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Sirtuin-activating compounds (STACs) alleviate D-galactosamine/lipopolysaccharide-induced hepatotoxicity in rats: Involvement of sirtuin 1 and heme oxygenase 1

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SHORT TITLE

STACs alleviate D-GalN/LPS-induced hepatotoxicity in rats

ABSTRACT

Sirtuin activating compounds (STACs) attenuate various type of liver insults through mechanisms which are not fully understood. In the present study, we investigated the ameliorative potential of quercetin (natural polyphenol) and SRT1720 (synthetic SIRT1 activator) against D-galactosamine/lipopolysaccharide-induced hepatotoxicity (an experimental model of acute liver failure). Moreover, we compared and contrasted the roles of stress responsive enzymes, sirtuin 1 (SIRT1) and heme oxygenase 1 (HO-1) in hepatoprotection/ hepatotoxicity. Liver injury was induced in male Wistar rats by intraperitoneal injection of D-galactosamine (400mg/kg) and lipopolysaccharide (10 μ g/kg). Some animals were pretreated with quercetin (50mg/kg i.p.) or SRT1720 (5mg/kg i.p.). Twenty-four hours later, the effects of these treatments were evaluated by biochemical studies and Western blot. D-GalN/LPS treatment upregulated HO-1 expression, downregulated SIRT1 expression, decreased AST: ALT ratio and markedly increased bilirubin, catalase and conjugated diene levels. Pretreatment of D-GalN/LPS rats with either quercetin or SRT1720 returned SIRT1 expression, HO-1 expression and all the aforementioned markers towards normal. Collectively, these findings suggest that elevated HO-1 and low SIRT1 expressions are involved in the pathogenesis of D-GalN/LPS-induced hepatotoxicity. Drugs that downregulate HO-1 and/or upregulate SIRT1 seem to have antihepatotoxic effects and need further exploration.

KEYWORDS

Quercetin; SRT1720; Sirtuin 1; Heme oxygenase 1; Hepatotoxicity

INTRODUCTION

Acute liver failure (ALF) refers to rapid deterioration in liver function leading to jaundice, coagulopathy and encephalopathy (Bernal and Wendon 2013). The etiology of ALF shows widespread geographic variation. In developed countries, infectious causes such as hepatitis A and B tend to be declining in incidence but drug-induced hepatotoxicity still predominates. For instance, in the United Kingdom, paracetamol overdose account for about 50% of the reported cases with as many as 90,000 patients presenting each year (Bateman et al. 2014). In about 15% of the cases, the cause remains indeterminate (Bernal et al. 2010). ALF is a medical emergency and requires prompt diagnosis and treatment. However, there are hardly any hepatoprotective drugs and the available few have doubtful efficacies.

Medicinal plants, such as *Silybum marianum* and *Glycyrrhiza glabra* have been used for many generations to treat liver afflictions (Wang et al. 2007). Currently, patients still self-medicate with herbal products, despite inadequate scientific evidence to support their efficacies (Jacobs et al. 2002). One way of verifying the therapeutic potential of plant remedies is by extracting/isolating their active ingredients and testing them in controlled experimental settings. Amongst the numerous phytochemicals found in herbal plants, polyphenols have received much attention due to their protective effects against heart attacks (Rimm et al. 1996), cancers (Keli et al. 1996) and many liver diseases (Aller et al. 2015). This may, in part, explain stunning biogeographic concepts such as the ‘French paradox’ and some of the health benefits ascribed to moderate consumption of red wine (Catalgol et al. 2012).

It is unclear how polyphenols mediate the aforementioned health benefits. Currently, it is known that polyphenols exhibit anti-oxidant (Gülçin 2010), anti-microbial (Daglia 2012) and

anti-inflammatory (García-Lafuente et al. 2009) properties. However, the molecular mechanism underlying these attractive features is still elusive. The widely accepted hypothesis is that polyphenols activate a histone deacetylase, SIRT1 (sirtuin 1). SIRT1 in turn acts as an ‘on-off’ transcription switch that regulates diverse array of substrates (including, but not limited to, p53, forkhead box proteins and DNA repair enzymes) which may be involved in the pathogenesis of many diseases (Farghali et al. 2013). These attractive features validate SIRT1 as a potential target in management of a wide spectrum of diseases.

In our institute, we have previously shown that dietary polyphenols have ameliorative effects against various liver insults such as high dose acetaminophen (APAP) and D-galactosamine/lipopolysaccharide (D-GalN/LPS). For instance, Černý et al. found curcumin pretreatment to have hepatoprotective effects in D-GalN/LPS treated rats through heme oxygenase 1 and nitric oxide synthase 2 modulation (Černý et al. 2011). Kemelo et al. demonstrated that EX-527 (SIRT1 inhibitor) diminishes the liver-protective effects of resveratrol against D-GalN/LPS-induced hepatotoxicity (Kemelo et al. 2014). Most recently, Wojnarova et al. found that resveratrol and CAY10591 (synthetic SIRT1 activator) increases SIRT1 activity and combat the cytotoxic effects of APAP both *in vivo* and *in vitro* (Wojnarová et al. 2015). Based on these promising results, we felt compelled to further clarify mechanisms underlying the cytoprotective effects of polyphenols.

The present study is an extension of our previous work, first reported in the European Review for Medical and Pharmacological Sciences (Kemelo et al. 2016). The study is designed to further shed light on the roles played by stress responsive enzymes, HO-1 and SIRT1, on the potential liver protective effects of putative SIRT1 activators, Quercetin and SRT1720.

MATERIALS AND METHODS

CHEMICALS

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one with HPLC purity of > 95%), D-galactosamine hydrochloride, Lipopolysaccharide from *Escherichia coli* K-235, mouse monoclonal anti- β -Actin antibody and anti-mouse IgG (whole molecule)-peroxidase antibody were purchased from Sigma-Aldrich (Prague, Czech Republic).

SRT1720 was purchased from Selleckchem (Munich, Germany). Heme oxygenase 1 and Stabilized Peroxidase conjugated goat anti-Mouse antibodies were purchased from Thermo Fisher Scientific (Prague, Czech Republic). SirT1 (1F3) mouse mAb antibody was from Cell Signaling Technology (Danvers, Massachusetts).

ANIMALS

Outbred male Wistar rats (Velaz-Lysolaje, Czech Republic) of 250-350g body weight were used. They were maintained under standard conditions (22 \pm 2°C temperature, 50 \pm 10% relative humidity, 12-hour light-dark cycle) and given standard granulated diet and water *ad libitum*. All rats received humane care in accordance with the ethical guidelines of the First Faculty of Medicine, Charles University.

EXPERIMENTAL DESIGN

The animals were randomly assigned into six groups of eight animals and treated as shown in **table 1**. All the drugs were administered intraperitoneally. The doses were selected based on our previous experiments with quercetin (Lekić et al. 2013) and SRT1720 (Kemelo et al. 2016).

After 24 hours, the animals were anesthetized with diethyl ether and euthanized by exsanguination. Blood samples were collected into heparinized tubes for biochemical analysis. Liver samples were excised and preserved in liquid nitrogen for Western blot study.

BIOCHEMICAL INVESTIGATIONS

Plasma levels of alanine aminotransferase (ALT), total bilirubin and aspartate aminotransferase (AST) were measured using customized diagnostic kits according to the manufacturer's instructions (Analyticon, Lichtenfels, Germany). Levels of catalase in plasma and conjugated dienes in liver homogenate were measured as previously described by Farghali et al. (2009).

IMMUNOBLOTTING

Liver samples were lysed and homogenized in NP40 lysis buffer. After centrifugation (12,000 rpm, 15 minutes, 4°C) of the homogenized samples, protein concentration of the supernatant was determined using BIO-RAD DC protein assay kit (BIO-RAD, Hercules, CA). Equal amounts of protein were fractionated on an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membranes were incubated in Tris-buffered saline (TBS) containing 5% non-fat milk (for 1 hour at room temperature) and then in primary antibodies overnight at 4°C: HO-1 (1: 1000), SIRT1 (1:1000) and Beta actin (1:5000). The following day, the membranes were washed in TBS-Tween and incubated for 1 hour at room temperature with the corresponding secondary antibody: goat anti-mouse stabilized peroxidase conjugate (1: 1000) or anti-mouse IgG (whole molecule)-peroxidase (1:80000). Bands were detected using Molecular Imager VersaDoc™ MP 5000 System and the protein density was measured using the Quantity One 1-D Analysis Software (Bio-Rad, Prague, Czech Republic).

STATISTICAL ANALYSIS

Data are expressed as mean \pm SEM (standard error of mean) of at least five animals per group. Significance of differences between the groups was determined by one-way ANOVA followed by Tukey-Kramer comparison test. $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Our first objective was to reconfirm the hepatotoxic effects of D-GalN/LPS (**Table 2 and Figure 1**). Compared to the negative control, D-GalN/LPS significantly decreased AST: ALT ratio. Moreover, D-GalN/LPS markedly increased bilirubin, catalase and conjugated diene levels. These findings suggest profound oxidative damage and lipoperoxidation in the liver.

Secondly, we investigated if quercetin and SRT1720 might have any protective effects against D-GalN/LPS-induced hepatotoxicity. Pretreatments of D-GalN/LPS rats with either quercetin or SRT1720 significantly increased the AST:ALT ratio towards normal and decreased bilirubin, conjugated diene and catalase levels, compared to the D-GalN/LPS treated group. This shows that quercetin and SRT1720 were effective in combating the liver toxic effects of D-GalN/LPS.

Thirdly, we investigated if quercetin, SRT1720 and D-GalN/LPS treatments might have any effect on HO-1 expression. For this, we performed Western blot analysis (**Figure 2**). Alone, quercetin and SRT1720 moderately increased HO-1 expression relative to the negative control by 4.2-fold and 2.9-fold respectively. D-GalN/LPS treatment caused an even greater upregulation of HO-1 expression (of 7.8-fold relative to the control). Pretreatment of D-GalN/LPS rats with quercetin and SRT1720 significantly decreased HO-1 expression relative to the D-GalN/LPS group. Quercetin pretreatment decreased HO-1 expression by 4.8-fold and SRT1720 by 4.7-fold.

Finally, we investigated how the aforementioned drugs might affect SIRT1 protein expression (**Figure 3**). Consistent with our previous findings (Kemelo et al. 2016), we found that quercetin and SRT1720 treatments markedly increased SIRT1 expression, compared to

the negative control. Conversely, D-GalN/LPS significantly downregulated SIRT1 protein expression relative to the negative control. Pretreatment of the D-GalN/LPS rats with either quercetin or SRT1720 restored SIRT1 expression to normal.

DISCUSSION

The present study highlights the capacity of quercetin and SRT1720 in ameliorating D-GalN/LPS-induced hepatotoxicity. As already reported elsewhere, D-GalN/LPS causes severe liver toxicity that closely resembles ALF seen clinically (Liu et al. 2008). In this model, lipopolysaccharide (LPS), an endotoxin, accumulates primarily in tissues rich in cells of the reticuloendothelial system such as the liver (Nolan 1981). It then interacts with hepatic macrophages and triggers local damage through a variety of cytotoxic agents such as interleukin-1, tumor necrosis factor (TNF) and reactive oxygen species (ROS) (Hsu and Wen 2002). D-Galactosamine (D-GalN), on the other hand, depletes uridine nucleotide pool, inhibits protein synthesis in hepatocytes and sensitizes the liver to the cytotoxic effects of LPS (Silverstein 2004). A cocktail of these two drugs produces extensive liver damage and markedly increases the aminotransferase levels in plasma. Another reliable index of hepatotoxicity is the AST:ALT ratio, first discovered by Fernando De Ritis in 1957 (Botros and Sikaris 2013). As previously demonstrated, D-GalN/LPS treatment increases both transaminases (Kemelo et al. 2016), with the greatest effect on ALT, hence the ratio plummets. In addition to transaminases, increase in conjugated dienes and catalase indicate lipoperoxidation and oxidative injury in the liver. Furthermore, lipopolysaccharide or ROS can induce HO-1 to produce peroxyl radical scavenging metabolites, biliverdin and bilirubin. It seems, therefore, reasonable to postulate that increase in HO-1 and bilirubin might be an adaptive mechanism to overcome stress. However, bilirubin can also be cytotoxic, especially at high concentrations. For instance, it can impair protein synthesis (Basu et al. 2014), disrupt membrane fluidity (Rodrigues et al. 2002), inhibit membrane-bound proteins such as ATPases (Kashiwamata et al. 1979) and trigger apoptosis (Rodrigues et al. 2002) (Oakes and Bend 2005). In particular, Molle et al. demonstrated that D-GalN/TNF challenge of mice upregulates HO-1, markedly increases total bilirubin and causes massive apoptosis of

hepatocytes. But, pretreatment with a HO-1 inhibitor, Sn (IV) protoporphyrin IX dichloride, lowers total bilirubin levels and significantly improves the outcome (Van Molle and Libert 2003). Consistent with our results, these findings provide a possible link between high bilirubin levels and liver toxicity. Alongside HO-1, SIRT1 activity can be affected by oxidative stress at different levels. For instance, ROS can decrease SIRT1 expression by covalently modifying and marking it for proteasomal degradation (Caito et al. 2010). Apart from gene expression, some studies have shown that oxidative stress can over-activate PARP (Poly [ADP-ribose] polymerase) , deplete cellular NAD⁺ stores and decreased SIRT1 deacetylase activity (Braidly et al. 2011). Collectively, this makes it most probable that D-GalN/LPS rats in our study were also deprived of potentially therapeutic SIRT1 effects such as activation of Ku70 (involved in DNA repair) (Jeong et al. 2007) and FoxO3 (which regulates antioxidant enzymes) (Brunet et al. 2004).

The antioxidant properties of quercetin are well-established. For instance, the ancient custom of preserving lard by mixing it with onion may be based on prevention of lipid oxidation by quercetin (Sun-Waterhouse et al. 2013). Moreover, the consumption of quercetin-rich diet have protective effects against oxidative stress-related diseases such as Alzheimer's and coronary heart diseases (Nishimuro et al. 2015). Numerous experimental studies have shown that quercetin can ameliorate different kinds of liver insults (carbon tetrachloride (Amália et al. 2007), paracetamol (Janbaz et al. 2004) and ethanol (Chen 2010)) through diverse mechanisms ranging from direct scavenging of free radicals (Jovanovic and Simic 2000) to interaction with various enzyme systems (Kobori et al. 2015). In this study, quercetin mitigated the cytotoxic effects of D-GalN/LPS, in part, through decreasing HO-1 expression and bilirubin level. Despite the fact that total bilirubin may be toxic at high levels, there is some evidence that it might be cytoprotective at micromolar concentrations. For instance, *in*

vitro, studies have shown that both conjugated and unconjugated bilirubin can protect cells against artificially generated peroxy radicals (Wu et al. 1996). *In vivo*, many experiments have shown that hepatoprotective drugs such as silymarin (Madkour and Abdel-Daim 2013) and *aspalathus linearis* (Ulicná et al. 2003) act, in part, through lowering total bilirubin levels towards normal. All these findings suggest that bilirubin is a ‘double-edged sword’ with concentration-dependent toxic and protective effects.

Liver-protective effects of quercetin may also be SIRT1 mediated. Like HO-1, but in reverse, quercetin pretreatment restored SIRT1 expression to normal. Taken together, a pattern appears to emerge: it seems as if quercetin triggers homeostatic mechanisms in D-GalN/LPS rats which ensures that both HO-1 and SIRT1 expressions return to reasonable physiologic limits. However, the controversy of SIRT1 activation by polyphenols has drawn much attention from the scientific community in the last couple of years. Whether polyphenols directly activate SIRT1 (Howitz et al. 2003), indirectly activate SIRT1 through other target molecules such as AMP-activated protein kinase (Kang et al. 2013) or act independent of SIRT1 (Zhang 2006) is still elusive. In order to address this uncertainty, we pretreated some rats with SRT1720, a structurally distinct but extremely potent STAC (Milne et al. 2007). To our surprise, SRT1720 similarly restored SIRT1 expression to normal in D-GalN/LPS rats. How these putative SIRT1 activators upregulate SIRT1 expression is not clear. However, some studies have shown that STACs activate FoxO1 (Huang et al. 2013), which can positively feedback SIRT1 expression (Xiong et al. 2006). These parallel liver-protective effects between quercetin and SRT1720 and their effects on SIRT1, as well as HO-1, expression strongly suggest a common mechanism of action.

In conclusion, we have demonstrated that Quercetin and SRT1720 pretreatments are ameliorative against D-GalN/LPS-induced hepatotoxicity. Excessively high HO-1 and concomitantly low SIRT1 expression levels seen in D-GalN/LPS treated rats are pathologic. Drugs that downregulate HO-1 and/or upregulate SIRT1, such as STACs, seems to be hepatoprotective and need further exploration.

CONFLICT OF INTEREST

The authors declare no conflicts of interest concerning this article.

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TABLES

Table 1

Group	Treatment
Group 1: Co	DMSO followed one hour later by physiologic solution
Group 2: Q	50 mg/kg of quercetin followed one hour later by physiologic solution
Group 3: SRT1720	5 mg/kg of SRT1720 followed one hour later by physiologic solution
Group 4: D-GalN/LPS	DMSO followed one hour later by 400 mg/kg of D-GalN and 10 μ g/kg of LPS
Group 5: Q + D-GalN/LPS	50 mg/kg of quercetin followed one hour later by 400 mg/kg of D-GalN and 10 μ g/kg of LPS
Group 6: SRT1720 + D-GalN/LPS	5 mg/kg of SRT1720 followed one hour later by 400 mg/kg of D-GalN and 10 μ g/kg of LPS

Table 1: Experimental design: Quercetin and SRT1720 were dissolved in pure DMSO (Dimethyl sulfoxide). D-GalN (D-Galactosamine) and Lipopolysaccharide (LPS) were dissolved in physiologic solution (0.9% NaCl). All drugs were administered intraperitoneally, in a volume of 1ml/kg body weight.

Table 2

Group	AST: ALT ratio	Catalase (nmol/l)	Conjugated dienes (nmol/mg protein)
<i>Co</i>	2.17 ± 0.45	41.2 ± 11.6	1.52 ± 0.37
<i>Q</i>	2.46 ± 1.03	37.8 ± 4.9	1.18 ± 0.32
<i>SRT1720</i>	2.21 ± 0.27	29.25 ± 7.2	2.07 ± 1.01
<i>D-GalN/LPS</i>	0.29 ± 0.11 *	130.2 ± 3.5 *	3.56 ± 0.89 *
<i>Q + D-GalN/LPS</i>	1.92 ± 0.45 #	79.2 ± 16.8 #	1.53 ± 0.45 #
<i>SRT1720 + D-GalN/LPS</i>	1.03 ± 0.49 #	99.8 ± 14.2 *#	1.88 ± 0.35 #

Table 2: Effects of quercetin and SRT1720 pretreatments in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats on AST: ALT ratio, the levels of catalase in plasma and conjugated dienes in liver homogenate. Co: negative control, vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 µg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. *

Indicates significant values ($p \leq 0.05$) compared to the negative control group (vehicle only);

Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means ± SEM, n = 5-8.

FIGURES

Figure 1.

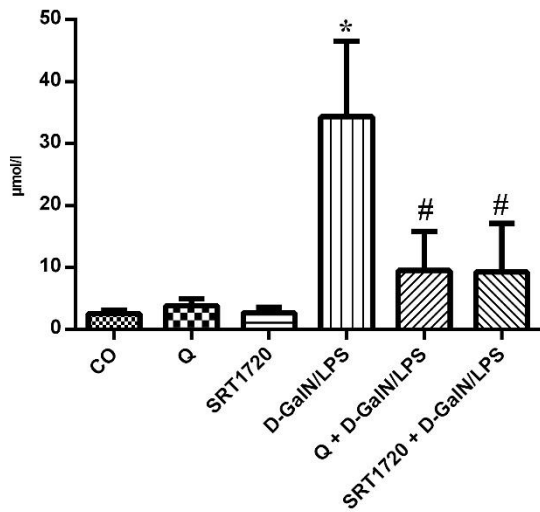


Figure 1: Effects of quercetin and SRT1720 pretreatments in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats on plasma levels of total bilirubin.

Co: negative control, vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 µg/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. * Indicates significant values ($p \leq 0.05$) compared to the negative control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means \pm SEM, n = 5-8.

Figure 2. A)

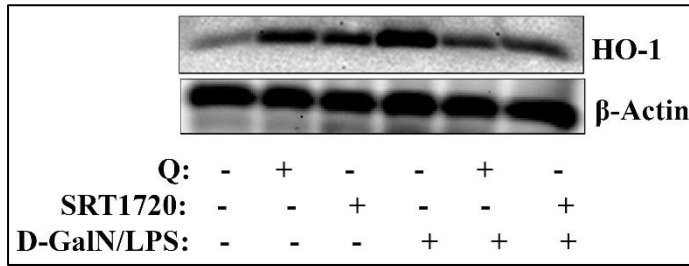


Figure 2. B)

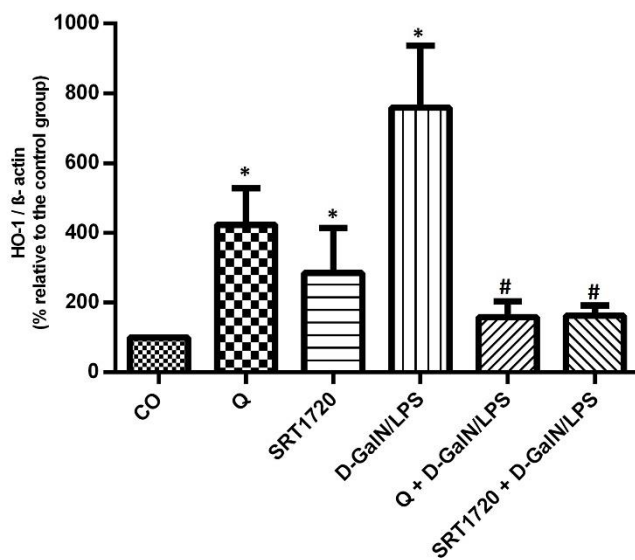


Figure 2: Effects of quercetin and SRT1720 pretreatments on HO-1 protein expression in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. Beta actin used as an endogenous control. (A) Representative Western blot image. (B) Protein expression relative to the control group. Co: negative control, vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μ g/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. * Indicates significant values ($p \leq 0.05$) compared to the negative control group (vehicle only); # Indicates

significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means \pm SEM, $n = 5$.

Figure 3. A)

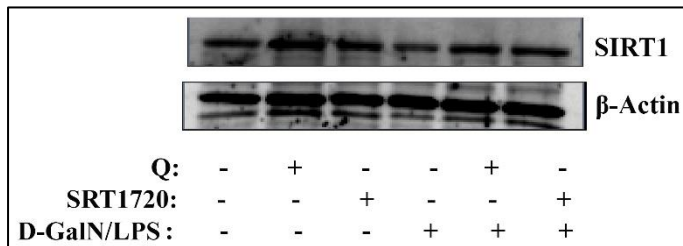


Figure 3. B)

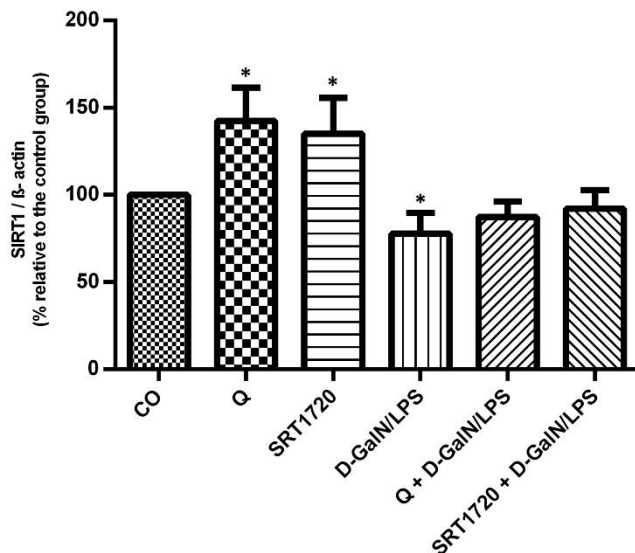


Figure 3: Effects of quercetin and SRT1720 pretreatments on SIRT1 protein expression in lipopolysaccharide-induced hepatitis in D-galactosamine sensitized (D-GalN/LPS) rats. Beta actin used as an endogenous control. (A) Representative Western blot image. (B) Protein expression relative to the control group. Co: negative control, vehicle only; Q: quercetin 50 mg/kg; SRT1720: SRT1720 5mg/kg; D-GalN/LPS: D-galactosamine 400 mg/kg + Lipopolysaccharide 10 μ g/kg; Q + D-GalN/LPS: Combination of Q and D-GalN/LPS; SRT1720 + D-GalN/LPS: Combination of SRT1720 and D-GalN/LPS. * Indicates significant

values ($p \leq 0.05$) compared to the negative control group (vehicle only); # Indicates significant values ($p \leq 0.05$) compared to the D-GalN/LPS group. The results are expressed as means \pm SEM, n = 5.