

# The potential antifibrotic impact of apocynin and alpha-lipoic acid in concanavalin A-induced liver fibrosis in rats: Role of NADPH oxidases 1 and 4

Mostafa R. Fayed<sup>1</sup>, Reem N. El-Naga<sup>2</sup>, El-Sayed Akool<sup>3,4</sup>, Ebtehal El-Demerdash<sup>2,\*</sup>

<sup>1</sup> Pharmacology and Toxicology Department, Faculty of Pharmacy, Kafrelsheikh University, Kafrelsheikh, Egypt;

<sup>2</sup> Pharmacology and Toxicology Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt;

<sup>3</sup> Pharmacology and Toxicology Department, Faculty of Pharmacy, El-Azhar University, Cairo, Egypt;

<sup>4</sup> Pharmacology and Toxicology Department, Faculty of Pharmacy, Egyptian Russian University, Cairo, Egypt.

## Summary

Liver fibrosis results from chronic inflammation that precipitates excessive accumulation of extracellular matrix. Oxidative stress is involved in its pathogenesis. This study aimed to elucidate the potential antifibrotic effect of the NADPH oxidase (NOX) inhibitor, apocynin against concanavalin A (ConA)-induced immunological model of liver fibrosis, and to investigate the ability of the antioxidant, alpha-lipoic acid ( $\alpha$ -LA) to potentiate this effect. Rats were treated with apocynin and/or  $\alpha$ -LA for six weeks. Hepatotoxicity indices, oxidative stress, insulin, NOXs, inflammatory and liver fibrosis markers were assessed. Treatment of animals with apocynin and  $\alpha$ -LA significantly ameliorated the changes in liver functions and histopathological architecture induced by ConA. Liver fibrosis induced by ConA was evident where alpha-smooth muscle actin and transforming growth factor-beta1 were elevated, which was further confirmed by Masson's trichrome stain and increased hydroxyproline. Co-treatment with apocynin and  $\alpha$ -LA significantly reduced their expression. Besides, apocynin and  $\alpha$ -LA significantly ameliorated oxidative stress injury evoked by ConA, as evidenced by enhancing reduced glutathione content, antioxidant enzymes activities and decreasing lipid peroxides. ConA induced a significant elevation in serum insulin level and inflammatory markers; tumor necrosis factor-alpha, interleukin-6 and nuclear factor kappa b. Furthermore, the mRNA tissue expression of NOXs 1 and 4 was found to be elevated in the ConA group. All these elevations were significantly reduced by apocynin and  $\alpha$ -LA co-treatment. These findings indicate that using apocynin and  $\alpha$ -LA in combination possess marked antifibrotic effects, and that NOX enzymes are partially involved in the pathogenesis of ConA-induced liver fibrosis.

**Keywords:** Concanavalin A, liver fibrosis, apocynin, alpha-lipoic acid, NADPH oxidase, NOX inhibitor

## 1. Introduction

Liver fibrosis results from chronic damage to the liver and accumulation of extracellular matrix (ECM) proteins, which is a characteristic of most types of

chronic liver diseases (1). There is compelling evidence of hepatic cellular recovery with possible remodeling of scar tissue (2). Oxidative stress has been identified as a key mechanism of fibrogenesis. Upon activation of kupffer cells, they secrete inflammatory and fibrogenic mediators. These mediators along with reactive oxygen species (ROS) activates hepatic stellate cells (HSCs) (3). NADPH oxidase (NOX) enzymes is indeed a main source of oxidative stress in hepatocytes and non-hepatocytes (4). They consist of seven transmembrane proteins (NOXs 1 to 5 and Duox 1 and 2), whose primary function is to catalyze the transfer of electrons

\*Address correspondence to:

Dr. Ebtehal El-Demerdash, Pharmacology & Toxicology Dept., Faculty of pharmacy, Ain Shams University, Cairo, Egypt.

E-mail: ebtehal\_dm@pharma.asu.edu.eg

from NADPH to O<sub>2</sub> generating superoxide and hydrogen peroxide (5). Evidence indicates a crucial role for NOX-mediated ROS generation in hepatic fibrogenesis (6,7).

Apocynin, isolated from *Picrorhiza kurroa* extracts, is a NOX inhibitor that was shown to interfere with membrane assembly of cytosolic subunits of the NOX complex. Apocynin reduced the expression of gp91<sup>phox</sup> (a NOX subunit), where it replenished cellular NADPH leading to alleviated hepatic oxidative injury, which may underlie its therapeutic potency (8). Besides, alpha-lipoic acid ( $\alpha$ -LA) was shown to possess a beneficial role in chronic liver diseases. This is mediated *via* its anti-inflammatory and antioxidant activities that inhibit the activation of HSCs (9). Moreover, administration of apocynin markedly enhanced the neuroprotection effect of  $\alpha$ -LA in a rat model of ischemia/reperfusion injury, a finding that may reflect the promising beneficial effect of using the two drugs together (10).

Accordingly, the present study was attempted to provide an update on the hepatoprotective effects and the undisclosed antifibrotic mechanisms of apocynin,  $\alpha$ -LA and their combination against concanavalin A (ConA)-induced liver fibrosis in rats. This model is considered as a murine model of autoimmune hepatitis (T-cell mediated) that resembles the pathological changes accompanying autoimmune and viral hepatitis in human. Also, this study aimed to assess the potential role that NOX-1 and NOX-4 might play in the pathogenesis of ConA-induced liver injury.

## 2. Materials and Methods

### 2.1. Drugs and chemicals

ConA, apocynin and  $\alpha$ -LA were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and formaldehyde (37%) were purchased from El-Gomhouria Chemical Co. (Cairo, Egypt). All other chemicals and solvents were of highest grade commercially available.

### 2.2. Animals

The study protocol was conducted according to the ethical guidelines (Faculty of Pharmacy, Ain Shams University, Egypt). Male Wistar rats (150-200 g) were obtained from Nile Co. for Pharmaceutical and Chemical Industries, Egypt. Rats were housed in an air-conditioned atmosphere, at a temperature of 25°C with alternatively 12 h light and dark cycles. Animals were acclimated for 2 weeks before experimentation and kept on a standard diet and water *ad libitum*.

### 2.3. Experimental design

Rats were divided into 7 groups ( $n = 15$ ) and treated

for 6 consecutive weeks. Group 1 received phosphate buffered saline (PBS) (once/week, *i.v.*) and DMSO (3 times/week, *i.p.*). Groups 2 and 3 received only apocynin or  $\alpha$ -LA, respectively (50 mg/kg, dissolved in DMSO, 3 times/week, *i.p.*). The doses of apocynin and  $\alpha$ -LA were chosen as a result of a pilot study in our lab. Group 4 received ConA (20 mg/kg, dissolved in PBS, once/week, *i.v.*). Group 5, 6 and 7 received ConA together with apocynin,  $\alpha$ -LA or both, respectively.

At the end of the 6 weeks, blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 1,000 g for 10 min and used for the assessment of liver functions and insulin level. Then, rats were sacrificed, and liver tissues were dissected, weighed and washed with ice-cold saline. Specimens from the three major lobes of each liver from the different treatment groups were fixed in formalin 10% for histopathological examination and detection of fibrosis markers; Masson's trichrome staining, alpha-smooth muscle actin ( $\alpha$ -SMA) and transforming growth factor-beta1 (TGF- $\beta$ 1). Other liver specimens were homogenized in ice-cold saline and the homogenate was used to assess oxidative stress markers; reduced glutathione (GSH) and lipid peroxides (MDA), antioxidant enzymes; superoxide dismutase (SOD) and catalase (CAT), inflammatory makers; nuclear factor kappa b (NF- $\kappa$ B), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ) and mRNA expression of NOX-1 and NOX-4.

#### 2.3.1. Assessment of hepatotoxicity indices

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined according to the method of Reitman and Frankel (11). Serum levels of total bilirubin, total cholesterol (TC), triglycerides (TG) and albumin were estimated using available commercial kits (Spectrum diagnostics, Cairo, Egypt). Liver index was calculated according to the formula: (liver weight/body weight)  $\times$  100.

#### 2.3.2. Histopathological examination

Autopsy samples were fixed in 10% formalin for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4  $\mu$ m thickness. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain (12).

#### 2.3.3. Assessment of oxidative stress markers and antioxidant enzymes

GSH and MDA content were determined according to the methods of Ellman (13) and Mihara and Uchiyama (14), respectively. The activities of SOD and CAT were determined using the methods of Nishikimi, Appaji Rao (15) and Aebi (16), respectively.

### 2.3.4. Assessment of inflammatory markers and insulin

Determination of IL-6, TNF- $\alpha$ , NF- $\kappa$ B and insulin levels were performed using commercial rat ELISA kits (Immuno-Biological Laboratories, Minnesota, USA), (RayBiotech, Inc., Norcross, Georgia, USA), (Cloud-Clone Corp., Texas, USA) and (Société de Pharmacologie et d'Immunologie-BIO, Montigny-le-Bretonneux, France), respectively, according to the manufacturer's instructions.

### 2.3.5. Assessment of liver fibrosis

Liver fibrosis was evaluated using Masson's trichrome stain and by measuring the hydroxyproline content according to the method of Reddy and Enwemeka (17). Liver content of  $\alpha$ -SMA and TGF- $\beta$ 1 were examined immunohistochemically with the following primary antibodies; mouse monoclonal to rat  $\alpha$ -SMA (A2547, Sigma-Aldrich Chemical Co., St Louis, MO, USA) and mouse monoclonal to rat TGF- $\beta$ 1 (T0438, Sigma-Aldrich Chemical Co., St Louis, MO, USA). The images were then quantified by using image analysis software (Image J, 1.46a, NIH, USA), and represented as the area percentage of the immunopositive reaction per field ( $\times 400$ ).

### 2.3.6. Assessment of NOX-1 and NOX-4 mRNA expression

NOX-1 and NOX-4 mRNA expression were estimated by quantitative real-time polymerase chain reaction (qRT-PCR). For RNA extraction, total RNA from liver tissue was extracted using the QIAzol and RNeasy mini kit (QIAGEN, California, USA), as recommended by the manufacturer. RNA samples were then reverse transcribed and processed for PCRs. The primers of NOX-1, NOX-4 and the internal control  $\beta$ -actin were designed and synthesized by Invitrogen, USA. qRT-PCR was performed using the Applied Biosystems 7500 RT-PCR system. PCR samples were activated at 94°C for 10 min followed 35 cycles that were performed at

94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The mRNA expression of NOX-1 and NOX-4 was calculated based on the method of  $2^{-\Delta\Delta C_t}$ , where  $C_t$  is cycle threshold. The primers were as follows: NOX-1 forward: 5'AACAACAGCACTACCAATG3', reverse: 5'TCAAGAAGGAAGCAAAGGG3', NOX-4 forward: 5'TCAACTGCAGCCTGATCCTTT3', reverse: 5'CTGTGATCCGCGAAGGTAAG3' and  $\beta$ -actin forward: 5'CCCAGCACAAATGAAGATCAAGATCAT3', reverse: 5'ATCTGCTGGAAGGTGGACAGCGA3'.

### 2.4. Statistical analysis

Data are presented as mean  $\pm$  S.D. Multiple comparisons were performed using one-way ANOVA followed by Tukey-Kramer as a post-hoc test, as appropriate. The 0.05 level of probability was used as the criterion for significance. All statistical analyses and graphs were performed using GraphPad Prism (ISI<sup>®</sup> software, USA) version 5 software

## 3. Results

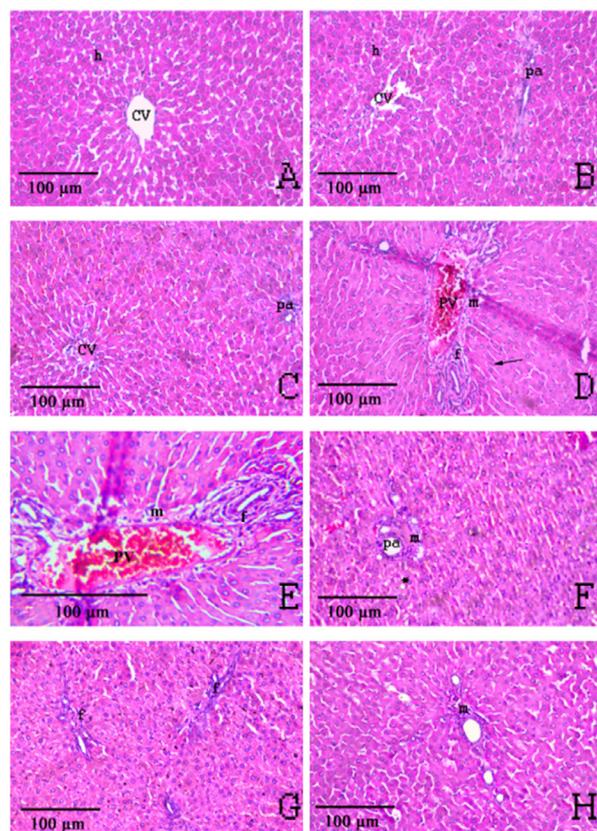
### 3.1. Hepatotoxicity indices

ConA intoxication significantly increased the levels of ALT and AST by 161 and 140%, respectively, when compared to the control group. In contrast, co-treatment with both apocynin and  $\alpha$ -LA significantly lowered the levels of ALT and AST by 59 and 52%, respectively, when compared to the ConA group. Also, ConA induced a significant decrease in albumin level by 34% and a significant increase in the levels of total bilirubin, TC, TG as well as the liver index by 269, 88 and 174 and 38%, respectively, as compared to the control group. Interestingly, the combination group showed a more pronounced increase in albumin level by 44% and a significant decrease in the serum levels of total bilirubin, TC, TG as well as the liver index accounting for 68, 43 and 56 and 27%, respectively, as compared to the ConA group (Table 1).

**Table 1. Effects of treatment with 50 mg/kg apocynin and/or 50 mg/kg  $\alpha$ -LA on liver function tests in ConA-induced liver injury in rats**

Treated group	Liver index (%)	ALT (IU/L)	AST (IU/L)	Total bilirubin (mg/dL)	Albumin (g/dL)	TC (mg/dL)	TG (mg/dl)
Control	3.6 <sup>b</sup> $\pm$ 0.29	27.4 <sup>b</sup> $\pm$ 5.7	107.7 <sup>b</sup> $\pm$ 17.5	0.22 <sup>b</sup> $\pm$ 0.06	4.33 <sup>b</sup> $\pm$ 0.63	57.2 <sup>b</sup> $\pm$ 8.70	37.4 <sup>b</sup> $\pm$ 14.3
Apocynin	3.4 <sup>b</sup> $\pm$ 0.13	24.6 <sup>b</sup> $\pm$ 6.5	104.2 <sup>b</sup> $\pm$ 11.1	0.20 <sup>b</sup> $\pm$ 0.06	4.33 <sup>b</sup> $\pm$ 0.50	61.7 <sup>b</sup> $\pm$ 21.3	22.8 <sup>b</sup> $\pm$ 7.20
$\alpha$ -LA	3.7 <sup>b</sup> $\pm$ 0.46	29.7 <sup>b</sup> $\pm$ 4.1	108.7 <sup>b</sup> $\pm$ 35.0	0.22 <sup>b</sup> $\pm$ 0.05	4.18 <sup>b</sup> $\pm$ 0.33	81.1 $\pm$ 9.60	24.7 <sup>b</sup> $\pm$ 6.10
ConA	4.9 <sup>a,c</sup> $\pm$ 0.29	71.4 <sup>a,c</sup> $\pm$ 4.9	258.7 <sup>a,c</sup> $\pm$ 60.1	0.83 <sup>a,c</sup> $\pm$ 0.15	2.85 <sup>a,c</sup> $\pm$ 0.56	107.7 <sup>a,c</sup> $\pm$ 25.7	102.7 <sup>a,c</sup> $\pm$ 23.5
ConA + Apocynin	4 <sup>b</sup> $\pm$ 0.17	35.4 <sup>b</sup> $\pm$ 11.2	159.2 <sup>b</sup> $\pm$ 23.9	0.40 <sup>b</sup> $\pm$ 0.19	3.98 <sup>b</sup> $\pm$ 1.13	62.8 <sup>b</sup> $\pm$ 8.70	37.1 <sup>b</sup> $\pm$ 14.2
ConA + $\alpha$ -LA	4.2 <sup>a,b,c</sup> $\pm$ 0.33	39.5 <sup>b</sup> $\pm$ 8.0	164.7 <sup>b</sup> $\pm$ 44.1	0.39 <sup>b</sup> $\pm$ 0.15	3.89 $\pm$ 0.33	96.8 <sup>a,c</sup> $\pm$ 19.5	44.6 <sup>b</sup> $\pm$ 4.20
ConA + Apocynin + $\alpha$ -LA	3.6 <sup>b</sup> $\pm$ 0.23	29.5 <sup>b</sup> $\pm$ 7.3	124.4 <sup>b</sup> $\pm$ 36.5	0.26 <sup>b</sup> $\pm$ 0.07	4.11 <sup>b</sup> $\pm$ 0.29	61.1 <sup>b</sup> $\pm$ 7.50	45.6 <sup>b</sup> $\pm$ 12.8

Data are presented as means  $\pm$  S.D. ( $n = 15$ ). <sup>a</sup>, <sup>b</sup> and <sup>c</sup>: Significantly different from control, ConA and ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test. AST, aspartate aminotransferase; ALT, alanine aminotransferase; TC, total cholesterol; TG, triglycerides.



**Figure 1. Representative photomicrographs of liver sections stained with haematoxylin and eosin (×400).** A, B and C: Sections taken from the livers of rats in control, apocynin or  $\alpha$ -LA, respectively, showing normal histological structure of the central vein (CV) and surrounding hepatocytes (h). D: Section taken from a liver of a rat intoxicated with ConA shows severe dilatation and congestion of the portal vein (PV) with fibrosis (f) and inflammatory cells infiltration (m) in the portal area surrounding the bile duct and diffused kupffer cells proliferation (arrow) in between the hepatocytes. E: Magnification at (×640) of the section of the liver shown in D. F: Section taken from the liver of a rat pretreated with apocynin showing few inflammatory cells infiltration (m) in the portal area (pa) with fatty changes (\*) in few hepatocytes. G: Section taken from a liver of a rat pretreated with  $\alpha$ -LA shows few fibroblastic cells (f) proliferation in the portal area. H: Section taken from a liver of a rat co-treated with both apocynin and  $\alpha$ -LA showing few inflammatory cells infiltration (m) in the portal area surrounding the bile duct.

### 3.2. Histopathological examination

Liver sections from the control, apocynin or  $\alpha$ -LA groups showed normal hepatic architecture (Figures 1A, 1B and 1C). Chronic intoxication with ConA induced fibroblastic cells proliferation with infiltrated inflammatory and kupffer cells in the portal area (Figures 1D and 1E). However, liver specimens from rats treated with ConA and either apocynin or  $\alpha$ -LA showed improvements in the histopathological changes (Figures 1F and 1G). Interestingly, co-treatment with both apocynin and  $\alpha$ -LA preserved the normal architecture of hepatic parenchyma (Figure 1H).

### 3.3. Oxidative stress markers and antioxidant enzymes

As expected, ConA-intoxicated rats showed significantly reduced content of GSH by 84% and elevation of MDA by 148%, as compared to the control group. Co-treatment of animals with both apocynin and  $\alpha$ -LA produced further improvement in oxidative stress markers than any of them alone, where GSH content was significantly increased by 226% and MDA level was reduced by 38% compared to ConA group (Table 2). In addition, ConA-intoxicated group showed a significant decrease in SOD and CAT activities by 85%, as compared to the control group. Remarkably, the combination of the two drugs resulted in a significant increase in the activity of SOD by 38% with respect to the apocynin only-treated group. Furthermore, this group showed a significant increase in the activities of SOD and CAT accounting to 82 and 64%, respectively, with respect to the  $\alpha$ -LA only-treated group (Table 2).

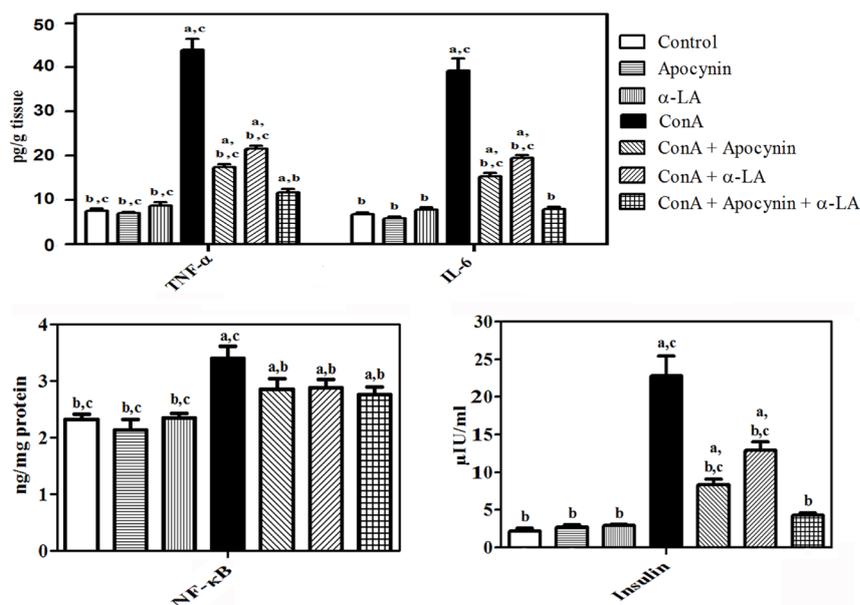
### 3.4. Inflammatory markers

It was found that the group injected with ConA showed about 5-fold increase in TNF- $\alpha$  and IL-6, as compared to the control group. In contrast, co-treatment with both apocynin and  $\alpha$ -LA revealed significantly lowered expression of TNF- $\alpha$  and IL-6 by 73 and 80%,

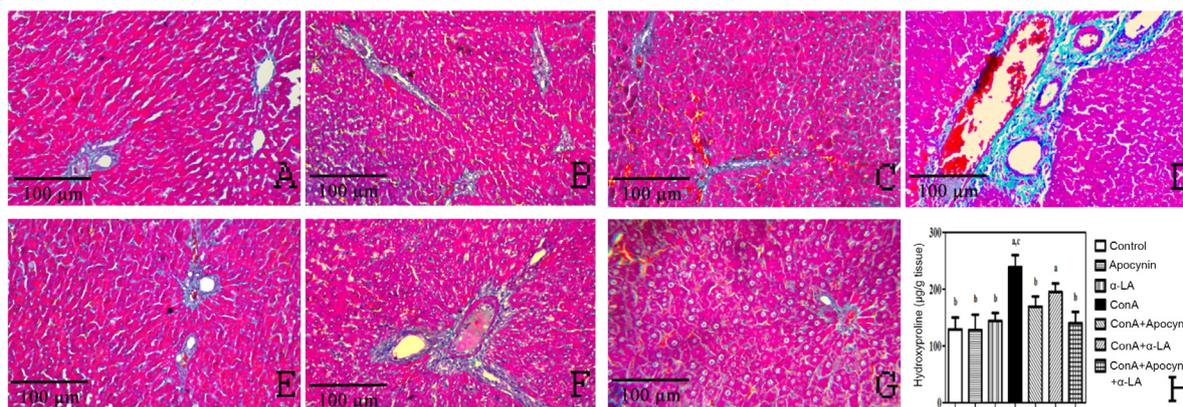
**Table 2. Effects of treatment with 50 mg/kg apocynin and/or 50 mg/kg  $\alpha$ -LA on oxidative stress markers and antioxidant enzymes in ConA-induced liver injury in rats**

Treated group	GSH ( $\mu$ mol/g tissue)	MDA (nmol/g tissue)	SOD (U/g tissue)	CAT (U/g tissue)
Control	4.0 <sup>b,c</sup> $\pm$ 0.2	54.6 <sup>b,c</sup> $\pm$ 11.3	13.2 <sup>b,c</sup> $\pm$ 0.5	13.5 <sup>b,c</sup> $\pm$ 0.6
Apocynin	3.8 <sup>b,c</sup> $\pm$ 0.2	55.3 <sup>b,c</sup> $\pm$ 9.3	12.2 <sup>b,c</sup> $\pm$ 0.8	12.7 <sup>b,c</sup> $\pm$ 1.2
$\alpha$ -LA	3.3 <sup>a,b,c</sup> $\pm$ 0.2	62.8 <sup>b,c</sup> $\pm$ 4.5	9.9 <sup>a,b,c</sup> $\pm$ 0.7	11.3 <sup>b,c</sup> $\pm$ 1.7
ConA	0.6 <sup>a,c</sup> $\pm$ 0.1	135.3 <sup>a,c</sup> $\pm$ 15.8	2.0 <sup>a,c</sup> $\pm$ 0.2	1.9 <sup>a,c</sup> $\pm$ 0.1
ConA + Apocynin	1.5 <sup>a,b,c</sup> $\pm$ 0.1	102.8 <sup>a,b</sup> $\pm$ 12.1	4.2 <sup>a,b,c</sup> $\pm$ 0.3	5.2 <sup>a,b</sup> $\pm$ 0.4
ConA + $\alpha$ -LA	1.2 <sup>a,b,c</sup> $\pm$ 0.1	120.1 <sup>a,c</sup> $\pm$ 14.7	3.1 <sup>a,c</sup> $\pm$ 0.2	4.1 <sup>a,c</sup> $\pm$ 0.2
ConA + Apocynin + $\alpha$ -LA	2.1 <sup>a,b</sup> $\pm$ 0.1	84.2 <sup>a,b</sup> $\pm$ 8.5	5.8 <sup>a,b</sup> $\pm$ 0.4	6.7 <sup>a,b</sup> $\pm$ 0.4

Data are presented as means  $\pm$  S.D. ( $n = 15$ ). a, b and c: Significantly different from control, ConA and ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test. GSH, reduced glutathione; MDA, lipid peroxides; SOD, superoxide dismutase; CAT, catalase.



**Figure 2.** Effect of treatment with 50 mg/kg apocynin and/or 50 mg/kg  $\alpha$ -LA on TNF- $\alpha$ , IL-6 and NF- $\kappa$ B tissue levels as well as serum insulin level in ConA-induced liver fibrosis in rats. Values are given as mean  $\pm$  S.D. TNF- $\alpha$ , IL-6 and insulin ( $n = 15$ ), NF- $\kappa$ B ( $n = 6$ ). a, b or c: Significantly different from the control, ConA or ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test. TNF- $\alpha$ , tumor necrosis factor-alpha; IL-6, interleukin-6; NF- $\kappa$ B, nuclear factor kappa b.



**Figure 3.** Representative photomicrographs of liver sections stained by Masson's trichrome ( $\times 400$ ). A, B and C: Sections obtained from livers of rats in control, apocynin or  $\alpha$ -LA, respectively, showing normal histological structure in the portal area with minimal collagen deposition. D: Section taken from a liver of a rat intoxicated with ConA showing severe congestion in the portal vein and excessive collagen fibers deposition along with pseudolobules formation in the portal area. E: Section taken from the liver of a rat pretreated with apocynin showing nearly an absence of pseudolobules with less collagen deposition. F: Section taken from a liver of a rat pretreated with  $\alpha$ -LA shows few fibroblastic cells proliferation in the portal area along with some collagen fibers deposition. G: Section taken from a liver of rats co-treated with both apocynin and  $\alpha$ -LA showing an absence of pseudolobules and minimal collagen fibers deposition. H: Liver hydroxyproline content in different groups. Values are given as mean  $\pm$  S.D. ( $n = 6$ ). a, b or c: Significantly different from the control, ConA or ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test.

respectively, as compared to the ConA group, which was more significant than that found in either apocynin or  $\alpha$ -LA only-pretreated groups (Figure 2). Moreover, ConA-intoxicated group showed a significant increase in NF- $\kappa$ B by 146%, as compared to the control group. Remarkably, co-treatment with apocynin and  $\alpha$ -LA revealed a significant decrease in NF- $\kappa$ B by 19% with respect to ConA group (Figure 2).

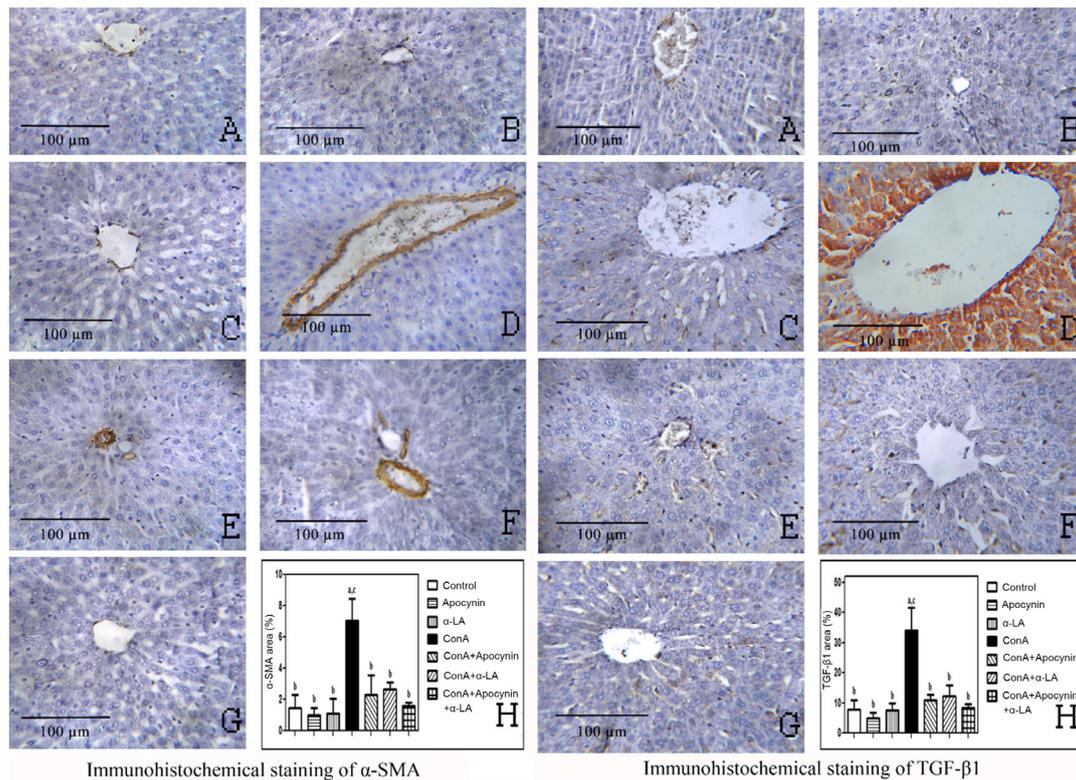
### 3.5. Insulin level

ConA injection showed a significant increase in serum insulin level by 9.5-fold, when compared to the control

rats. Considering co-treatment with both apocynin and  $\alpha$ -LA, serum insulin level was reduced as compared to ConA-intoxicated group (by 81%) or the groups treated with either apocynin (by 49%) or  $\alpha$ -LA (by 67%) (Figure 2).

### 3.6. Liver fibrosis markers

Masson's trichrome stain showed that collagen fibers were not demarcated around the classical hepatic lobules in liver sections from the control, apocynin or  $\alpha$ -LA only-treated groups (Figures 3A, 3B, and 3C). In contrast, the collagen fibers were heavily deposited in sections



**Figure 4. Expression of  $\alpha$ -SMA and TGF- $\beta$ 1 antigens by immunohistochemical staining ( $\times 400$ ).** A, B and C: Sections of livers obtained from rats in control, apocynin or  $\alpha$ -LA, respectively, shows that  $\alpha$ -SMA and TGF- $\beta$ 1 antigens were minimally detected in the walls of the central vein. D: Sections taken from livers of rats intoxicated with ConA shows extensive  $\alpha$ -SMA and TGF- $\beta$ 1 expression (brown color). E: Sections taken from livers of rats pretreated with apocynin showing mild  $\alpha$ -SMA and TGF- $\beta$ 1 expression. F: Sections taken from livers of rats pretreated with  $\alpha$ -LA shows moderate expression of  $\alpha$ -SMA and mild expression of TGF- $\beta$ 1. G: Sections taken from livers of rats co-treated with both apocynin and  $\alpha$ -LA showing limited  $\alpha$ -SMA and TGF- $\beta$ 1 expression (brown color). H: Quantitative image analysis expressed as percentage of area of immunopositive reaction. Values are given as mean  $\pm$  S.D. ( $n = 10$ ). a, b or c: Significantly different from the control, ConA or ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test.  $\alpha$ -SMA, alpha-smooth muscle actin; TGF- $\beta$ 1, transforming growth factor-beta1.

taken from ConA-intoxicated group (Figure 3D), while  $\alpha$ -LA pretreated group showed moderate collagen fibers deposition (Figure 3F). Remarkably, pretreatment with either apocynin alone or along with  $\alpha$ -LA markedly counteracted these changes (Figures 3E and 3G). As expected, ConA-intoxicated rats showed significantly increased hydroxyproline content by 185%, as compared to the control group. Co-treatment of animals with both apocynin and  $\alpha$ -LA revealed reduction in hydroxyproline content by 41%, compared to ConA group (Figure 3H).

Immunohistochemical staining revealed minimal  $\alpha$ -SMA and TGF- $\beta$ 1 expression in the liver sections from the control, apocynin or  $\alpha$ -LA only-treated rats (Figures 4A, 4B, and 4C). However, ConA-intoxicated group showed significantly raised expression of  $\alpha$ -SMA and TGF- $\beta$ 1 by about 4 and 3-fold, respectively, as compared to the control (Figure 4D). Compared with ConA intoxicated group, liver sections of rats pretreated with apocynin alone,  $\alpha$ -LA alone or both of them showed a marked reduction in  $\alpha$ -SMA expression by about 68, 62 and 77%, respectively, as well as a reduction in TGF- $\beta$ 1 expression by about 68 and 64 and 75%, respectively (Figures 4E, 4F, and 4G). Figure 4H represents the percentage of area of immunopositive reaction.

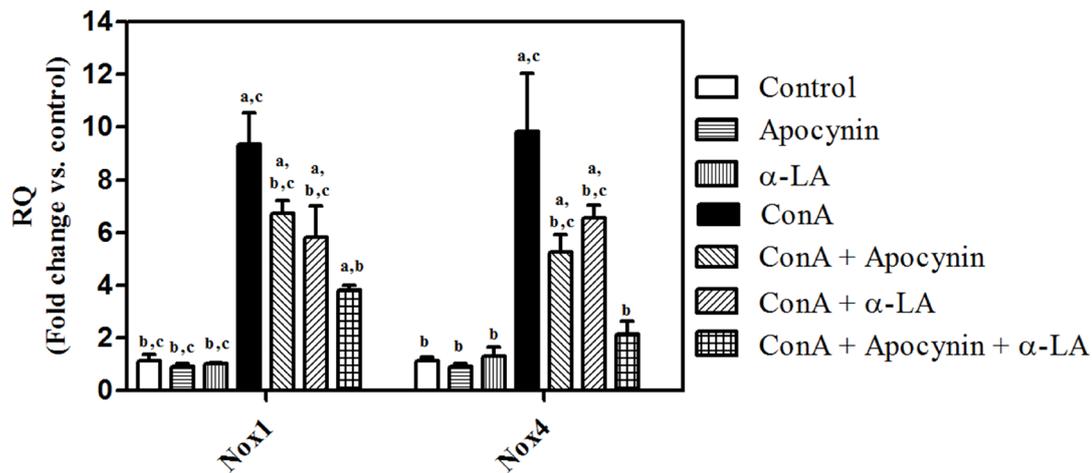
### 3.7. NOX-1 and NOX-4 gene expression

NOX-1 and NOX-4 mRNA tissue expression showed about 7.5 and 8-fold increase, respectively, in ConA-injected group, as compared to the control. While in the combination group, NOX-1 and NOX-4 mRNA tissue expression were significantly lowered by 59 and 78%, respectively, when compared to the group intoxicated with ConA, which was also more significant than either apocynin or  $\alpha$ -LA only-pretreated groups (Figure 5).

## 4. Discussion

The ConA model is a typical and well established one for investigating T-cell and macrophage dependent liver injury in rodents, which closely resembles the pathogenesis mechanisms of viral and autoimmune hepatitis in humans. ConA is purified from *Canavalia brasiliensis*, after its *i.v.* injection, hepatic CD4<sup>+</sup> T-cells recognize the ConA-modified major histocompatibility complex structures of kupffer cells and become activated, followed by the release of inflammatory mediators in the blood (18).

Injection of ConA for six consecutive weeks was



**Figure 5.** Effect of treatment with 50 mg/kg apocynin and/or 50 mg/kg  $\alpha$ -LA on NOX-1 and NOX-4 mRNA expression in ConA-induced liver fibrosis in rats. Values are given as mean  $\pm$  S.D. ( $n = 15$ ). a, b or c: Significantly different from the control, ConA or ConA + apocynin +  $\alpha$ -LA group, respectively, at  $p < 0.05$  using ANOVA followed by Tukey-Kramer as a post-hoc test. NOX-1, NADPH oxidase-1; NOX-4, NADPH oxidase-4.

found to significantly increase serum ALT and AST levels that is attributed to increased enzymes release from damaged liver parenchymal cells into the blood stream (19). Meanwhile, serum levels of TC, TG and total bilirubin were significantly increased in ConA-intoxicated group, while albumin level was significantly reduced. Pretreatment of apocynin along with ConA significantly ameliorated these changes. In this context, the hepatoprotective effects of apocynin in a rat model of diet-induced hypercholesterolaemia were reported by a previous study (8). Interestingly, the group co-treated with both apocynin and  $\alpha$ -LA showed a more pronounced hepatoprotective effect as compared to that treated with apocynin alone. Beside hepatotoxicity indices; histopathological examination revealed severe inflammatory cells infiltration as well as severe fibrosis in the portal area induced by ConA which is in accordance with previous studies (20,21). Remarkably, the combination of apocynin and  $\alpha$ -LA preserved normal liver tissue architecture with few inflammatory cells infiltration.

Liver fibrosis is characterized by both quantitative and qualitative alteration of hepatic ECM, as a consequence of HSCs activation towards myofibroblast-like cells. This is characterized by increased liver content of  $\alpha$ -SMA as a marker for activated HSCs, and accumulation of ECM mainly collagen, which is stimulated by the multifactorial growth factor TGF- $\beta$  (22). In the present study, histopathological examination of collagen fibers using Masson's trichrome stain revealed severe fibroblastic cells proliferation in liver samples of ConA intoxicated group as well as elevated hydroxyproline content. It is known that hydroxyproline is the main characteristic compound in collagen that indicates increased *de novo* synthesis of liver collagen (23). Nil fibroblastic cells proliferation was obvious in the liver tissues isolated from rats pretreated with either

apocynin alone or together with  $\alpha$ -LA. These findings were further confirmed by the decreased content of hydroxyproline within the same groups.

Furthermore, the distribution of  $\alpha$ -SMA and TGF- $\beta$ 1 positive hepatic cells were significantly upregulated in the ConA-injected group. Moreover,  $\alpha$ -SMA has been directly related to experimental liver fibrogenesis (24). Also, the ongoing inflammation in the liver is associated with the formation of the profibrogenic cytokine TGF- $\beta$ 1 (25) that has been shown to regulate multiple fundamental cellular processes, including cell growth, migration, adhesion, ECM deposition and apoptosis (26,27). The increased  $\alpha$ -SMA and TGF- $\beta$ 1 expression was counteracted by pretreatment with either apocynin or  $\alpha$ -LA, however, co-treatment with apocynin and  $\alpha$ -LA showed a further significant reduction of  $\alpha$ -SMA and TGF- $\beta$ 1 expression. Indeed,  $\alpha$ -LA reduced them in experimental models of hepatic fibrosis in rodents induced by and dimethylnitrosamine (28) and bile duct ligation (29).

The next step was to explore the mechanism underlying the hepatoprotective and antifibrotic effects of apocynin and  $\alpha$ -LA in combination. More evidence links oxidative stress involvement in ConA-induced liver injury (30,31), where SOD, GSH and CAT protects against the deleterious effects of ROS (32,33), while MDA is used as an indicator of cellular oxidation status (34). In the present study, ConA significantly lowered the liver tissue content of GSH and activities of SOD and CAT while it induced a significant increase in liver MDA content. These results are in accordance with previous studies (22,35). Apocynin pretreatment significantly counteracted ConA-induced oxidative stress in accordance with earlier studies performed in the heart and vascular tissue of rats (36,37). Paradoxically, pretreatment with  $\alpha$ -LA significantly increased only the content of GSH. Meanwhile, co-

treatment with apocynin and  $\alpha$ -LA along with ConA proved to be the most effective in significantly counteracting the changes occurring in the assessed oxidative stress markers.

T-cell activation elicited by ConA resulted in the elevation of the cytokines TNF- $\alpha$  and IL-6, which play critical roles in the development of ConA-induced hepatic injury (38,39). Furthermore, TNF- $\alpha$  stimulates the release of cytokines and promotes oxidative stress causing liver damage (40). Indeed, NF- $\kappa$ B is crucial during hepatic fibrogenesis through regulating hepatocyte injury, inflammatory signals and fibrogenic responses in HSCs (41). NF- $\kappa$ B activation in response to liver injury results in production and secretion of proinflammatory cytokines such as TNF- $\alpha$  and IL-6 (42). Pretreatment with apocynin succeeded in significantly decreasing the serum levels of TNF- $\alpha$  and IL-6 induced by ConA intoxication. The anti-inflammatory activity of apocynin was supported by previous studies conducted on rodents (43,44). Also,  $\alpha$ -LA pretreatment with ConA produced a significant reduction of TNF- $\alpha$  and IL-6, which coincides with previous studies on different models of liver injury in rats (45,46). Once again concomitant treatment with apocynin and  $\alpha$ -LA led to a further significant decrease in the serum levels of TNF- $\alpha$  and IL-6.

In addition, Fartoux *et al.* (47) demonstrated that insulin resistance and increased circulating insulin is a cause rather than a consequence of liver fibrosis. Moreover, antioxidants can alleviate insulin resistance through ROS-scavenging activity (48). Further, Sukumar, Viswambharan (49) identified a NOX enzyme as a contributor in insulin resistance-mediated oxidative stress and postulated that its pharmacological inhibition may possess a novel therapeutic target in insulin resistance-related diseases. In this context, rats injected with ConA showed a significant serum hyperinsulinemia in accordance with Francisco-DoPrado, Zambelli (50). Pretreatment with either apocynin or  $\alpha$ -LA significantly counteracted the effect of ConA on serum insulin. Earlier studies have reported the effect of apocynin on insulin in a rat model (48). Interestingly, co-treatment with both apocynin and  $\alpha$ -LA almost restored the normal level of insulin being significantly lower than the groups treated with ConA only or along with apocynin or  $\alpha$ -LA.

Finally, we tried to explore the possible role of NOXs in ConA-induced liver hepatitis. It was found that, a significant elevation of liver NOX-1 and NOX-4 gene expression after ConA administration was evident. Thus, NOX enzymes could play a role in ConA induced liver injury. TGF- $\beta$  in fetal rat hepatocytes was shown to generate ROS through activation of NOX enzymes and down-regulation of antioxidant genes (51). This was found to be coincident with the increase in Rac-1 protein level, a well-known activator of NOX-1 (52). As a NOX inhibitor, apocynin pretreatment resulted in

a significant reduction of NOX-1 and NOX-4 mRNA expression. This protective effect of apocynin coincides with a previous experiment conducted on rat hepatoma cells (53). Also, pretreatment with  $\alpha$ -LA reduced NOX-1 and NOX-4 expression, which was in agreement with a previous study where  $\alpha$ -LA inhibited the activation of NOX and thus, reduced ROS production in *H. pylori*-infected gastric adenocarcinoma AGS cells (54). The combining treatment of apocynin and  $\alpha$ -LA along with ConA in the present study significantly reduced NOX-1 and NOX-4 expression compared to any of the singly-pretreated groups.

Collectively, this study highlights the crucial role that NOX enzymes play in the pathogenesis of ConA-induced liver fibrosis. It was also shown that using apocynin and  $\alpha$ -LA in combination possess a marked antifibrotic effect. This was attributed to the reduction of NOX enzymes in liver tissue, therefore, limiting the production of free radicals, inflammation, insulin and subsequent chronic hepatic fibrosis.

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