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Therapeutic potential of morin against liver fibrosis in rats: Modulation of oxidative stress, cytokine production and nuclear factor kappa B

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ABSTRACT

Therapeutic potential of morin, a member of flavonoid family, against carbon tetrachloride (CCl_4)-induced liver fibrosis in rats was investigated and compared with that of silymarin. Results show that treatment with morin (30 mg/kg/day) revealed attenuation in liver index and serum biomarkers of liver function that were enhanced by chronic CCl_4 intoxication. Further, morin inhibited the elevated levels of malondialdehyde and nitric oxide and restored hepatic reduced glutathione to its normal level. The increased production of hepatic hydroxyproline content by CCl_4 was markedly decreased by administration of morin. In addition, treatment with morin significantly attenuated the inflammatory responses caused by CCl_4 as evident by the decreased hepatic tumor necrosis factor-alpha (TNF-α) level, immunohistochemical expressions of inducible nitric oxide synthase and nuclear factor kappa B. Collectively, this study indicates that morin possesses antifibrotic effect in the CCl_4 model of fibrosis via reducing oxidative stress, inflammatory responses and fibrogenic markers.

1. Introduction

Liver fibrosis, a common pathological process of chronic hepatic diseases, can lead to irreversible cirrhosis, and involves multiple cellular and molecular events that ultimately result in accumulation of collagen and extra cellular matrix protein (Pinzani and Rombouts, 2004). It was reported that fibrosis is a dynamic process and may be reversible prior to the establishment of advanced architectural changes to the liver (Thompson and Patel, 2010). Several lines of evidence suggest that oxidative stress plays an important role in the pathogenesis of liver fibrosis (Parola and Robino, 2001; Steib et al., 2007). Moreover, collective evidences indicated that liver fibrosis incorporates uncontrolled inflammation as a part of its etiology. Kupffer cells which act as resident macrophages in the liver represent the primary inflammatory effectors which initiate the inflammatory cascade leading to tissue remodeling and fibrosis (Steib et al., 2007).

Nuclear factor kappa B (NF-κB) is an inducible transcription factor that exists in the cytoplasm in an inactive form and is associated with regulatory proteins called IkB. After stimulation, it is translocated to the nuclei and bound to decameric DNA sequences, and activates transcription of target genes (O'Dea et al., 2007). Carbon tetrachloride (CCl_4)-induced oxidative stress can stimulate the phosphorylated effect of IκB,

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and degradation of IκB leads to the translocation of NF-κB to the nucleus (Orfila et al., 2005). This process further regulates the expression of inducible inflammatory mediators such as tumor necrosis factor-alpha (TNF-α), nitric oxide (NO), and interleukin-1β (IL-1β) (Racanelli and Rehermann, 2006). Consequently, prolonged activation of NF-κB results in the perpetuation of inflammatory responses (Luedde and Schwabe, 2011). In vivo studies using rodent models of liver diseases and cell-targeted modulation of NF-κB activity have revealed complex and multicellular functions in hepatic inflammation, fibrosis, and the development of hepatocellular carcinoma – a process termed as "inflammation–fibrosis–cancer axis". Hence, NF-κB is regarded as potential target for the treatment of inflammatory-induced diseases including hepatic damage (Luedde and Schwabe, 2011).

Natural products and their active principles, as sources for new drug discovery and treatment of diseases, have attracted attention in recent years. Many natural products have been clinically available as potent hepatoprotective agents against commonly occurring liver diseases (Zhang et al., 2013). Morin (2′,3,4′,5,7-pentahydroxyflavone) is a member of the flavonoid family which consists of a yellowish pigment found in many herbs and fruits. It has been shown that morin acts as a potent antioxidant and has metal ion chelating capacities. It is worth noting that morin possesses various biological and biochemical effects including anti-oxidant, anti-inflammatory (Zhang et al., 2011), cardioprotective (Al-Numair et al., 2012), and anticancer activities (Sivaramakrishnan and Devaraj, 2010). In addition, it was reported that morin could modulate the activities of the metabolic enzymes, including cytochrome P450, and protect various human cells, like myocytes, endothelial cells, hepatocytes and erythrocytes against oxidative damages (Kitagawa et al., 2004). Previous studies indicated that morin might be beneficial in ameliorating alcohol-induced oxidative damage and CCl_4-induced acute hepatotoxicity in rats (Lee et al., 2008; Shankari et al., 2010). Accordingly, the present study aims to investigate the therapeutic effect of morin against liver fibrosis induced by chronic administration of CCl_4 in rats and the possible protective mechanisms involved with the use of silymarin as a standard hepatoprotective drug.

2. Materials and methods

2.1. Drugs and chemicals

Morin, silymarin, CCl_4, hydroxyproline, chloramine-T, p-dimethylaminobenzaldehyde, bovine serum albumin and thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N-butanol, dipotassium hydrogen phosphate, potassium dihydrogen phosphate and trichloroacetic acid were purchased from El-Nasr Chemical Co. (Cairo, Egypt). All other chemicals and solvents were of highest grade commercially available.

2.2. Animals and experimental design

Adult male albino rats (180–200 g) were obtained from the animal house of Faculty of Agriculture, Minia University, El-Minia, Egypt. Animals were used after 2 weeks of acclimatization to the animal house conditions (12 h lighting cycle and 25 ± 2 °C temperature) and had free access to standard rodent chow and water. The experiments were conducted according to ethical guidelines of Minia University, Egypt. Rats were randomly divided into 5 groups with 8 animals each as follows:

(1) Control group: healthy rats were injected with the respective vehicle (1 ml/kg corn oil in saline, i.p.) twice weekly for 8 weeks.
(2) Control Morin group: healthy rats were injected with the respective vehicle (1 ml/kg corn oil in saline, i.p.) twice weekly and treated daily with morin (30 mg/kg, orally) for 8 weeks.
(3) CCl_4 group: This group was given CCl_4 (1 ml/kg, 1:1 mixture with corn oil, i.p.), twice weekly for 8 weeks to induce liver fibrosis.
(4) CCl_4 + morin group: This group was given both CCl_4 and daily morin treatment (30 mg/kg, orally) for 8 weeks. CCl_4 was injected 30 min after oral treatment with morin.
(5) CCl_4 + silymarin group: This group was given both CCl_4 and daily silymarin treatment (100 mg/kg, orally) for 8 weeks. CCl_4 was injected 30 min after oral treatment with silymarin.

The doses of the drugs were chosen according to previous studies (Subash and Subramanian, 2009; Yuvaraj and Subramoniam, 2009). At the end of 8 weeks, rats were anesthetized and blood samples were collected from the retro-orbital plexus and allowed to clot. Serum was separated by centrifugation at 1000 × *g* for 10 min and used for the assessment of liver function tests. Then, rats were sacrificed and liver tissues were dissected, washed with ice-cold saline, weighed and stored at −80 °C. Thereafter, liver tissues were homogenized in saline and the homogenate was used for assessment of oxidative stress markers [reduced glutathione (GSH), total NO and lipid peroxides], as well as a fibrotic marker, hydroxyproline content, hepatic TNF-α level and NF-κB mRNA expression with semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). In addition, specimens from liver tissues were fixed in 10% formalin for histopathological examination and immunohistochemical detection of NF-κB (p65) and inducible nitric oxide synthase (iNOS) expressions.

2.3. Assessment of liver index and serum biomarkers for liver function tests

Liver index was calculated according to the formula: (liver weight/body weight) × 100.

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) activities were measured using available commercial kits (Spectrum Diagnostics, Cairo, Egypt). Total serum bilirubin was determined spectrophotometrically using kits purchased from Randox Laboratory Ltd., UK.

Table 1 – Effects of morin and silymarin on liver index and serum biomarkers for liver function tests in rats subjected to chronic CCl_4 intoxication.

	Liver index (%)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	Total bilirubin (μmol/L)
Control	2.48 ± 0.32	20 ± 1.85	57 ± 10.5	71 ± 1.3	0.74 ± 0.06
Control morin	2.51 ± 0.38	21 ± 3.45	60 ± 9.4	73 ± 0.62	0.83 ± 0.32
CCl_4	3.56 ± 0.51[#]	39 ± 3.14[#]	147 ± 19.6[#]	173 ± 9.2[#]	2.45 ± 0.18[#]
CCl_4 + morin	2.87 ± 0.42[*]	24 ± 4.66[*]	103 ± 21.4[#,*,○]	89 ± 5.3[#,*,○]	1.33 ± 0.09[#,*,○]
CCl_4 + silymarin	2.65 ± 0.37[*]	23 ± 5.16[*]	81 ± 16.4[#,*]	75 ± 8.1[*]	0.92 ± 0.05[#,*]

Data represent the mean ± S.E.M. of observations from 8 rats.
[#] Significantly different from control group at $P < 0.05$.
[*] Significantly different from CCl_4 group at $P < 0.05$.
[○] Significantly different from CCl_4 + Silymarin group at $P < 0.05$.

2.4. Assessment of oxidative stress parameters

Hepatic lipid peroxidation was measured by the method of Ohkawa et al. (1979) using 2-thiobarbituric acid reacting substances and is expressed as malondialdehyde (MDA) nmol/g tissue using 1,1,3,3-tetramethoxypropane as standard. GSH was determined according to the method described earlier by Beutler et al. (1963). The procedure is based on the reduction of 2-nitrobenzoic acid by glutathione to produce a yellow compound which was measured spectrophotometrically at 405 nm. NO level was measured as total nitrite/nitrate, the stable degradation products of NO, by reduction of nitrate into nitrite using copperized cadmium, followed by color development with Griess reagent in acidic medium (Sastry et al., 2002).

2.5. Determination of hydroxyproline as a representative for liver tissue collagen

As an index of liver fibrosis, hydroxyproline was estimated in the liver using the method of Woessner (1961). Briefly, 0.5 ml of 20% liver homogenate was digested in 1 ml of 6 N HCl at 120 °C for 8 h. An aliquot of digested homogenate (25 μl) was added to 25 μl citrate–acetate buffer and finally 500 μl of chloramine T solution was added then the mixture was left at room temperature for 20 min. Then, 500 μl Ehrlich's solution was added and the mixture was incubated at 65 °C for 15 min. After cooling for 10 min, the color developed was measured spectrophotometrically at 550 nm. Results were expressed as μg/g of wet tissue.

2.6. Determination of TNF-α level

TNF-α was assayed using ELISA kit (Genzyme Diagnostics, Cambridge, MA, USA). Briefly, liver tissues were homogenized in PBS (pH 7.4) for 30 s. They were then centrifuged at 12,000 rpm for 20 min. 100 μl of the supernatant was added to the 96-well microtiter plate pre-coated with monoclonal TNF-α antibody (coupled to horse radish peroxidase) and incubated for 2 h at 37 °C. After thorough washing, the substrate solution was added. Color development was allowed for 10 min and the reaction was stopped by application of stop solution. Color absorbance was read in a microplate reader (Dynex Technologies, Chantilly, VA, USA) at 450 nm. Protein was determined using the Lowry method (Lowry et al., 1951).

2.7. Immunohistochemical detection of iNOS and NF-κB (p65) expressions

Paraffin embedded tissue sections of 4 μm thickness were rehydrated in xylene and then in graded ethanol solutions. The slides were then blocked with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) for 2 h. The sections were then immunostained with one of the following primary antibodies; rabbit polyclonal IgG to rat iNOS (Santacruz Biotechnology, Cat No. sc-651) and rabbit polyclonal IgG to rat NF-κB p65 (Santacruz Biotechnology, Cat No. sc-372), at a concentration of 1 μg/ml containing 5% BSA in TBS and incubated overnight at 4 °C. After washing the slides with TBS, the sections were incubated with goat anti-rabbit secondary antibody. Sections were then washed with TBS and incubated for 5–10 min in a solution of 0.02% diaminobenzidine containing 0.01% H_2O_2. Counter staining was performed using hematoxylin, and the slides were visualized under a light microscope.

2.8. RT-PCR analysis of NF-κB

Total RNA was extracted from liver samples using RNA extraction kit according to the manufacturer's instructions (Bio Basic Inc., Markham, ON, Canada). RNA (5 mg) was then transcribed using revert aidTM first strand cDNA synthesis kit (Ferments life science, Fort Collins, CO, USA). The cDNA products were amplified by PCR in a total volume of 50 μl containing 2.5 U Taq DNA polymerase and 10 pmol of the upstream and downstream primers for NF-κB (Forward: 5′-GTCATCAGGAAGAGGTTTGGCT-3′; Reverse: 5′-TGATAAGCTTAGCCCTTGCAGC-3′). As an internal control, we also estimated the expression of β-actin mRNA using the following primers sequences: Forward: 5′-TCACCCTGAAGTACC CCATGG AG-3′; Reverse: 5′-TTGGCCTTGGGG TTCAGGGGG-3′. The predicted sizes of the amplified NF-κB and β-actin DNA products were 286 bps and 374 bps, respectively. Amplified products (5 μl) were loaded onto 1.5% agarose gels previously stained with 0.5 μg/ml ethidium bromide, electrophoresed at 100 V for 30 min and then examined under a UVP gel imaging system (UVP Co., USA). Images were analyzed with the Gel-Pro Analyzer Version 3.0, and the semi-quantitative measure of mRNA expression was expressed as the ratio of the optical density (OD) of NF-κB to that of β-actin.

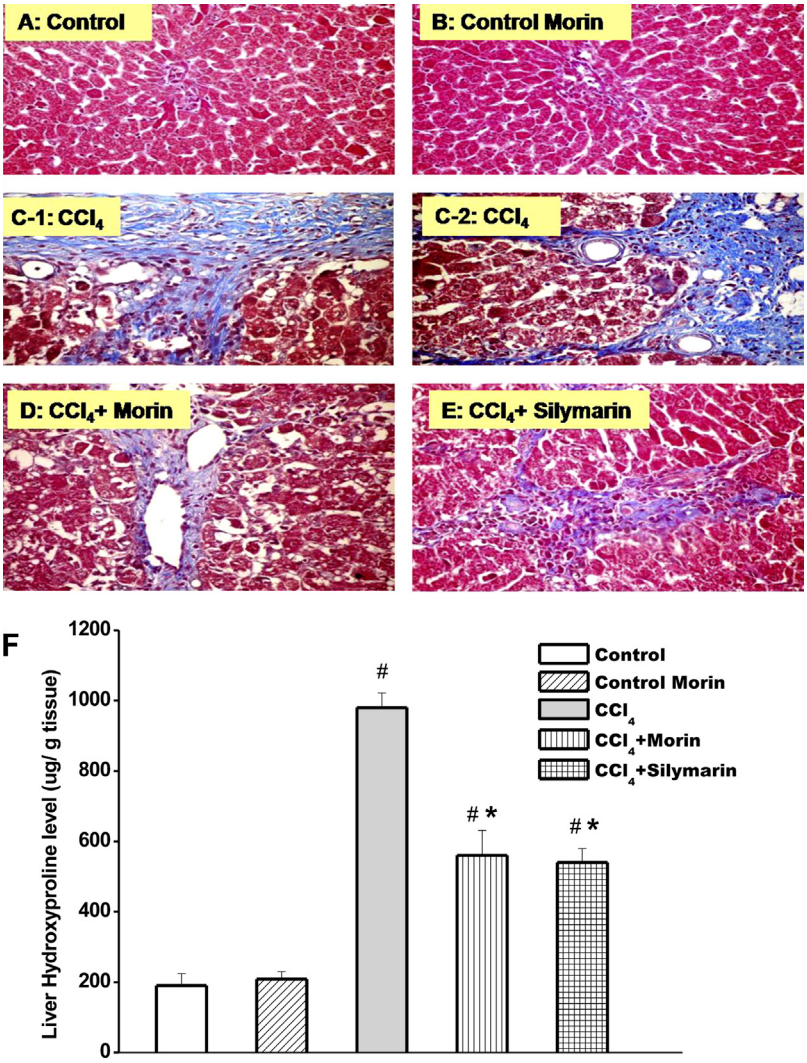

Fig. 1 – Representative photomicrographs of liver sections stained by Masson's trichrome (200×). (A) Section obtained from a liver of control rat and (B) section obtained from a liver of morin treated rat and show complete absence of collagen fibers, stained blue, between hepatic lobules. (C-1 and C-2) Sections obtained from liver of CCl_4-intoxicated rats show excessive collagen fibers deposition and pseudolobules formation (bridging fibrosis). (D and E) Sections obtained from liver of rats intoxicated with CCl_4 and treated concomitantly with morin and silymarin, respectively, show minimal deposition of collagen fibers and absence of pseudolobules. (F) Liver hydroxyproline content expressed as μg/g tissue. Data represent the mean ± S.E.M. of observations from 8 rats. # and *: Statistically significant from control and CCl_4 group, respectively, at $P < 0.05$. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

2.9. *Histopathological examination*

Liver specimens were fixed in 10% formalin and processed for paraffin sections of 4 μm thickness. Sections were stained with hematoxylin and eosin for routine histopathological examination and Masson's trichrome for demonstration of collagen fibers.

2.10. *Statistical analysis*

Data are presented as mean ± S.E.M. Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a post hoc test. The 0.05 level of probability was used as the criterion for significance. All analysis was made with the statistical software Microcal Origin (version 6, Microcal Software Inc., Northampton, USA).

3. Results

3.1. *Effect on liver index and biochemical markers of hepatic injury*

The relative liver weight significantly increased in the CCl_4-intoxicated group by 1.4-fold as compared to the control group. On the other hand, treatment with either morin or silymarin concurrently with CCl_4 significantly lowered the liver index similar to the control value. However, morin alone did not

modify the liver index when compared to the control group (Table 1). Serum levels of ALT, AST, ALP, and total bilirubin, were used as biochemical markers for evaluation of hepatic injury and were significantly elevated in the CCl_4-treated animals (Table 1). Treatment of CCl_4-intoxicated group with either morin or silymain significantly reduced the levels of serum biomarkers of liver function tests as compared to CCl_4 group. Nevertheless, treatment with morin alone did not show any significant changes in all markers when compared to the control group (Table 1).

3.2. *Effect on oxidative stress parameters*

CCl_4-induced oxidative stress in rat liver was evaluated by assessing lipid peroxides, NO and GSH levels. As shown in Table 2, CCl_4 significantly increased lipid peroxides and NO levels by 93% and 68%, respectively, and decreased GSH level by 51% as compared to the control group. Whereas, treatment of animals with either morin or silymain concomitantly with CCl_4 afforded significant protection against CCl_4-intoxication and maintained levels of both lipid peroxides and NO at near basal levels with significant increase in GSH level when compared to CCl_4-treated group. Furthermore, animals treated with morin alone did not show any significant alterations in oxidative stress markers as compared to the control group.

3.3. *Effect on collagen accumulation in Masson's trichromic stain and hydroxyproline content*

Liver fibrosis was biochemically evaluated by measuring hydroxyproline content as an index of collagen accumulation and histologically by visualizing the blue color of collagen fibers using Masson's trichromic stain. In sections stained with Masson's trichrome, the connective tissue septa, containing collagen fibers, were not demarcated around the classical hepatic lobules in normal control and control morin groups (Fig. 1A and B, respectively). In contrast, the collagen fibers were heavily deposited around portal tracts and central veins in CCl_4-intoxicated group and extended from central vein to portal tract resulting in formation of pseudolobules (Fig. 1C-1 and C-2). Interestingly, either morin or silymain when administered along with CCl_4 prevented the abnormal collagen deposition and induced a significant decrease in the hydroxyproline content as compared to the CCl_4-intoxicated group

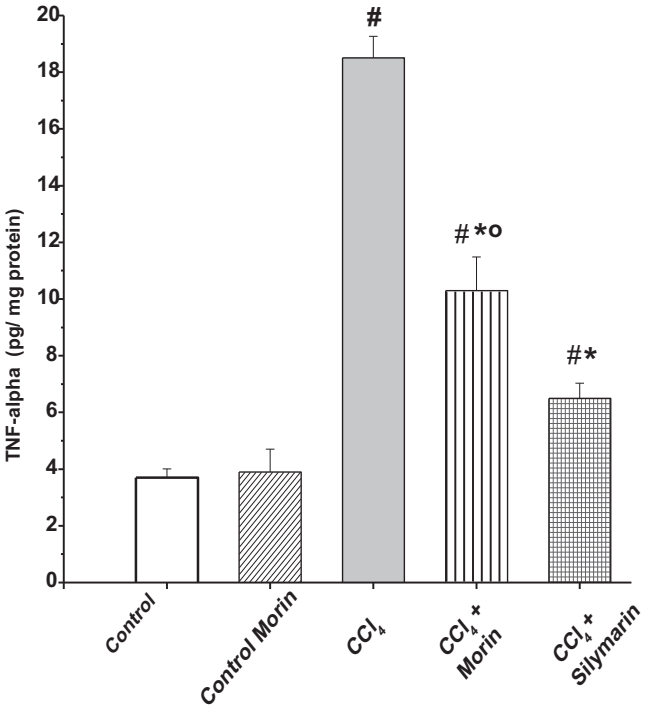

Fig. 2 – Effects of morin and silymarin on hepatic TNF-α level (pg/mg protein) in chronic CCl_4-treated rats. Data represent the mean ± S.E.M. of observations from 8 rats. #, * and °: Statistically significant from control, CCl_4 and CCl_4 + Silymarin groups, respectively, at $P < 0.05$.

(Fig. 1D and E). Biochemical estimation of hydroxyproline supported these results in which CCl_4 induced a significant increase in the liver hydroxyproline content reaching 5-folds as compared to the control group, while concomitant treatment with either morin or silymain reduced this elevation to a large extent (Fig. 1F).

3.4. *Effect on inflammatory and fibrogenic markers: TNF-α, iNOS and NF-κB (p65)*

CCl_4 showed pro-inflammatory responses as evidenced by significant increase in TNF-α level (Fig. 2) and expression of the pro-inflammatory enzyme, iNOS (Fig. 3C) in liver tissue

Table 2 – Effects of morin and silymarin on hepatic oxidative stress parameters in rats subjected to chronic CCl_4 intoxication.

	MDA (nmol/g wet tissue)	NO (nmol/g wet tissue)	GSH (μmol/g wet tissue)
Control	57.1 ± 7.61	147 ± 9.1	7.5 ± 0.61
Control morin	63.4 ± 8.64	151 ± 12.4	7.3 ± 1.34
CCl_4	110 ± 3.41[#]	248 ± 30.4[#]	4.1 ± 1.16[#]
CCl_4 + morin	75.4 ± 12.3[#,*]	187 ± 21.3[#,*,°]	6.2 ± 0.32[#,*]
CCl_4 + silymarin	70.3 ± 8.52[#,*]	162 ± 15.2[#,*]	6.7 ± 0.83[#,*]

Data represent the mean ± S.E.M. of observations from 8 rats.
[#] Significantly different from control group at $P < 0.05$.
[*] Significantly different from CCl_4 group at $P < 0.05$.
[°] Significantly different from CCl_4 + Silymarin group at $P < 0.05$.

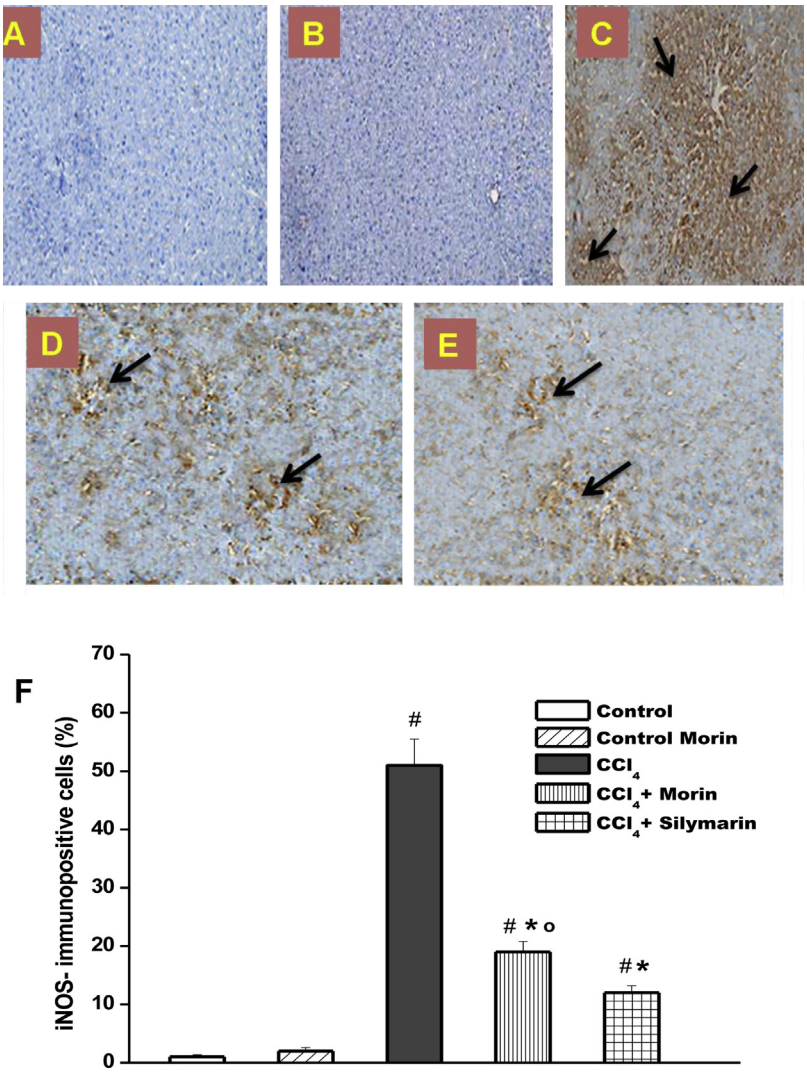

Fig. 3 – Effects of chronic CCl_4 alone and in combination with either morin or silymarin on hepatic immunohistochemical expression of iNOS (magnification 100×). (A and B) Sections of livers obtained from control and control morin rats, respectively, show minimal degree of iNOS expression (brown color). (C) Section of a liver obtained from CCl_4-intoxicated rat shows extensive iNOS expression (brown color) in tissue. (D and E) Sections obtained from liver of rats intoxicated with CCl_4 and treated concomitantly with morin and silymarin, respectively, and show limited iNOS expression (brown color). (F) Quantification of iNOS staining represents the number of stained (positive) cells per 20× fields for each rat section. Values are given as mean ± S.E.M. #, * and °: Statistically significant from control, CCl_4 and CCl_4 + Silymarin groups, respectively, at $P < 0.05$. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

reaching 5 and 12 folds, respectively, as compared to the control group. In contrast, concurrent treatment with CCl_4 and either morin or silymain exhibited anti-inflammatory effects by significant reduction in liver TNF-α level and iNOS expression to lower levels compared to CCl_4-intoxicated group. It was of interest to observe that the ability of morin to decrease TNF-α level and iNOS expression was lower than that of silymain.

Furthermore, the expression of pro-fibrotic marker, NF-κB was assessed by detecting the activated subunit p65 using immunohistochemical staining in liver tissue. The staining was quantified and represented in Fig. 4. Normal control and control morin groups showed minimal immunostaining for NF-κB-p65 subunit (Fig. 4A and B). On the other hand, CCl_4 elevated the expression of NF-κB (p65) as shown by the intense brown staining (Fig. 4C), while concomitant treatment with either morin or silymain reduced this elevation to a large extent (Fig. 4D and E). In addition, RT-PCR analysis of NF-κB supported these results in which CCl_4 induced a significant increase of NF-κB mRNA expression compared with normal control group. Whereas, concomitant treatment with CCl_4 and either morin or silymain reduced this altitude compared with CCl_4-intoxicated group (Fig. 5).

3.5. *Histopathological examination*

Histopathological examination of rat livers from normal control and control morin groups showed normal hepatic lobular architecture (Fig. 6A and B). On the other hand, sections

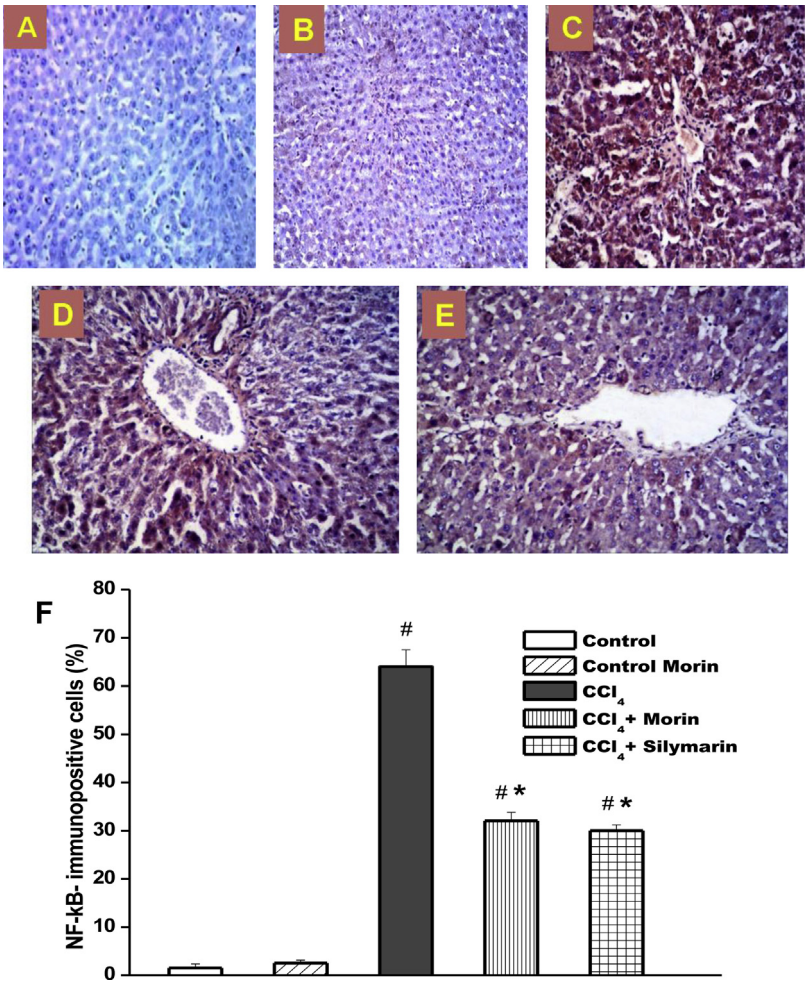

Fig. 4 – Effects of chronic CCl_4 alone and in combination with either morin or silymarin on hepatic immunohistochemical expression of NF-κB-p65 subunit (magnification 100×). (A and B) Sections of livers obtained from control and control morin rats, respectively, show minimal degree of NF-κB (p65) expression. (C) Section of a liver obtained from CCl_4-intoxicated rat shows extensive NF-κB (p65) expression (brown color) in tissue. (D and E) Sections obtained from liver of rats intoxicated with CCl_4 and treated concomitantly with morin and silymarin, respectively, and show limited NF-κB (p65) expression. (F) Quantification of NF-κB (p65) staining represents the number of stained (positive) cells per 20× fields for each rat section. Values are given as mean ± S.E.M. # and *: Statistically significant from control and CCl_4 groups, respectively, at $P < 0.05$. (For interpretation of the references to color in text, the reader is referred to the web version of this article.)

taken from liver of rats intoxicated with CCl_4 showed marked hepatocellular damage in the form of severe macrovesicular steatosis, centrilobular hepatocellular necrosis and periportal inflammation with sever fibrosis (Fig. 6C-1 and C-2). Nevertheless, these findings were ameliorated by treatment with morin and silymarin which showed fatty degeneration of hepatocyte, Kupffer cell activation and moderate fibrosis (Fig. 6D and E).

4. Discussion

Morin is a flavonoid that has been reported to show various beneficial activities, including antioxidant (Subash and Subramanian, 2009), anti-mutagenesis (Sivaramakrishnan and Devaraj, 2010) and anti-inflammatory effects (Zhang et al., 2011). The present study elucidated a potential antifibrotic effect of morin in an experimental model of chronic CCl_4-induced liver fibrosis in rats via its effects on different oxidative stress and inflammatory markers as well as the expression of profibrotic marker, NF-κB. Such effects have been further compared with a known hepato-protective agent, silymarin, whose efficacy is clinically established in different liver diseases (Saller et al., 2001).

CCl_4 is considered one of the most commonly used hepatotoxins in the experimental study of liver diseases. Hepatic responses in rats to chronic CCl_4 stimulation are shown to be superficially similar to human cirrhosis (Johnston and Kroening, 1998). Serum ALT, AST, ALP and total bilirubin are reliable markers of liver function. In the present study, CCl_4 caused a significant elevation in serum ALT, AST, ALP and total bilirubin reflecting hepatic necrosis and inflammation,

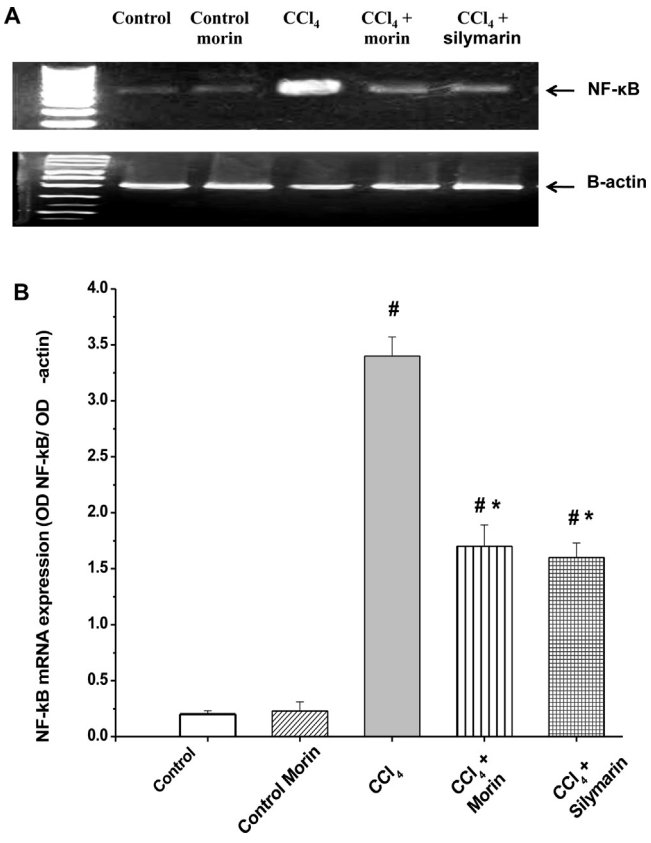

Fig. 5 – Effects of chronic CCl_4 alone and in combination with either morin or silymarin on hepatic NF-κB mRNA expression (RT-PCR). Data are reported as: (A) representative electrophoretic analysis of RT-PCR products; (B) a graph presents the ratio of densitometric measurements (optical density, OD) of samples to the corresponding reporter gene (β-actin). # and *: Statistically significant from control and CCl_4 groups, respectively, at $P < 0.05$.

and markedly increased the relative liver weight thus confirming the induction of liver hypertrophy (Yachi et al., 2010). Co-treatment of animals with morin significantly prevented these changes and implies that morin effectively attenuated the progression of CCl_4-induced liver injury. This gives a support that morin is able to condition the hepatocytes, accelerate regeneration of parenchyma cells, protect against membrane fragility and decrease leakage of the enzymes into circulation (Prahalathan et al., 2012). Therefore, morin acted by the same mode of action of silymarin. Our results are in accordance with earlier studies that showed the ability of morin to reduce/inhibit the hepatotoxicity induced by several chemicals (Lee et al., 2008; Shankari et al., 2010; Sivaramakrishnan and Devaraj, 2010).

CCl_4 metabolism in the liver results in the stimulation of lipid peroxidation and the production of free radicals (Sancheti et al., 2013), which causes necrosis of hepatocytes, induces inflammation, and further promotes progression of hepatic fibrogenesis. The present study showed that administration of CCl_4 leads to parallel increase in oxidative stress markers, MDA and NO with subsequent decrease in GSH, while treatment with morin had the opposite effect. These findings suggest that the antifibrotic effect of morin may be caused, at least in part, by its radical scavenging action and its antioxidant activity.

Collagen amount can be reflected by the content of hydroxyproline, the main characteristic compound in collagen, so its elevated level reflects an increase in the *de novo* synthesis of liver collagen (Ali and Mann, 2004). In the present study, chronic administration of CCl_4 resulted in a significant increase in the liver content of hydroxyproline which by co-treatment with either morin or silymarin was reduced to near the control levels. In addition, histopathological examination of collagen deposition using Masson's trichromic stain supported these findings.

Furthermore, assessment of different inflammatory markers revealed that CCl_4 intoxication induced a significant increase of hepatic NF-κB and iNOS expressions together with significant elevation in tissue TNF-α level indicating amplified inflammatory responses during chronic hepatic injury. Sustained hepatic inflammation provoked by long-term administration of CCl_4 is believed to be through NF-κB pathway (Son et al., 2007). In this context, activation of many kinases involved in NF-κB pathway is shown to be dependent on oxidative stress. Reactive oxygen species (ROS) are shown to cause prolonged NF-κB DNA binding activity and antioxidants have been shown to diminish this activity. Kupffer cells display powerful NF-κB activation in response to liver injury by CCl_4, resulting in production and secretion of proinflammatory cytokines such as TNF-α and IL-6 that are strongly implicated as promoters of fibrosis (Racanelli and Rehermann, 2006). Moreover, induction of NF-κB during liver injury has also been reported in hepatocytes after CCl_4 administration in rats (Orfila et al., 2005). Accordingly, persistently elevated levels of NF-κB promote the secretion of inflammatory and chemotactic factors in hepatocytes and thereby worsen hepatic inflammation and fibrosis (Luedde and Schwabe, 2011). In this context, several studies showed that downregulation of NF-κB expression and subsequent inflammatory cascade is capable of alleviating CCl_4-induced hepatic fibrogenesis (Orfila et al., 2005; Tipoe et al., 2010).

In the present study, co-treatment with morin significantly reduced the expression of NF-κB and hence inhibited the downstream inflammatory cascade as evidenced by decreasing the expression of iNOS as well as the hepatic levels of TNF-α and NO, so morin provides satisfactory anti-inflammatory effects. Our results coincided with previous studies which showed that morin reduced the expression of inflammatory mediators like metalloproteinases, cyclooxygenase-2 and NF-κB during diethylnitrosamine-induced hepatocarcinogenesis in rats (Sivaramakrishnan and Devaraj, 2009, 2010) and in human chondrocytes (Chen et al., 2012). In addition, it was reported that morin could protect acute liver damage by CCl_4 in rats by inhibiting the production of TNF-α, IL-1β, IL-6, and iNOS expressions (Lee et al., 2008). Moreover, Kim et al. (2010) further confirmed that morin modulates oxidative stress-induced NF-κB pathway through its anti-oxidant activity.

The results of histopathological study also support the results of biochemical parameters and are additional evidence

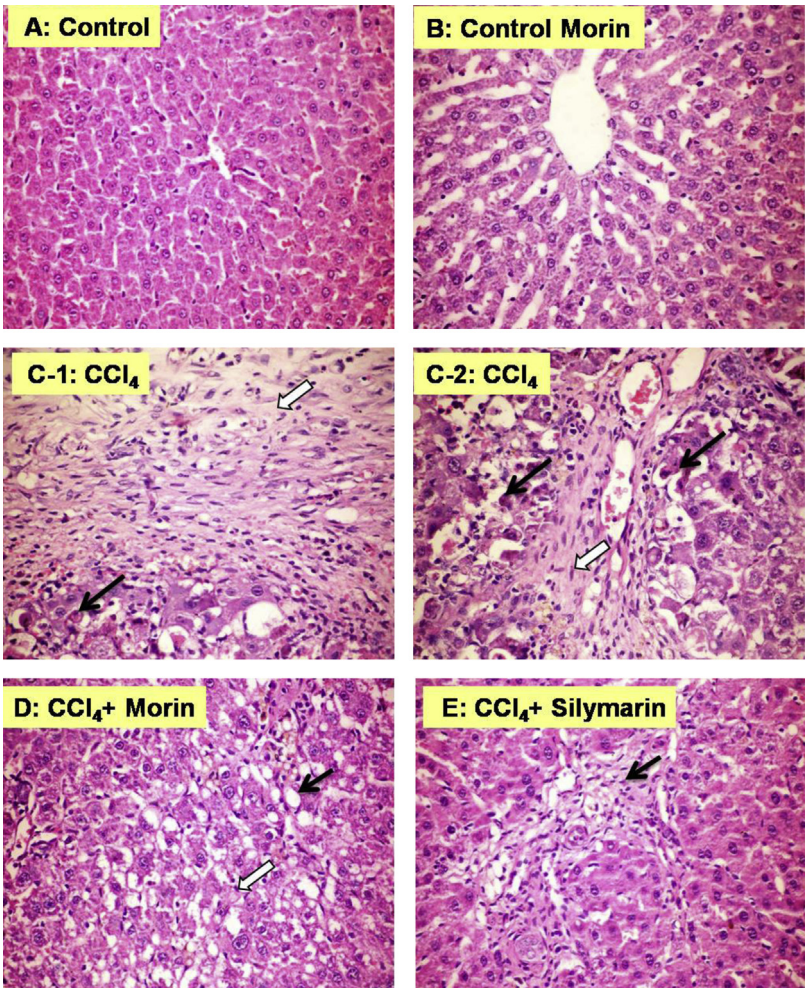

Fig. 6 – Representative photomicrographs of liver sections stained by H&E (magnification 200×). (A) Section taken from a liver of control rat and (B) section taken from a liver of morin-treated rat show normal hepatic architecture and hepatocyte structure. (C-1 and C-2) Sections taken from liver of rats intoxicated with CCl_4 show Glissonian's cirrhosis (C-1: white arrow), necrosis of subcapsular hepatocytes (C-1: black arrow), apoptosis of hepatocyte (C-2: white arrow) and multilobular fibroblasts (C-2: black arrow) with sever fibrosis. (D and E) Sections taken from livers of rats intoxicated with CCl_4 and treated concomitantly with morin and silymarin, respectively, showing fatty degeneration of hepatocyte (D: black arrow), Kupffer cell activation (D: white arrow) and portal fibrosis (E: black arrow) with moderate fibrosis.

of hepatoprotective effect of morin. There was marked hepatocellular damage in CCl_4-intoxicated group. Simultaneous treatment with either morin or silymarin obviously mitigated the histopathological changes and exhibited less damage to the hepatic cells compared to rats treated with CCl_4 alone. A notable finding is that the reduction in cellular damage that was seen in morin-treated group was morphologically similar to that of silymarin-treated group.

In conclusion, the present study provides an evidence for the hepatoprotective and antifibrotic effects of morin. The mechanisms underlying these promising effects of morin could be through attenuating oxidative stress as well as decreasing the expression of NF-κB and subsequent proinflammatory cytokines production. Therefore, morin might be considered a potential therapeutic tool to ameliorate liver tissue injury and fibrosis encountered with various forms of etiological factors.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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