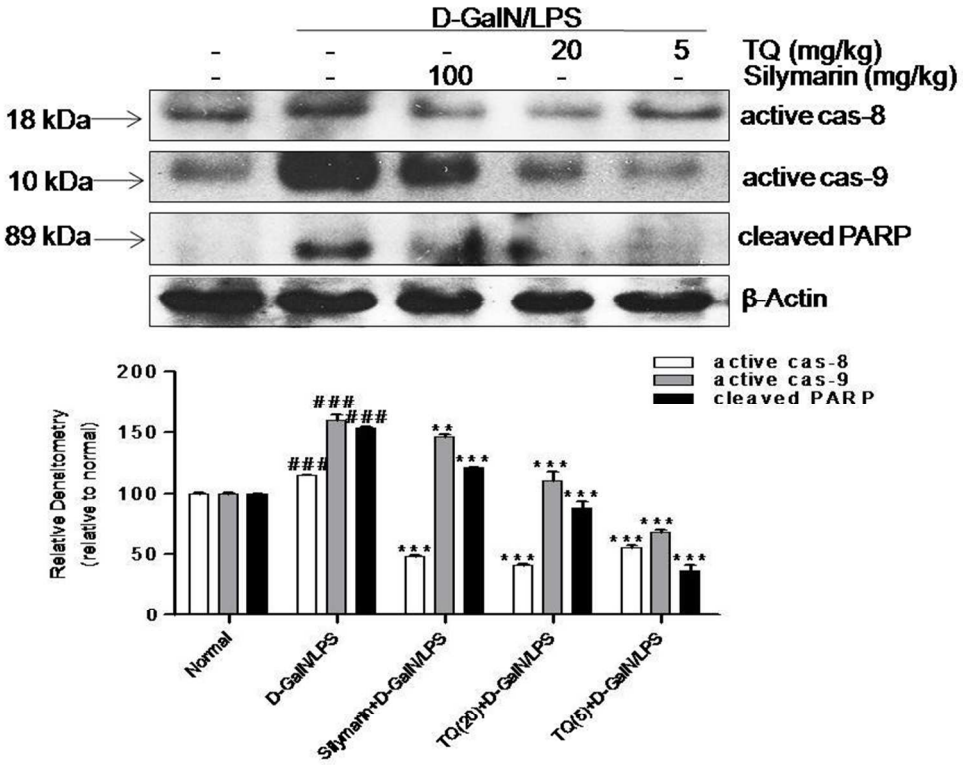

Fig 4

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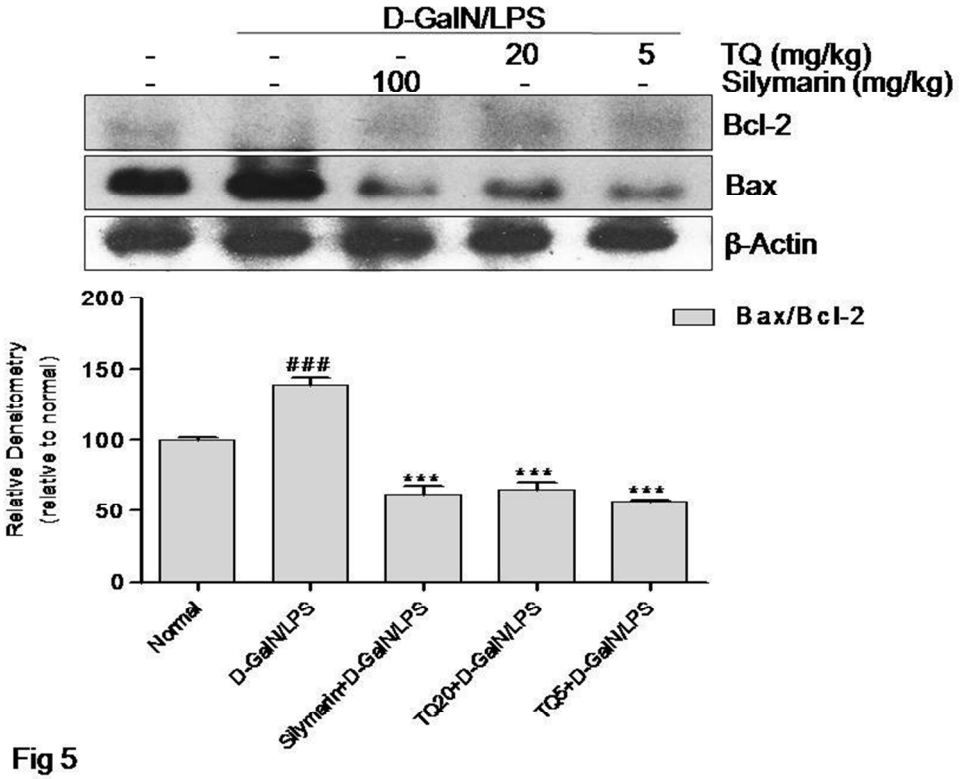

Fig 5

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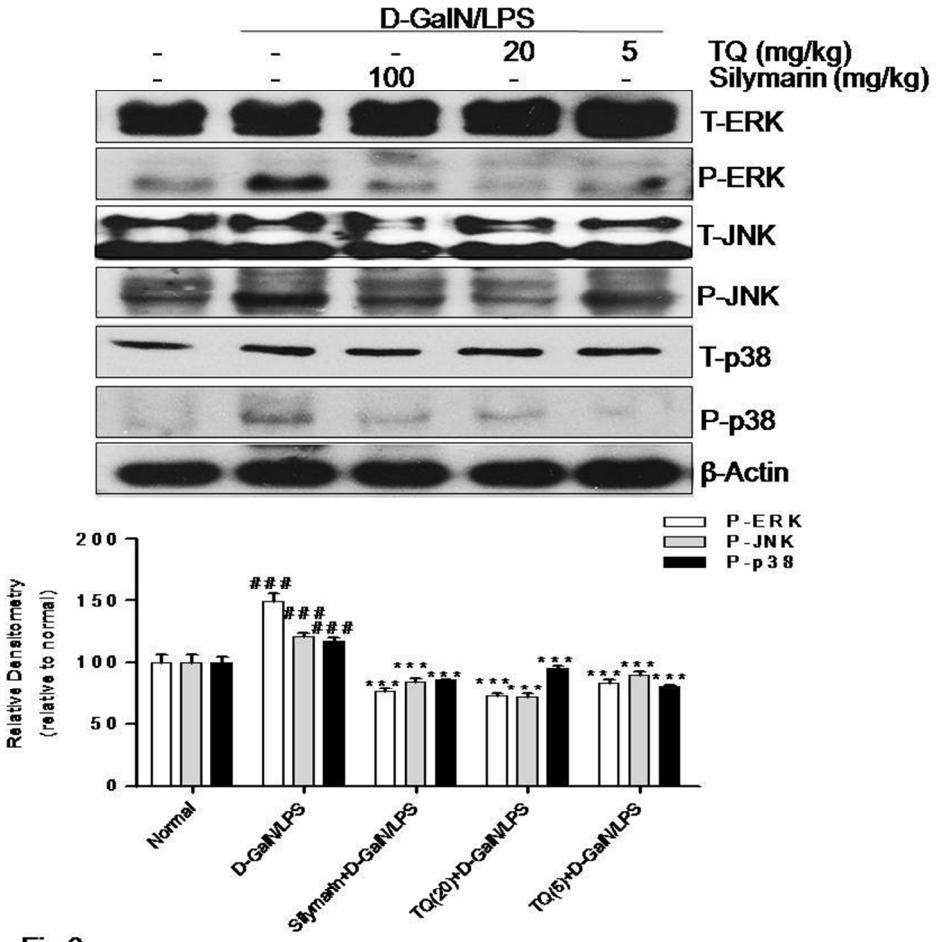

Fig 6

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