COMPARATIVE STUDY ON THERAPEUTIC POTENTIAL OF CAFFEIC ACID AND SILYMARIN IN PARACETAMOL-INDUCED HEPATOTOXICITY: EFFECT ON HO-1, OXIDATIVE STRESS, HEPATIC INFLAMMATION AND NEUTROPHILS INFILTRATION

Mona F. El-Azab

Department of Pharmacology and Toxicology, Faculty of Pharmacy, Suez Canal University, Ismailia 41522, Egypt

ABSTRACT

Paracetamol is a widely used analgesic and antipyretic drug, but at high doses it leads to undesirable side effects, mainly hepatotoxicity. The hepatoprotective effect of caffeic acid has been previously reported, however the mechanism of this protective effect is not fully explored. The current study aimed to investigate the therapeutic potential of caffeic acid vs. silymarin in paracetamol-induced hepatic damage. To better understand the mechanisms by which these phenolic compounds confer their protective effects, parameters of lipid peroxidation, hepatic inflammation and neutrophils infiltration were evaluated. In addition, liver toxicity markers and hepatic antioxidants (enzymatic as well as non-enzymatic) were measured.

Daily administration of paracetamol (700 mg/kg, p.o.) caused hepatic injury that was manifested as increased hepatic levels of malonaldehyde (MDA), tumor necrosis factor-alpha (TNF-α) and heme oxygenase (HO-1) with marked increase in myeloperoxidase (MPO) activity, depletion of liver reduced glutathione, and diminished catalase and super-oxide dismutase (SOD) activities. Furthermore, serum liver enzymes (ALT, AST, GGT) were significantly increased along with hepatocellular degeneration and steatosis. Co-treatment with caffeic acid or silymarin alleviated paracetamol-induced oxidative stress, blunted TNF-α levels and MPO activity, while enhanced HO-1 levels with restoration of antioxidant enzymes activities. This was accompanied by normalization of liver transaminases and improvement of hepatic architecture. Interestingly, the effect of caffeic acid on HO-1, TNF-α, and MPO was more prominent when compared to silymarin.

The present study provide an evidence on the multiple mechanisms by which caffeic acid as well as silymarin grant their hepatoprotective activities mainly through the induction of HO-1 in addition to the reduction of oxidative stress burden in hepatocytes. The powerful anti-oxidant effect was accompanied by a significant anti-inflammatory activity.

Keywords: caffeic acid, heme oxygenase-1, hepatotoxicity, oxidative stress, paracetamol, silymarin

1. INTRODUCTION

Paracetamol is a commonly used analgesic and antipyretic agent which is usually safe and well tolerated when used at therapeutic doses. However, acute paracetamol overdose causes severe and fatal hepatotoxicity **(Maddrey, 2005)**. Paracetamol toxicity is one of the most common causes of poisoning worldwide, mainly causing liver injury and acute liver failure **(Larson *et al.*, 2005; Ryder and Beckingham, 2001)**. Damage to the liver results from one of

paracetamol metabolites, that is N-acetyl-p-benzoquinoneimine (NAPQI). NAPQI is a highly reactive intermediate that depletes hepatic glutathione and binds covalently to intracellular proteins including mitochondrial proteins **(James *et al.*, 2003)**. Consequently, enormous hepatocellular necrosis occurs as a result of mitochondrial dysfunction, oxidative and nitrative stress, and inflammatory reactions **(Jaeschke and Bajt, 2006)**.

Several studies have documented the effective protection afforded by antioxidants and anti-inflammatory agents against paracetamol-induced hepatotoxicity **(Girish *et al.*, 2009; Nagi *et al.*, 2010; Yan *et al.*, 2009)**. However most of these studies reported the protective effect of various agents only when administered prior to paracetamol insult. Hence, there is a need to find agents that can stop or even reverse the hepatotoxicity when administered following paracetamol-induced injury.

Caffeic acid and silymarin are phenolic compounds widely present in plant kingdom **(Duke, 1992)**. Caffeic acid has been reported to posses many pharmacological activities including anti-inflammatory **(Moreira *et al.*, 2000)**, anti-tumor **(Soleas *et al.*, 2002)**, anti-allergic **(Kimata *et al.*, 2000)**, anti-ulcer **(Al-Sereiti *et al.*, 1999)**, cardioprotective **(Cornicelli and Trivedi, 1999)** and immunomodulatory **(Russo *et al.*, 1999)** activities. Likewise, silymarin was reported to exhibit several biological activities including antioxidant action **(Lieber, 2004)**, chemopreventive **(Chlopcikova *et al.*, 2004)**, and immunomodulatory effects **(Schumann *et al.*, 2003; Yoo *et al.*, 2004)** in addition to its well established hepatoprotective activity **(Berger and Kowdley, 2003; Lieber *et al.*, 2003)**. Although the beneficial effect of caffeic acid in models of chemically-induced hepatic injury has been previously reported **(Janbaz *et al.*, 2004)**, the mechanism of its hepatoprotective effect needs further investigation.

The main objectives of the current study were to evaluate the protective effects of caffeic acid concomitant treatment in comparison with silymarin against paracetamol-induced hepatic injury in rats and to investigate the possible mechanism(s) underlying these effects. Therapeutic potential was assessed by monitoring liver injury through estimation of serum alanine aminotransferase (sALT), aspartate aminotransferase (sAST), and gamma-glutamyl transferase (GGT) as well as hepatic lipid peroxidation expressed as malondialdehyde (MDA). In addition, the activities of hepatic endogenous antioxidants including reduced glutathione (GSH), catalase, and superoxide dismutase (SOD), hepatic levels of the pro-inflammatory marker TNF-α, and the neutrophils infiltration marker myeloperoxidase (MPO) were evaluated.

2. MATERIALS AND METHODS:

2.1. Drugs, kits, and chemicals

Caffeic acid was purchased from sigma (St. Louis, MO, USA). *Paracetamol* and *Silymarin* were provided by Egyptian Int. Pharmaceutical Industries Co. and Pharaonia Pharmaceuticals Co., respectively. Kits for *alanine aminotransferase* (ALT, or sGPT), *aspartate aminotransferase* (AST, or sGOT), and *gamma-glutamyl transferase* (GGT) were purchased from (bioMérieux, France). Commercially available kits (Bio-Diagnostic, Egypt) were used for determining the enzymes *superoxide-dismutase (SOD)* and *catalase*, *reduced glutathione (GSH)*, and the lipid-peroxide marker *malondialdehyde (MDA)*. Liver *heme oxygenase* (HO-1) levels were determined using Rat HO-1 Enzyme Linked Immunosorbent Assay (ELISA) Kit (Stressgens, MI,USA). *Rat tumor necrosis factor-alpha (TNF-α) Immunoassay Kit [Quantikine, ELISA]* was purchased from R&D systems (Minneapolis, USA) and *myeloperoxidase* kit was

purchased from Northwest Co. (Canada). All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise mentioned.

2.2. Animals

Male Wister rats weighing 120-150g were obtained from the Egyptian Organization for Biological Products and Vaccines (Egypt) and housed under controlled temperature (25 ± 1°C) on a 12 h light/dark cycle. Rats were allowed to acclimatize for one week before starting the experiment. Food and water were allowed *ad libitum* during the study period.

2.3. Experimental design

Thirty two rats were randomly divided into four groups of eight rats each and treated as follows:

- 1st group: rats that served as the untreated (normal) control and were administered single daily dose of 5 ml/kg body weight of distilled water orally for 14 days.
- 2nd group: rats that served as the model control and were administered single daily dose of paracetamol suspension at 700 mg/kg body weight for 14 days.
- 3rd group: rats were co-treated daily with single oral dose of caffeic acid at 40 mg/kg body weight 1 hour after oral administration of paracetamol suspension (700 mg/kg) for 14 days.
- 4th group: rats were co-treated daily with single oral dose of silymarin at 100 mg/kg 1 hour after oral administration of paracetamol suspension (700 mg/kg) for 14 days.

2.4. Sample preparation

At the end of experiment, 6 rats from each group were selected randomly and sacrificed by cervical dislocation, blood samples were collected, left for 60 min to clot, and then centrifuged for 10 min at 5000 rpm to obtain clear sera which were stored at −20°C.

The livers were excised, divided into two portions, one portion was washed with ice-cold saline, and subsequently homogenized in cold potassium phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 14000 rpm for 15 min at 4°C and the resulting supernatant was stored at −80°C. The other portion was kept for histopathological evaluation.

2.5. Biochemical determination of liver enzymes

Liver enzymes (ALT, AST, and GGT) were determined in serum samples spectrophotometrically using appropriate kits. Protocols used were according to the recommended manufacturer procedures.

2.6. Measurements of lipid peroxidation and antioxidants in liver

Liver homogenate from different groups were used for measuring MDA, GSH, and the activities of SOD and catalase using standard spectrophotometric assays. Levels of MDA as thiobarbituric acid-reactive substances (TBRS) were measured by the method of **Ohkawa et al. (1979).** GSH was determined based on the use of Ellman's reagent **(Beutler *et al.*, 1963)**. SOD and catalase activities were measured as described previously **(Aebi, 1984; Nishikimi *et al.*, 1972)**.

2.7. Determination of liver TNF-α

Levels of TNF-α in liver homogenate were determined using ELISA following the manufacturer's protocol (R&D systems). In brief, to a 96-well microplate pre-coated with polyclonal antibody specific for rat TNF-α, 50 µl assay diluent and 50 µl samples were added. Recombinant rat TNF-α was used to set up the corresponding standard curve. After incubation for 2h at room temperature, the wells were washed and polyclonal anti-rat TNF-α antibody conjugated to horseradish peroxidase was added. Incubation was continued for 2h, plates were washed; substrate solution was added to each well and incubated for 30 min. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The O.D at 450nm (correction wavelength set at 570 nm) was measured using microplate reader (Metertech, M960).

2.8. Determination of liver HO-1

Levels of HO-1 in liver homogenate were determined using ELISA following the manufacturer's protocol (Stressgen). Briefly, to a 96-wellmicroplate pre-coated with monoclonal antibody specific for rat HO-1, 100 ml standards, sample diluent, or samples were added to appropriate wells. Recombinant rat HO-1was used to setup the standard curve. After incubation for 1h at room temperature, the wells were washed and polyclonal antibody specific for rat HO-1 was added followed by incubation for another 1h. After washing, 100 ml of rat HO-1 horseradish peroxidase conjugate was added. Incubation was continued for 30min at room temperature, plates were washed; tetramethylbenzidine substrate solution was added to each well and incubated for 15min in dark. The enzyme reaction yielded a blue product that turned yellow when the stop solution was added. The O.D at 450nm was measured using microplate reader (Metertech, M960).

2.9. Determination of MPO activity in liver

Myeloperoxidase (MPO) activity was determined as described by Weiss and coworkers (1982). Briefly, hypochlorous acid (HOCl) was formed from MPO catalyzed reaction between chloride and hydrogen peroxide. HOCl was rapidly trapped by amino acid taurine to form a stable oxidant taurine chloramine. After incubation for 30min, the MPO catalyzed reaction was stopped by adding catalase to eliminate hydrogen peroxide. Taurine chloramine was then allowed to react with the chromophore 5-thio-2-nitrobenzoic acid (TNB) to give a colorless reaction product 5-5-dithiobis(2-nitrobenzoic acid) or DTNB. By following the decrease in absorbance at 412 nm, MPO activity was measured. One unit is the amount of MPO that can produce 1.0 nmole of taurine chloramine (hypochlorous acid) at pH 6.5 and 25°C during 30 minutes in the presence of 100 mM chloride and 100 µM of hydrogen peroxide.

2.10. Histopathological examination of liver tissue

The other liver portion from each animal was fixed in 10% formalin, dehydrated in ascending grades of alcohol and embedded in paraffin. Sections were cut at 4 µm, stained with hematoxylin and eosin (H&E) and examined under a light microscope for histopathologic evaluations.

2.11. Statistical analysis

All data were expressed as mean ± S.E.M. Statistical significance was tested by one way analysis of variance (ANOVA) followed by Bonferroni post hoc analysis. A P value <0.05 was considered statistically significant.

3. RESULTS

3.1. Effect of caffeic acid vs. silymarin on hepatic toxicity markers

Treatment with paracetamol (700 mg/kg, daily) for 2 weeks disrupted liver function as reflected by marked (2- to 6-fold) rises in serum alanine aminotransferase **(Pestka *et al.*, 2004)**, aspartate aminotransferase **(Camacho-Barquero *et al.*, 2007)**, and gamma-glutamyl transferase (GGT) (Fig. 1). The treatment with caffeic acid concomitantly with paracetamol significantly ($P \leq 0.05$) inhibited paracetamol-induced increases in serum aminotransferases and kept their levels close to normal values. The effect of silymarin, the known hepatoprotective agent, on ALT and AST was comparable to caffeic acid. However, the level of GGT after silymarin treatment stayed slightly higher than normal levels.

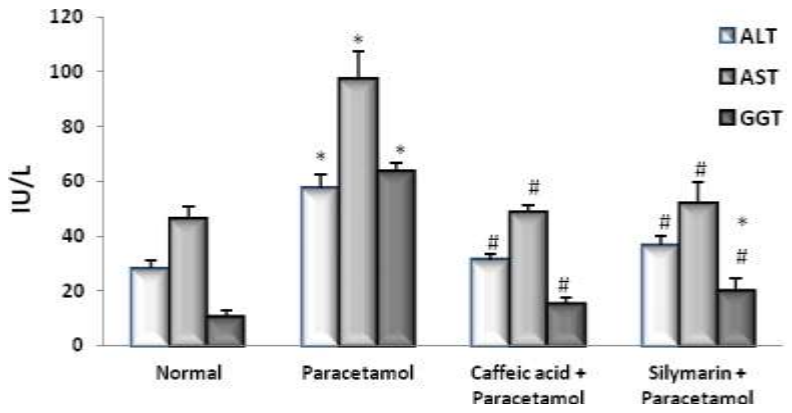

Fig 1. Changes in serum hepatic toxicity markers (ALT, AST, GGT) after treatment with paracetamol (700 mg/kg, p.o., for 14 consecutive days) either alone or in combination with caffeic acid (40 mg/kg, p.o.) vs. silymarin (100 mg/kg, p.o.). Values are expressed as mean ± S.E.M. All data were analyzed using one-way ANOVA followed by Bonferroni post *hoc* test. $^{*}P \leq 0.05$ vs. normal control, $^{\#}P \leq 0.05$ vs. paracetamol. (n=6)

3.2. Effect of caffeic acid vs. silymarin on lipid peroxidation and antioxidants in liver

Oxidative stress and endogenous antioxidant levels were evaluated to gain insights into the hepatic molecular and cellular changes occurring following paracetamol treatment. Fig 2A demonstrates that paracetamol resulted in 4-fold increase in MDA levels in liver cell homogenates indicating lipid peroxidation. This was accompanied by 63% reduction in GSH levels (Fig 2B). In addition, the activities of antioxidant enzymes, catalase and SOD, were depleted in liver following paracetamol treatment as evident by 69% and 47% reduction, respectively (Fig 2C-D). Treatment with caffeic acid or silymarin significantly ($P \leq 0.05$) suppressed hepatic lipid peroxidation and prevented the reductions of GSH level and catalase as well as SOD activities resulting from paracetamol administration.

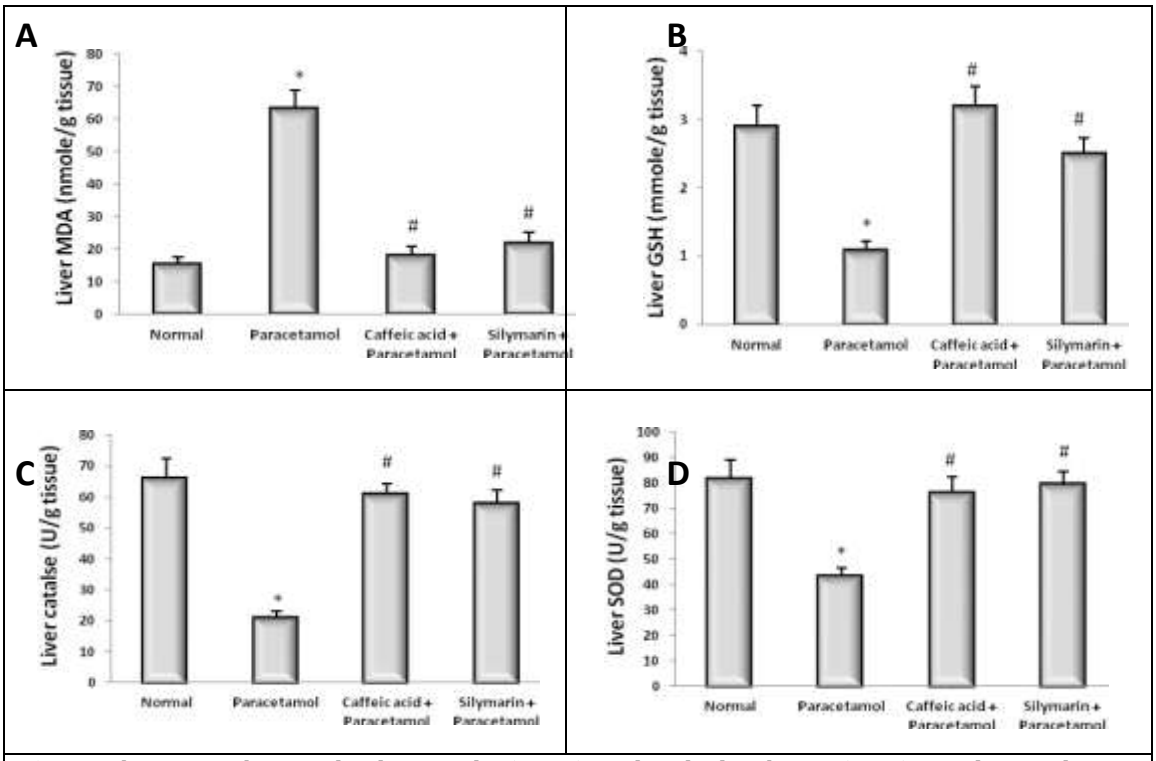

Fig 2. Changes in hepatic lipid peroxide (MDA), reduced glutathione (GSH), catalase and SOD activities after treatment with paracetamol (700 mg/kg, p.o., for 14 consecutive days) either alone or in combination with caffeic acid (40 mg/kg, p.o.) vs. silymarin (100 mg/kg, p.o.). Values are expressed as mean ± S.E.M. All data were analyzed using one-way ANOVA followed by Bonferroni post *hoc* test. $^{*}P\leq0.05$ vs. normal control, $^{\#}P\leq0.05$ vs. paracetamol. (n=6)

3.3. Effect of caffeic acid vs. silymarin on HO-1 levels in liver

Following treatment with paracetamol for 2 weeks, a marked (15-fold) increase in heme oxygenase-1 (HO-1) protein was observed in liver cell homogenate suggesting a stress response by hepatocytes. The co-treatment with caffeic acid resulted in a further 5-fold increase in HO-1 expression while the concomitant treatment with silymarin caused only 3.5-fold increase in HO-1 compared to paracetamol treated group (Fig. 3).

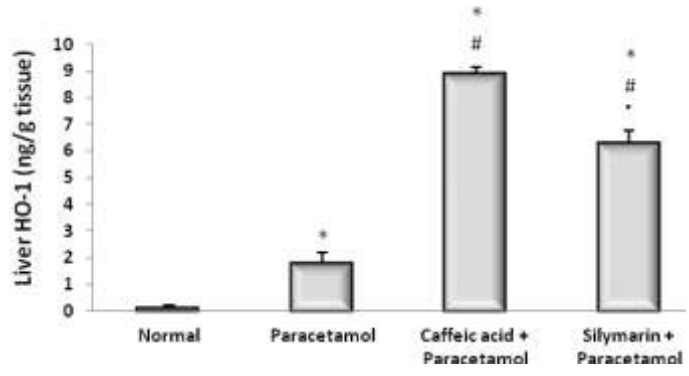

Fig 3. Changes in liver heme oxygenase (HO-1) after treatment with paracetamol (700 mg/kg, p.o., for 14 consecutive days) either alone or in combination with caffeic acid (40 mg/kg, p.o.) vs. silymarin (100 mg/kg, p.o.). Values are expressed as mean ± S.E.M. All data were analyzed using one-way ANOVA followed by Bonferroni post *hoc* test. $^{*}P\leq0.05$ vs. normal control, $^{\#}P\leq0.05$ vs. paracetamol, $^{\bullet}P\leq0.05$ vs. silymarin + paracetamol.

(n=6)

3.4. Effect of caffeic acid vs. silymarin on paracetamol-induced hepatic inflammation

To gain more insights into the mechanisms by which caffeic acid and/or silymarin confer their hepatoprotective effects against paracetamol-induced hepatotoxicity, TNF-α and MPO were evaluated as markers for hepatic inflammation and neutrophils infiltration, respectively. Paracetamol significantly ($P \leq 0.05$) increased the hepatic TNF-α level (3.6-fold) compared with control rats. Caffeic acid or silymarin co-administration with paracetamol significantly ($P \leq 0.05$) reduced paracetamol-induced hepatocytic production of TNF-α by 66 % and 50 %, respectively (Fig. 4A). Similarly, paracetamol markedly (7-fold) increased MPO activity in liver homogenate compared with normal control group. Co-administration of caffeic acid or silymarin significantly ($P \leq 0.05$) reduced paracetamol-induced MPO activity in liver cell homogenates by 78 % and 68%, respectively, compared with paracetamol control group (Fig. 4B).

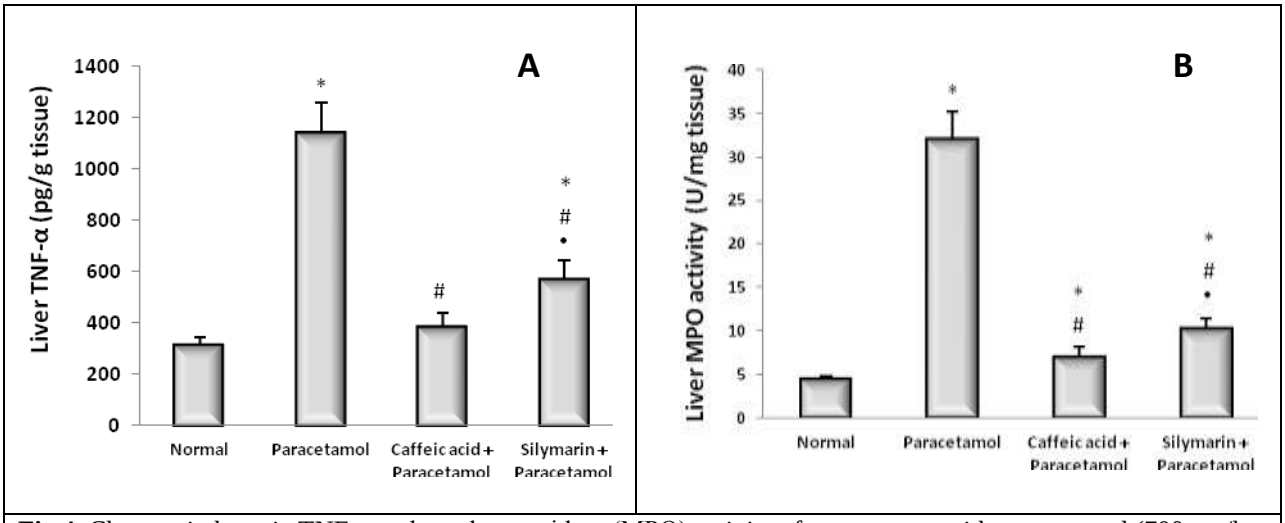

Fig 4. Changes in hepatic TNF-α and myeloperoxidase (MPO) activity after treatment with paracetamol (700 mg/kg, p.o., for 14 consecutive days) either alone or in combination with caffeic acid (40 mg/kg, p.o.) vs. silymarin (100 mg/kg, p.o.). Values are expressed as mean ± S.E.M. All data were analyzed using one-way ANOVA followed by Bonferroni post *hoc* test. $^{*}P \leq 0.05$ vs. normal control, $^{\#}P \leq 0.05$ vs. paracetamol, $^{\bullet}P \leq 0.05$ vs. caffeic acid + paracetamol. (n=6)

3.5. Histopathological evaluation

The microscopic examination of liver of control rats showed normal architecture without any signs of vascular or inflammatory changes (Fig. 2A). Histopathological examination revealed that paracetamol induced diffuse cytoplasmic vacuolation, centrilobular necrosis, vascular congestion and nuclear pyknosis of the hepatocytes which are indicative of acute hepatocellular injury (Fig. 2B) that came in line with the increased levels of serum aminotranferases. Whereas, caffeic acid or silymarin co-treatment preserved the liver histology close to the normal architecture with minimal small focal necrosis and no evidence of degeneration or steatosis (Fig. 2C-D) which is consistent with the amelioration of pathologic anomalies by both compounds.

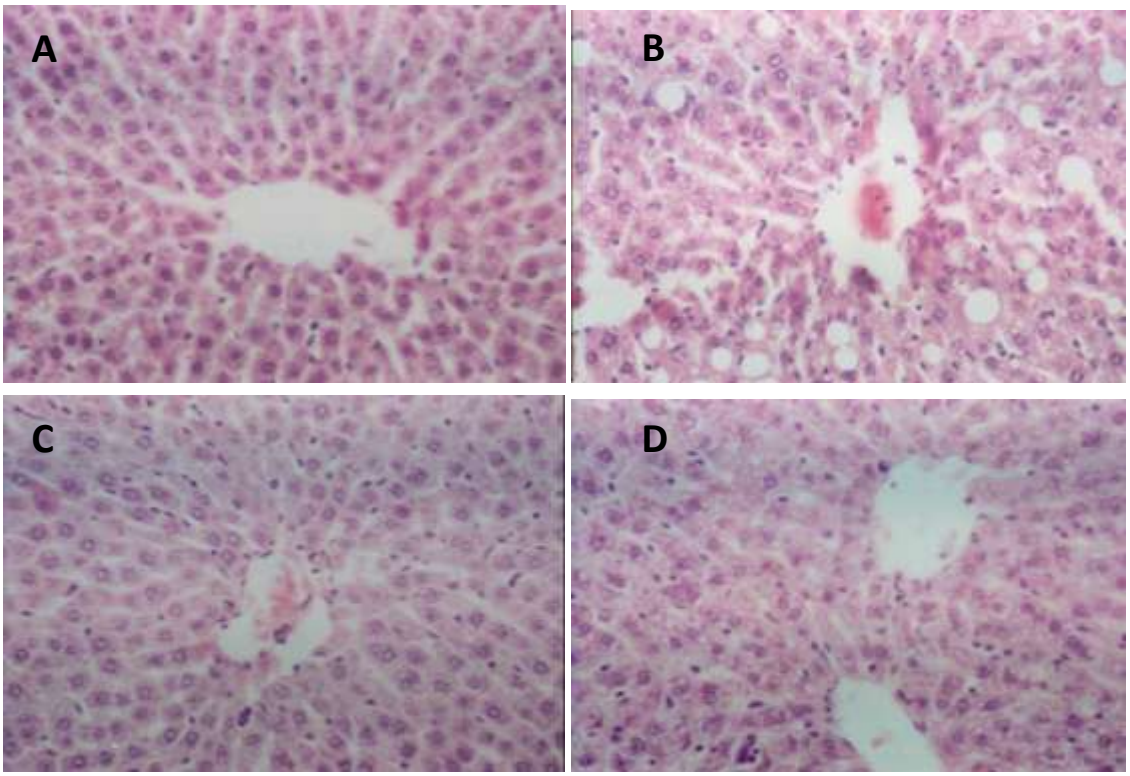

Fig 5. Photomicrographs of rat liver (H&E, 400×) from: (A) normal control group showing normal hepatic architecture; (B) paracetamol group showing cytoplasmic vacuolization and ballooning degeneration of hepatocytes; (C and D) acetaminophen plus caffeic acid or silymarin, respectively, showing a histological picture comparable to that of the control group.

4. DISCUSSION

Caffeic acid, one of the most widely distributed flavonoids, has been demonstrated to have potent antioxidant ability **(Jayanthi and Subash, 2010; Rice-Evans and Miller, 1996)** and has been reported to protect cells from oxidative damages caused by a diverse range of oxidative stresses, including paracetamol-mediated toxicity **(Janbaz *et al.*, 2004)**. However, to the best of our knowledge, the present study is the first to investigate the therapeutic potential of "post-treatment" with caffeic acid against paracetamol-induced hepatotoxicity in rats.

Cancer patients on chemotherapy generally receive higher doses of paracetamol to relieve pain **(Nassar *et al.*, 2009)**. Hepatic damage as a result of paracetamol overdoses in human is fairly common **(Nelson, 1995)**. At therapeutic doses, paracetamol is metabolized primarily in the liver via glucuronidation and sulfation reactions, and results in water-soluble metabolites that are excreted via kidney. As a result of the metabolic conversion of paracetamol by the microsomal CYP-450 enzyme system, a highly reactive intermediate, N-acetyl-p-benzoquinoneimine (NAPQI) is produced **(Mitchell *et al.*, 1973)**. NAPQI directly reacts with GSH and at overdoses of paracetamol, depletion of cellular GSH occurs allowing NAPQI to bind to cellular proteins especially in the mitochondria. The resulting mitochondrial oxidative and nitrative stress leads to mitochondrial dysfunction and eventually hepatocellular necrosis **(Jaeschke *et al.*, 2011)**.

Indeed, histopathological evaluation confirmed the hepatic damage associated with high doses of paracetamol in the current model as was evident by hepatic necrosis and steatosis. In addition, the elevated levels of serum transaminases observed in this study support the previous observation. Our results are in accordance with previous reports showing that hepatic damage resulting from paracetamol overdoses is always associated with increased serum liver enzymes as marker for hepatotoxicity **(Heard, 2008; Larson *et al.*, 2005)**

Paracetamol also induces the release of numerous cytokines and signaling molecules from activated Kupffer cells, including TNF-α which in turn up-regulates the iNOS enzyme **(Gardner *et al.*, 2002; Morris and Billiar, 1994)** resulting in increased hepatic production of nitric oxide **(Jaeschke *et al.*, 2011)**. Excess nitric oxide depletes intracellular GSH that increases the susceptibility to oxidative stress **(Clancy and Abramson, 1995)**. Moreover, excess nitric oxide reacts with superoxide anion to generate peroxynitrite radical that causes further cell damage by oxidizing and nitrating cellular macromolecules. In agreement with previous studies **(Girish *et al.*, 2009; Nagi *et al.*, 2010; Oz and Chen, 2008; Yan *et al.*, 2009)**, current results demonstrated that oxidative stress accompanied by increased lipid peroxidation, undermined antioxidant defenses and increased release of pro-inflammatory cytokines which are the hallmarks of paracetamol hepatotoxicity. The antioxidant effect of caffeic acid was evident in the this study through the restoration of catalase and SOD activities as well as GSH with blunting of lipid peroxidation. Our results came in line with previous reports that demonstrated similar effect of caffeic acid in different models **(Jayanthi and Subash, 2010; Kus *et al.*, 2004)**. Silymarin produced a comparable effect to caffeic acid in the current study. The antioxidant activities of both polyphenols were associated with an improved liver architecture and normalized serum transaminases.

There is an emerging evidence that caffeic acid, as well as other polyphenols, may exert modulatory actions on the endogenous antioxidative defense system by interactions with intracellular signaling cascades in addition to their ability of quenching ROS **(Williams *et al.*, 2004)**. Heme oxygenase-1 (HO-1), a redox-sensitive inducible protein that provides efficient cytoprotection against oxidative stress, has attracted much attention for its up-regulation in a variety of stress-related circumstances **(Dore, 2002; Ryter *et al.*, 2007)**. Growing evidences indicate that HO-1 plays a key role in defense mechanisms against oxidative damages **(Nakahira *et al.*, 2003; Ryter *et al.*, 2007)**. The induction of the HO-1 gene has been proposed to be a beneficial adaptive response to oxidative stress **(Applegate *et al.*, 1991)**. Further, Alum et al. (1995) have shown HO-1 gene to be induced by reactive oxygen species. It is known that both biliverdin and bilirubin, the byproducts of heme metabolism by HO-1, exhibit antioxidant properties. In the light of these observations, our findings suggest that the reactive oxygen species released from the damaged liver as a result of paracetamol overdose induced HO-1 expression. Indeed, activation of HO-1 at the transcriptional level has been previously reported in acetaminophen-induced hepatitis which confirm the current finding **(Bauer *et al.*, 2000; Ishida *et al.*, 2006)**. On the other hand, preceding findings showed that caffeic acid markedly increased heme oxygenase activity and HO-1 protein in astrocytes **(Scapagnini *et al.*, 2002)**. Similarly, silymarin was reported to up-regulate HO-1 mRNA in human hepatoma cells **(Bonifaz *et al.*, 2009)**. However, as far as we know, the current study is the first to investigate the effect of caffeic acid and silymarin on liver HO-1 in the paracetamol-induced hepatotoxicity. Both caffeic acid and silymarin enhanced the production of HO-1 by the liver far beyond the effect of paracetamol alone suggesting an additional mechanism by which these polyphenols confer a protective effect against paracetamol-induced toxicity.

TNF-α is a pro-inflammatory cytokine that is associated with liver injury in many experimental models **(Camacho-Barquero *et al.*, 2007; Cover *et al.*, 2006)** and human diseases **(Felver *et al.*, 1990)**. In the current study, treatment of rats with paracetamol resulted in a 3.6-fold increase in hepatic TNF-α. Previous reports showed that paracetamol-induced liver injury resulted in a significant increase in TNF-α which is in accordance with our results **(Cover *et al.*, 2006; Sener *et al.*, 2006; Teng *et al.*, 2012)**. However, the treatment of paracetamol-intoxicated rats with caffeic acid or silymarin significantly inhibited the rise in TNF-α indicating anti-inflammatory activity of the currently studied polyphenols. Preceding studies showed the inhibitory effect of caffeic acid or silymarin on TNF-α expression in different models support our finding **(Chao *et al.*, 2010; Song *et al.*, 2006)**.

It is well established that free radicals play a role in the recruitment of neutrophils by injured tissues as a potential source of free radicals and activating cytotoxic enzymes, namely MPO **(Lau *et al.*, 2005)**, which in turn maintain the vicious cycle of generating more free radicals resulting in lipid peroxidation. The infiltration of polymorphnuclear leukocytes that generates oxygen radicals and induces direct tissue injury has been previously reported with indomethacin treatment **(Tanaka *et al.*, 2001)**. This effect can result in increased lipid peroxidation that may exacerbate the already existing damage. In the present study, the elevated hepatic MDA concentrations along with increased hepatic MPO activity following paracetamol intoxication indicated the contribution of neutrophils infiltration and the impact of free radical generation on oxidative hepatic damage. While the co-treatment with caffeic acid or silymarin abrogated hepatic neutrophils infiltration as was evident by the significant reduction in MPO activity. This was consistent with our histopathological findings that showed normal architecture in the liver tissues from caffeic acid- or silymarin-treated animals and this could be attributed to the known antioxidant and anti-inflammatory activities of both compounds **(Jayanthi and Subash, 2010; Lieber, 2004; Muriel, 2009)**.

It is noteworthy that the HO system abates inflammation through several mechanisms including the suppression of macrophage-infiltration and abrogation of oxidative/inflammatory transcription factors like NF-κB, JNK and activating protein-1 **(Ndisang, 2010)**. HO-1 is stimulated by a wide variety of different stimuli including oxidative and inflammatory insults **(Jonas *et al.*, 2003; Mohri *et al.*, 2006)**, hyperglycemia **(Jonas *et al.*, 2003)**, hypertension **(Ndisang *et al.*, 2003)**, and dyslipidaemia **(Landar *et al.*, 2006)**. Therefore, HO-1 may be considered a sensitive index that is triggered in the onset of pathophysiological changes. However, in most cases the pathophysiological activation of HO-1 results only in a transient or marginal increase of HO-1 that falls below the threshold necessary to activate the downstream signaling components of the HO system **(Ndisang *et al.*, 2004; Ndisang *et al.*, 2003)** through which the HO system elicits its effects of restoring tissue homeostasis **(Farombi and Surh, 2006; Ndisang *et al.*, 2003)**. Therefore, a more vigorous enhancement of HO-1 would be needed to surmount that threshold, an effect that could be achieved by pharmacological agents capable of inducing HO **(Ndisang and Jadhav, 2009)**. This may explain the results observed in the current study that although hepatic level of HO-1 was induced after paracetamol insult, it wasn't sufficient to subside the inflammatory response elicited by paracetamol reactive metabolites. On the other hand, the co-administration of caffeic acid or silymarin in this study abrogated the paracetamol-induced inflammation and neutrophils infiltration as was evident by significant reduction of hepatic TNF-α levels and MPO activities through the robust enhancement of hepatic HO-1levels and subsequent resume of liver homeostasis.

CONCLUSION

The current study lends an additional evidence to paracetamol-induced hepatotoxicity which was manifested as increased serum liver enzymes and histopathological anomalies. The hepatotoxic effect involved disturbed antioxidant defense along with marked inflammation. Caffeic acid as well as silymarin treatment significantly ameliorated the liver injury induced by paracetamol overdoses in rats through multiple mechanisms including the restoration of antioxidant machinery, up-regulation of HO-1, and abrogation of inflammation and neutrophils infiltration. These data reinforce the concept of using natural agents with therapeutic potential for the protection against drug-induced hepatotoxicity.

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دراسة مقارنة في الإمكانية العلاجية لحمض الكافييك مقابل سيليمارين في تسمم الكبد المستحث بواسطة باراسيتامول: التأثير في الهيم أوكسيجيناز، الإجهاد التأكسدي، الإلتهاب الكبدي، وإرتشاح العدلات

منى فرج العزب*

قسم الأدوية و السموم ، كلية الصيدلة ، جامعة قناة السويس

يستخدم عقار باراسيتامول على نطاق واسع كمسكن وخافض للحرارة ، ولكن بجرعات عالية فإنه يؤدي إلى آثار جانبية غير مرغوب فيها ، و بشكل رئيسي تسمم الكبد. بالرغم من أن التأثير الوقائي لحمض الكافييك قد سبق ذكره ، ولكن آلية هذا التأثير الوقائي لم تستكشف بالكامل. تهدف الدراسة الحالية إلى تحقيق الإمكانات العلاجية لحمض الكافييك مقابل سيليمارين في تلف الكبد الناجم عن باراسيتامول. لتحقيق فهم أفضل للآليات التي بواسطتها تضفي هذه المركبات الفينولية آثارها الوقائية، تم تقييم علامات فوق تأكسد الدهون ، الإلتهاب الكبدي ، وإرتشاح العدلات (neutrophils). بالإضافة إلى ذلك تم قياس دلالات تسمم الكبد ومضادات الأكسدة (الإنزيمية، وكذلك غير الإنزيمية). تناول باراسيتامول يومياً (٧٠٠ ملغ / كلغ ، عن طريق الفم) تسبب في إصابة الكبد والذي تجلى في زيادة مستويات البيروكسيدات الدهنية ، عامل نخر الورم (TNF-α) ، والهيم أوكسيجيناز (HO-1) بالكبد ، مع زيادة ملحوظة في نشاط مايلو بيرأكسيداز (MPO) myloperoxidase ونضوب الجلوتاثيون المختزل (GSH) ، وكذلك تضاؤل أنشطة إنزيمات الكاتالاز (catalase) والسوبرأوكسيد ديسميوتاز (SOD) في الكبد. بالإضافة إلى ذلك، فإن إنزيمات الكبد بمصل الدم (ALT, AST, GGT) قد زادت زيادة ذات دلالة إحصائية ، جنبا إلى جنب مع تَنَكُّسٌ دُهْنِيّ لخلايا الكبد. العلاج المصاحب بحمض الكافييك أو سيليمارين خفف من الإجهاد التأكسدي المستحث بالباراسيتامول ، قلل مستويات عامل نخر الورم (TNF-α) ونشاط مايلو بيرأكسيداز (MPO) myloperoxidase ، في حين عزز مستويات الهيم أوكسيجيناز(HO-1) مع إستعادة أنشطة الإنزيمات المضادة للأكسدة. وقد رافق ذلك عودة إنزيمات الكبد للمستوى الطبيعي مع تحسين بنية الكبد. ومن المثير للإهتمام أن تأثير حمض الكافيـيك في كلٍ من الهيم أوكسيـجيناز (HO-1)، عامل نخر الورم (TNF-α) ، و مايلو بيرأكسيداز (MPO) myloperoxidase كان أكثر وضوحاً بالمقارنة مع سيليمارين. تقدم هذه الدراسة دليلاً على الآليات المتعددة والتي من خلالها يمنح كل من حمض الكافييك و سيليمارين أنشطتهما الوقائية للكبد وذلك بشكل رئيسي من خلال تحفيز الهيم أوكسيجيناز (HO-1) ، بالإضافة إلى الحد من عبء الإجهاد التأكسدي في خلايا الكبد. وقد كان التأثير القوي المضاد للأكسدة مصحوباً بنشاط مضاد للإلتهابات ذو دلالة إحصائية.