Effect of silibinin and vitamin E on the ASK1-p38 MAPK pathway in D-galactosamine/lipopolysaccharide induced hepatotoxicity

Reem M Hashem¹, Kamel MA Hassanin², Laila A Rashed³, Mohamed O Mahmoud¹ and Mohamed G Hassan¹

¹Department of Biochemistry, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt; ²Department of Biochemistry, Faculty of Veterinary Medicine, Minia University, El Minia, Egypt; ³Department of Biochemistry and Molecular Biology, Faculty of Medicine, Cairo University, Cairo, Egypt

Corresponding author: Mohamed G Hassan. Email: mohamed011159@pharm.bsu.edu.eg

Abstract

Apoptosis signal-regulating kinase 1 (ASK1), a redox-sensor mitogen-activated protein kinase kinase kinase (MAPKKK) that activates p38 MAPK pathways in oxidative stress-induced hepatotoxicity in D-galactosamine/lipopolysaccharide (D-GalN/LPS) model, is a key central pathway in which specific targeting of ASK1 deactivation is of a great therapeutic potential. We tested the effect of silibinin and vitamin E in curative and prophylactic manner of treatment on the negative modulators of ASK1, thioredoxin1 (Trx1), thioredoxin reductase1 (TrxR1), and the protein phosphatase (PP5), whereas they have previously proven to have hepatoprotective effect. Either curative or prophylactic silibinin and vitamin E groups significantly decreased ASK1 and p38 MAPK levels through increasing the gene expression of Trx1, TrxR1, and PP5 to reduce the oxidative stress as demonstrated by decreasing the levels of NADPH oxidase 4 (NOX4), TBARS and conjugated diene with a concomitant increase in the levels of GSH, CAT, and SOD. These results were confirmed by histopathology examination which illustrated progressive degenerative changes of hepatocytes such as hydropic degeneration, vacuolation, pyknosis, karyolysis, and loss of architecture of some cells in D-GalN/LPS treatment, and these features were alleviated with silibinin and vitamin E administration. In conclusion, silibinin and vitamin E decreased ASK1-p38 MAPK pathway through deactivating the upstream signalling ASK1 molecule via increasing the levels of Trx1 and TrxR1 as well as the PP5 to alleviate in D-GalN/LPS induced hepatotoxicity.

Keywords: ASK1, hepatotoxicity, p38 MAPK, PP5, Trx1, TrxR1

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Introduction

Apoptosis signal-regulating kinase1 (ASK1) plays a major role in reactive oxygen species (ROS)-induced cell death in various types of cells and has been implicated in the pathogenesis of oxidative stress-related diseases such as neurodegenerative diseases, cardiovascular diseases,^{1,2} and hepatic disorders as acetaminophen hepatotoxicity,³ hepatocarcinogenesis and in alcohol-induced liver injury.⁴ ASK1 is a serine/threonine kinase belonging to mitogen-activated protein kinase kinase kinase (MAPKKK) family that activates the MAP kinase kinase (MAPKK) and consequently activates c-Jun N-terminal kinase (JNK) and p38 MAPK.⁵ It is evolutionarily conserved among eukaryotes and regulates various cellular responses such as apoptosis, differentiation, cell survival, cell death, and inflammation. ASK1 is activated by various stresses such as ROS, tumor necrosis factor- α (TNF- α), endoplasmic reticulum stress, and lipopolysaccharide (LPS).⁶

A wide variety of antioxidant proteins and redox-sensitive signaling systems is implicated in maintaining the intracellular homeostasis of redox status.⁷ Thioredoxin (Trx) antioxidant system consists of Trx, thioredoxin reductase (TrxR), and NADPH.⁸ Trx exists in cells in two isoforms, cytosolic Trx1 and mitochondrial Trx2 whose active site contain thiol group of cysteine residue.^{9,10} The reduced form of Trx interacts with N-terminal of ASK1 *in vitro* and *in vivo* thereby inhibiting the activity of MAPK family.¹¹ Moreover, dephosphorylation of ASK1 by protein phosphatase 5 (PP5) is another important mechanism implicated in ASK1 deactivation. PP5 belongs to the phosphoprotein phosphatase family, and it acts by dephosphorylation of various substrates in response to hormone and stress.^{12,13}

Many recent studies using inhibitor compounds have revealed that the components of the ASK1-MAPK pathways have potential as therapeutic targets for the diseases in which ASK1 is involved.⁶ Although silibinin and vitamin

E are well-documented antioxidants; their effects on ASK1 and downstream signals p38 MAPK are not fully elucidated. Recent studies have documented that the control of ASK1 facilitates the precise regulation of stress responses through the JNK and p38 pathways and thus provides less-toxic pharmaceutical treatment options.⁶ Silibinin and vitamin E is commonly used as hepatoprotective antioxidants in various hepatic disorders.^{14,15} Silibinin is the main active ingredient of silymarin, isolated from the fruits and seeds of milk thistle, *Silybum marianum*.¹⁶ They act as potent oxidant scavenger of most free radicals and inhibit lipid peroxidation.^{17–19}

The current study has been undertaken to reveal the effect of those antioxidants in a curative and prophylactic manner of treatment on deactivating the upstream signaling kinases, ASK1 and modulating the internal Trx antioxidant system, Trx1 and TrxR1 as well as PP5 in ROS-activated ASK1-MAPK pathways in attempting to provide a new mechanism centered on ASK1 deactivation and precise downregulation of the downstream signaling kinase p38 MAPK in D-GalN/LPS induced hepatotoxicity.

Materials and methods

Chemicals

Silibinin, vitamin E, D-galactosamine (D-GalN), and LPS (serotype E.coli 0111:4) were purchased from Sigma Aldrich Chemical Co., St Louis, MO, USA. Alanine transaminase (ALT) and aspartate transaminase (AST) kits were purchased from Human Co. (Wiesbaden, Germany). Thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), Glutathione (GSH) and catalase (CAT) colorimetric kits obtained from Biodiagnostic Co. (Giza, Egypt).

Animals

Sixty male Albino Wister rats weighting 180–230 g were used in the present study. They were obtained from the animal house of research institute of ophthalmology (Giza, Egypt). Rats had free access to water *ad libitum* and a standard laboratory diet; all animals were subjected to controlled conditions of temperature and illumination. All studies were conducted in accordance with the Animal Care and Use Committee of the Biochemistry Department, Faculty of Pharmacy, Beni-Suef University.

Experimental design

The rats were kept on standard chow diet and divided randomly into six groups of 10 rats each; the **Control Group** received single dose of normal saline (0.5 ml intraperitoneally (i.p)), the **D-GalN/LPS group** were injected with single dose of D-GalN (500 mg/kg i.p), followed by another one of LPS (50 µg/kg i.p),²⁰ the **Curative Silibinin group** administered a single dose of D-GalN (500 mg/kg i.p) and LPS (50 µg/kg i.p) and treated by silibinin (100 mg/kg i.p) once daily for two weeks,²¹ the **Curative Vitamin E group** received a single dose of D-GalN (500 mg/kg i.p) and LPS (50 µg/kg i.p) and orally administered vitamin E (400 mg/kg) once daily for two weeks,²² the **prophylactic**

Silibinin received silibinin (100 mg/kg i.p) once daily for two weeks, followed by single dose of D-GalN (500 mg/kg i.p) and LPS (50 µg/kg i.p), the **Prophylactic Vitamin E group** received vitamin E (400 mg/kg) once daily for two weeks, followed by D-GalN (500 mg/kg i.p) and LPS (50 µg/kg i.p).

After 18 h of overnight fasting, the rats were sacrificed and the blood samples were collected for separation of serum which was kept at –20°C for determination of ALT and AST. The livers were excised and divided into three parts, the first part of them were preserved at –80°C for Western blot studies and RT-PCR. A second part of the liver was preserved in 10% formalin solution for histopathological examination. The third part of the liver was homogenized in 5 ml phosphate buffered saline by using tissue homogenizer (Yellow line DI 18 basic, IKA, Germany). The liver homogenates were centrifuged at 10,000 rpm for 15 min. The supernatants were collected and used directly for measurement of TBARS, GSH, CAT, and SOD levels.

Biochemical analysis

Serum ALT and AST activities were measured by kinetic methods (Human, Wiesbaden, Germany). Liver TBARS and reduced GSH levels were measured using colorimetric kits (Biodiagnostic, Giza, Egypt). CAT and SOD activities were measured using colorimetric kits (Biodiagnostic, Giza, Egypt).

Western blot analysis for detection of phosphorylated ASK1 and p38 MAPK

Liver segment was suspended in homogenization buffer (25 mM Tris-HCl, pH=7.0, 10% Triton-X100) containing protease inhibitors and phosphatase inhibitors (protease and phosphatase inhibitor cocktail, Calbiochem), and homogenized using a tissue homogenizer (Yellow line DI 18 basic, IKA, Germany). The liver tissue homogenates were centrifuged at 10,000 rpm for 15 min. The supernatant was collected and used to prepare protein for Western blot analysis. The amount of protein was determined by the Bio-Rad protein assay method. Expression of ASK1 and p38 MAPK was analyzed by Western blot. Thirty micogram protein of each sample was heated at 100°C for 5 min with a loading buffer containing 0.125 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol, and 0.002% bromophenol blue. It was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel and the Bio-Rad minigel system. The proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA). Blotting membranes were incubated with 3% bovine serum albumin in tris-buffered saline with tween (TBST) (10 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20) and probed with corresponding primary antibodies of anti-ASK1 and anti-p38 MAPK (CST, Beverly, MA, USA) at 4°C overnight at a dilution 1:500 or 1:1000. Blotting membranes were washed three times with phosphate buffered saline and then incubated with horseradish peroxidase-coupled secondary anti IgG monoclonal antibody

(Santa Cruz biotechnology, USA) for 2 h at room temperature. Membranes were exposed to films, and the bands were visualized using an enhanced chemiluminescence reagent (AmershamTM, UK). These bands were quantitated by densitometry (UVP Upland, CA, USA). In all Western blot experiments, β -actin was used as an internal control for equal protein loading. Western blots were repeated three times for each protein.

Detection of Trx1, TrxR1, PP5 and NOX4 gene expression using real time-polymerase chain reaction (RT-PCR)

RNA extraction. Total RNA was isolated from liver tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. The purity (A260/A280 ratio) and the concentration of RNA were detected by using spectrophotometry (Gene Quant 1300, Uppsala, Sweden). RNA quality was confirmed by gel electrophoresis.

cDNA synthesis. First-strand cDNA was synthesized from 4 μ g of total RNA using an oligo (dT) 12-18 primer and SuperScriptTM II RNase Reverse Transcriptase. This mixture was incubated at 42°C for 1 h. The kit was supplied by SuperScript Choice System (Life Technologies, Breda, The Netherlands).

Real-time quantitative polymerase chain reaction. Real-time PCR (RT-PCR) amplification was carried out using 10 μ L amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM of each primer as shown in the Table 1. PCR reactions consisted of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles) were performed on step one plus real-time PCR system (Applied Biosystems). Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA, USA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH gene.²³

Table 1 The sequences of primers used in RT-PCR

Primer	Sequence
Trx1	Forward primer 5'-GCTCACGGTTGGTCTGTTTG-3'
	Reverse primer 5'-TGAATTGTCCATCCGGCA T-3'
TrxR1	Forward primer 5'-TCCTTCGCCAGATCACAGTCA-3'
	Reverse primer 5'-CCTTGCTGTCATCCACATTGG-3'
PP5	Forward primer 5'-CTGAAAGCCAGCTGGCTGTA-3'
	Reverse primer 5'-TGGCACCCAAGTCCTGATAG-3'
Nox4	Forward primer 5'-GGAAATAGAAAGTTGACTGGCCCC-3'
	Reverse primer 5'-GTATGAGTGCCATCCAGAGCAG-3'
GAPDH	Forward primer 5'-CTCCCATTCTCCACCTTTG-3'
	Reverse primer 5'-CTTGCTCTCAGTATCCTTGC-3'

Histopathological examination

Liver sections were prepared and stained with routine Hematoxylin and Eosin (H&E) staining according to the method of Bancroft and Gamble²⁴ by the histopathology laboratory, Faculty of Veterinary Medicine, Beni-Suef University. The liver was kept in well-sealed containers in 10% formalin solution prepared in saline till becoming hard enough to be sectioned. A section of 5 μ m thick was prepared using paraffin blocks. The prepared section was then deparaffinized using xylol then hydration was performed using dehydrated in graduated ethyl alcohol (50%, 70%, 95% and 100%). The hydrated deparaffinized section was stained in Hematoxylin for 10 min then counterstained in Eosin for 1 min followed by rapid rinsing with distilled water. Finally, a section was dehydrated, cleared, mounted in Canada balsam and photographed using a digital camera attached to a light microscope.

Statistical analysis

All experiments were performed in six groups of 10 rats each. The results were expressed as mean $\pm$ standard error of mean (SEM). The statistical analysis was performed using one-way analysis of variance (ANOVA), followed with Tukey multiple comparisons *post hoc* test. *P* values less than 0.05 were considered significant. All calculations were made using SPSS 22 (SPSS, Chicago, IL, USA). Data were graphed by using Graphpad prism 6 (GraphPad software, Inc., USA).

Results

Serum markers of hepatic injury

Alanine transaminase and AST activities were significantly ($P < 0.05$) increased in D-GalN/LPS treated group when compared to the normal control group as shown in Table 2. Treatment or prophylaxis of either silibinin or vitamin E significantly ($P < 0.05$) reduced ALT and AST elevation to reveal a remarkable improvement in liver function. Curative and prophylactic silibinin groups showed a non-significant decrement in ALT and AST activities when compared to curative and prophylactic vitamin E groups.

Detection of oxidative stress pattern

D-galactoseamine/lipopolysaccharide induced a potential oxidative stress as evidenced by the significant increment in the level of TBARS by 3.3 folds at $P < 0.05$ in Comparison to the normal group. Curative treatment of silibinin or vitamin E resulted in a considerable statistical antioxidative effect as indicated by the significant decrement in the levels of TBARS up to 8.4 and 5.8 folds at $P < 0.05$, respectively, as compared to D-GalN/LPS-treated group. As a result of prophylactic treatment, silibinin or vitamin E groups statistically reduced TBARS by 4 and 2.9 folds at $P < 0.05$, respectively, as compared to D-GalN/LPS treated group. Curative and prophylactic silibinin groups showed non-significant decrement in TBARS level when compared to curative and prophylactic vitamin E groups (Table 3).

Table 2 The effect of curative and prophylactic silibinin or vitamin E administration on the liver function in D-GalN/LPS-induced hepatotoxicity in rats

	Control	D-GalN/LPS	Curative silibinin	Curative vitamin E	Prophylactic silibinin	Prophylactic vitamin E
ALT (U/L)	31.2 ± 1.5	91.8 ± 2.3 ^a	22.9 ± 2 ^b	31.7 ± 2.7 ^b	41 ± 5.3 ^b	40.7 ± 2.5 ^b
AST (U/L)	60.4 ± 1.6	190.2 ± 16.2 ^a	66 ± 8.6 ^b	74.9 ± 9 ^b	79.6 ± 5.7 ^b	81 ± 9 ^b

Note: Values are means ± SEM.

^a*P* < 0.05 compared with control group.

^b*P* < 0.05 compared with D-GalN/LPS treated group.

Table 3 The effect of curative and prophylactic silibinin or vitamin E administration on the level of TBARS, GSH, CAT and SOD in D-GalN/LPS-induced hepatotoxicity in rats

	Control	D-GalN/LPS	Curative silibinin	Curative vitamin E	Prophylactic silibinin	Prophylactic vitamin E
TBARS (nmol/g)	72 ± 7	239 ± 36 ^a	35 ± 4 ^b	41 ± 4 ^b	59 ± 7 ^b	82 ± 8 ^b
GSH (mg/g)	193.4 ± 9.8	72 ± 2.9 ^a	182 ± 3.1 ^b	167 ± 4.3 ^b	155 ± 4.6 ^b	134 ± 4 ^b
CAT (U/g)	4825 ± 115	2713 ± 38 ^a	4233 ± 139 ^b	3865 ± 129 ^{a,b}	3272 ± 145 ^{a,b}	3025 ± 117 ^{a,b}
SOD (U/g)	1167 ± 82	343 ± 69 ^a	1063 ± 25 ^b	969 ± 66 ^{a,b}	884 ± 23 ^{a,b}	814 ± 53 ^{a,b}

Note: Values are means ± SEM.

^a*P* < 0.05 compared with control group.

^b*P* < 0.05 compared with the D-GalN/LPS-treated group.

Figure 1 Liver ASK1 and p38 MAPK activities in groups treated with either silibinin or vitamin E compared to the D-GalN/LPS-treated group. In addition to protein blotting and immunodetection of expressed ASK1, p38 MAPK and β -actin in liver tissue of various studied groups. Values are mean $\pm$ SEM. (a) Significant difference from the normal control group. (b) Significant difference from the D-GalN/LPS-treated group. (c) Significant difference from the curative silibinin group. *P* < 0.05 (using one-way ANOVA followed by Tukey's *post hoc* test)

Detection of antioxidant pattern

Consumption of antioxidant system was observed in D-GalN/LPS-induced hepatotoxicity as manifested by the significant (*P* < 0.05) decrease in the levels of GSH and activities of CAT and SOD when compared to the normal control group. Treatment or prophylaxis with either silibinin or vitamin E recorded a significant (*P* < 0.05) increase in

the levels of GSH and activities of CAT and SOD when compared to D-GalN/LPS-treated group (Table 3).

Liver ASK1, p38 MAPK and NOX4

The gene expression of liver ASK1, p38 MAPK, and NOX4 was significantly (*P* < 0.05) increased in the D-GalN/

LPS-treated group when compared to the normal control group. Treatment or prophylaxis with silibinin or vitamin E resulted in a significant decrease in the gene expression of liver ASK1, p38 MAPK, and NOX4. Curative silibinin group showed a significant ($P < 0.05$) decrement in liver NOX4 activity when compared to vitamin E groups in curative and prophylactic manner. Also, curative silibinin group showed a significant ($P < 0.05$) decrement in liver ASK1, p38 MAPK, and NOX4 activities when compared to prophylactic silibinin- and vitamin E-treated groups as shown in Figures 1 and 2.

Molecular antioxidant defense system

The gene expression of liver Trx1, TrxR1, and PP5 were significantly ($P < 0.05$) decreased in D-GalN/LPS treated group when compared to those of the normal control

group. Either treatment or prophylaxis with silibinin or vitamin E significantly ($P < 0.05$) increased liver Trx1, TrxR1, and PP5 when compared to D-GalN/LPS-induced hepatotoxicity. Curative silibinin group showed a significant ($P < 0.05$) increment in liver Trx1 level and TrxR1 activity when compared to prophylactic vitamin E group. Also, curative silibinin group showed a significant increment ($P < 0.05$) in liver Trx1 level and TrxR1 activity when compared to prophylactic silibinin group (Figure 3).

Histopathological examination

Histopathological examination of sections from rat livers from normal control group showed normal architecture with intact central vein and hepatocyte appear normal (A & B). While examined, liver sections from rats which received D-GalN/LPS showed highly vacuolated hepatic lobules, which lost their endothelial cells. They also showed progressive degenerative changes of hepatocytes which were ranging from cloudy swelling, hydropic degeneration, vacuolation, pyknosis, karyolysis, and loss of architecture of some cells (C & D). Examination of liver sections of curative and prophylactic silibinin groups showed intact central vein, and hepatic plates appeared to be moderate normal. Hepatocytes mostly appeared moderate normal with increased Kupfer cells (E, F, I & J). A photomicrograph of liver sections of curative and prophylactic vitamin E group showed approximately normal hepatic lobules with a slightly affected central vein. Hepatocytes showed slightly vacuolation, dilated hepatic sinusoid with increased Kupfer cells (G, H, K & L) (Figure 4).

Discussion

ASK1 and p38 MAPK are the important downstream signalling molecules of toll-like receptor 4 (TLR4) signalling pathway²⁵ and they have emerged as a key cell death pathway in response to oxidative stress.^{26,27} The current study successfully recorded a significant increase in the expression of both ASK1 and p38 MAPK in D-GalN/LPS group and severe hepatotoxicity as manifested by the

Figure 2 Liver NOX4 activity in groups treated with either silibinin or vitamin E compared to D-GalN/LPS treated group. Values are mean $\pm$ SEM. (a) Significant difference from the normal control group. (b) Significant difference from the D-GalN/LPS-treated group. (c) Significant difference from the curative silibinin group. $P < 0.05$ (using one-way ANOVA followed by Tukey's *post hoc* test)

Figure 3 Liver Trx1, TrxR1 and PP5 levels in groups treated with either silibinin or vitamin E compared to D-GalN/LPS-treated group. Values are mean $\pm$ SEM. (a) Significant difference from the normal control group. (b) Significant difference from the D-GalN/LPS-treated group. (c) Significant difference from the curative silibinin group. $P < 0.05$ (using one-way ANOVA followed by Tukey's *post hoc* test)

Figure 4 Sections of rat liver from the control group (A (H&E stain -X 200) & B (H&E stain -X 400)) showing normal architecture with intact central vein (V) from which hepatic plates are mediated (H), hepatocytes appeared normal with wide irregular blood spaces sinusoids (S) lined by Kupfer cells (K). D-GalN/LPS group (C (H&E stain -X 200) & D (H&E stain -X 400)) showing highly vacuolated hepatic lobules and central vein (V) lost its endothelial cells, progressive degenerative changes of hepatocytes (H), and some hepatic sinusoids are dilated (S). Curative silibinin group (E (H&E stain -X 200) & F (H&E stain -X 400)) showing intact central vein (V) with most hepatocytes appearing moderate normal while few cells have slight degeneration. Curative vitamin E group (G (H&E stain -X 200) & H (H&E stain -X 400)) showing slightly affected central vein (V) with slightly normal hepatic lobules showing slightly vacuolated hepatocytes (H), dilated hepatic sinusoids (S) with slightly increased Kupfer cells (K). Prophylactic silibinin group (I (H&E stain -X 200) & J (H&E stain -X 400)) showed moderate normal hepatic plates (H) with intact slightly dilated central vein (V) and have some dilated hepatic sinusoids (S) with marked increased in Kupfer cells (K). Prophylactic vitamin E group (K (H&E stain -X 200) & L (H&E stain -X 400)) showed slightly affected central vein (V) and the periphery of the hepatic lobules suffered from degenerative change (a color version of this figure is available in the online journal.)

deterioration of liver function tests; ALT and AST. Histopathological findings illustrated progressive degenerative changes of hepatocytes such as cloudy swelling, hydropic degeneration, vacuolation, pyknosis, karyolysis, and loss of architecture of some cells.

This hepatotoxicity was attributed to the achieved severe oxidative stress in D-GalN/LPS group. Increased levels of TBARS and conjugated diene in addition to decreased antioxidant; GSH, CAT, and SOD have been established in accordance with other reports.^{25,28,29} The current study reported a significant increase in the gene expression of NOX4 in accordance with other studies.^{30,31} Direct interaction of TLR4 with NOX4 is pivotal in LPS-mediated ROS generation in hepatocytes.^{32,33} Indeed, Liang and his colleagues reported that LPS, the prototypical agonist of TLR4, stimulates the production of ROS in cultured endothelial cells, and this response is reduced by silencing RNAs for NOX4.³⁴

We compared the effects of two antioxidant vitamin E and silibinin. The treatment with silibinin and vitamin E in both curative and prophylactic manner demonstrated a substantial decrease in the levels of NOX4.³⁵⁻³⁷ Moreover, the curative groups demonstrated more significant effect than that showed by the prophylactic ones. Reducing the expression levels of NOX4 attenuated the ASK1-MAPK signalling pathway. Certainly, the curative-treated groups, either silibinin or vitamin E, revealed much more significant reduction in the levels of ASK1 and p38 MAPK than the prophylactic ones in collaboration with others.³⁸ Curative silibinin revealed more significant reduction than vitamin E in the levels of ASK1 as silibinin interacts with amino acid residues (Lys152, Ser154, and His174) of p38 MAPK and occupies the ATP binding pocket of p38 MAPK.³⁸

Recent studies have shown that ASK1-MAPK signalling pathway has a key role in human diseases induced by the dysfunction of cellular stress responses. Consequently, ASK1 has a potential therapeutic target for human diseases.⁵ We conducted this study to examine and compare the effects of silibinin and vitamin E on deactivation of ASK1 molecule to throw the light upon novel mechanism that silibinin and vitamin E may have exerted on ASK1 and the downstream signalling p38-MAPK.

We are the first study that showed that silibinin and vitamin E downregulated ASK1-MAPK signalling pathway through deactivation of ASK1 molecule, and we demonstrated significant increase in the expression levels of Trx antioxidant system; Trx1, TrxR1 as well as PP5 in curative and prophylactic groups subjected to D-GalN/LPS treatment. It is noteworthy to mention that the curative silibinin group established a non-significant increase than the curative vitamin E in the gene expression of the ASK1 deactivating molecules, Trx1, TrxR1, and PP5. Trx system is to maintain several intracellular proteins in their reduced state.³⁹ In doing so, the Trx active site is converted to the oxidized (disulfide) form. TrxR regenerates reduced (active) Trxs.⁴⁰

Saitoh and his colleagues documented that Trx forms a complex with ASK1 through the N-terminal region of ASK1, and inhibits ASK1's kinase activity, whereas Trx1 blocks ASK1 activation by autophosphorylation in Thr838 in response to oxidative stimuli. Additionally, under oxidative stress conditions, the reactive cysteine residues within Trx are oxidized and form an intramolecular disulphide bond⁴¹ and the oxidized form of Trx is released from

ASK1^{42–44} to enhance the transautophosphorylation of the threonine residue within the activation loop of the kinase domain of ASK1, which is essential for ASK1 activation.⁴³ TrxR regenerates reduced (active) Trx.⁴⁰

Although Trx1 and TrxR1 proteins are pivotal molecules in the regulation of ROS-induced activation of ASK1, PP5 is demonstrated as a potential negative regulator of ASK1. PP5 interacts with the activated form of ASK1 in response to ROS and dephosphorylates the essential phosphothreonine residue, thereby inhibiting the kinase activity of ASK1 and oxidative stress- and ASK1-dependent apoptosis. Thus, PP5 is one of the key molecules that determine the cell fate in the oxidative stress response.⁴⁵

Conclusion

In conclusion, silibinin and vitamin E as antioxidants proved a new mechanism in ROS-activated ASK1-p38 MAPK pathway in D-GalN/LPS-induced hepatotoxicity. Both of them modulate the ASK1-p38 MAPK pathway through deactivating the ASK1 molecule, and they increased the levels of Trx1 and TrxR1 as well as PP5, this and direct action on ASK1 molecule is pivotal in blocking the down streaming effector signalling p38 MAPK and alleviating hepatotoxicity.

Authors' contributions: RMH designed research; LAR and MGH performed experiments; KMA and MOM analyzed data; RMH and MGH wrote the paper. All authors read and approved the final manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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