PHARMACOLOGY AND CELL METABOLISM

Chrysin Modulates Ethanol Metabolism in Wistar Rats: A Promising Role against Organ Toxicities

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Abstract — Aims: Hepato- and nephro-protective efficacy of chrysin was investigated against sequential increase of ethanol intake on the alteration of alcohol metabolizing enzymes–alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP 2E1), xanthine oxidase (XO) and oxidant/anti-oxidant status. **Methods:** Thirty female Wistar rats segregated into five groups, each with six animals, were put to different doses. Group I as control followed by Group II, III and IV were treated with ethanol (5,8,10 and 12g/kg body weight per week respectively) for 4 weeks. While Group III and IV were administered with chrysin at 20 mg (D1) and 40 mg/kg body weight (D2), respectively, prior to ethanol administration. Group V was given only chrysin (D2). Various oxidative stress and ethanol metabolizing enzymes were estimated in hepatic and renal tissues. **Results:** Ethanol administration significantly induced CYP 2E1, ADH and XO in liver and kidneys, respectively, along with an enhancement in levels of malondialdehyde and serum alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine and lactate dehydrogenase when compared with the control group and this enhancement is significantly normalized with chrysin administration. Oxidative stress markers: reduced glutathione, glutathione peroxidase, catalase and glutathione reductase were significantly ($P < 0.001$) depleted in the ethanol-treated group, while chrysin administration significantly restored all of these. Only chrysin administration did not show any adverse effect. **Conclusion:** Results demonstrate that chrysin administration prevents the liver and kidney of Wistar rats against oxidative damage during chronic ethanol consumption by inhibiting the activities of ADH, CYP 2E1, XO and catalase.

INTRODUCTION

Alcohol-related disorders are one of the current challenging health problems associated with the socio-economical consequences. Alcoholic liver disease remains one of the most common causes of chronic liver disease in the world (Diehl, 2002). Alcohol consumption is associated with the toxicity to various organs of the body including liver, brain, kidney etc. (Lieber, 1988). It has been demonstrated that even in patients with the alcohol dependence syndrome, the liver damage implicates immunological impairment showing modulation of key parameters such as the CD4/CD8 ratio, IgG, IgM etc. The extent of immunological impairment was found to be related to the duration of alcohol abuse (Leksowski *et al.*, 2000). Oxidative stress is known to play an important role in the pathogenesis of ethanol-induced liver injury (Lindros, 1995; Rodrigo, *et al.,* 1998; Zima *et al.*, 2001). Ethanol administration can elicit disturbances in the delicate balance between the pro- and anti-oxidant system of the body, therefore leading to oxidative stress. Several studies have shown the involvement of cytokines and oxidative stress in alcohol-mediated liver damage. Ethanol is known to induce generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which stimulate the release of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1B (IL-1B), interleukin-6 (IL-6) etc. These cytokines in turn further enhance oxidant generation in hepatic parenchyma and Kupffer cells, overwhelming them to result in their necrotic death. (Blonska *et al.,* 2002, Hoek and Pastorino, 2002). Alcohol-induced oxidative stress is associated with the metabolism of ethanol. Ethanol metabolism occurs primarily in the liver, which sustains the greatest amount of organ damage from excessive drinking (Lieber, 1994, 2004). Besides the liver, other organs such as the kidney, brain, and heart were also affected by ethanol consumption (Dinu *et al.,* 2005). Three major

pathways play a key role in ethanol-induced toxicity, namely, alcohol dehydrogenase (ADH), microsomal ethanol oxidation system and catalase (Lieber, 2005). All these pathways lead to the production of free radicals, which cause tissue injury.

Increased generation of ethanol-derived free radicals has been observed at the microsomal level (particularly at the ethanol-inducible cytochrome P450 isoform), the cytosolic ADH as well as through the mitochondrial respiratory chain (Nordmann *et al.*, 1992). In the cells, ethanol is converted to acetaldehyde, and there is increased evidence that acetaldehyde rather than ethanol is responsible for the carcinogenic and toxic effect (Seitz *et al.,* 2001). In cells, ethanol is catabolized to highly toxic acetaldehyde by enzyme ADH, and this acetaldehyde is responsible for ethanol-mediated toxicity, mutagenicity and carcinogenicity (Seitz and Oneta, 1998). Acetaldehyde can interact with the macromolecules like proteins and nucleic acids, and thus lead to the adduct formation. Numerous *in vitro* and *in vivo* studies have shown that acetaldehyde has a direct mutagenic and carcinogenic effect. It causes point mutation in human lymphocytes, induces sister chromatid exchanges and gross chromosomal aberrations (Obe *et al.,* 1986; Dellarco, 1988; Helander and Lindahl-Kiessling, 1991).

Nowaday, there is growing interest in elucidating the role and mechanism of the phytochemicals as free radical scavengers and inhibitors of oxidative stress. In fact, the pharmacological effects of many traditional drugs have been ascribed to the presence of flavonoid compounds (Kuehnau, 1976; Pietta, 2000), due to their ability to inhibit certain enzymes and their anti-oxidant activity. Chrysin (5, 7-dihydroxyflavone), which is the focus of present study, is a flavone. The flavonoid chrysin is present at high levels in honey, propolis and many plant extracts (Siess *et al.*, 1996; Willams *et al.*, 1997). Further, it has been demonstrated that the bee propolis scavenges ROS in phorbolmyristate acetate

(PMA)-activated neutrophils, and has been implicated in its anti-inflammatory potential (Krol *et al.*, 1996). Chrysin has been shown to possess several beneficial pharmacological properties, such as an anti-oxidant (Lapidot *et al.*, 2002), anti-hypertensive (Villar *et al.*, 2002), anti-diabetogenic (Lukacinova *et al.*, 2008), anti-inflammatory (Cho *et al.*, 2004), anti-cancer (Habtemariam, 1997; Cardenas *et al.*, 2006), anti-estrogenic (Machala *et al.*, 2001) and anxiolytic (Wolfman *et al.*, 1994). Chrysin has recently shown to be a potent inhibitor of aromatase (Sanderson *et al.*, 2004) and of human immunodeficiency virus activation in models of latent infection (Critchfield *et al.*, 1996). Moreover, it has been recently shown that chrysin exhibits potent hepato-protective and anti-oxidant activity against d-galactosamine-induced hepatitis in rats by inhibiting the liver toxicity markers (Pushpavalli *et al.*, 2010). On the basis of these considerations, the present study was conducted to elucidate the influence of chrysin on the ethanol-metabolizing enzymes and to evaluate its nephro- and hepato-protective efficacy against ethanol-mediated kidney and liver injury.

MATERIALS AND METHODS

Chemicals

Glutathione reductase (GR), oxidized (GSSG) and reduced glutathione (GSH), 1,2-dithio-bis-nitrobenzoic acid (DTNB), 1-chloro-2, 4-dinitrobenzene, bovine serum albumin (BSA), oxidized and reduced nicotinamide adenine dinucleotide phosphate (NADP), (NADPH), chrysin, flavine adenine dinucleotide, 2,6-dichlorophenolindophenol, thiobarbituric acid (TBA) etc: were obtained from Sigma-Aldrich, USA. Sodium hydroxide, ferric nitrate, trichloroacetic acid (TCA) and perchloric acid (PCA) etc were purchased from CDH, India. All other reagents used are of highest purity and commercially available.

Animals

Male Wistar rats (150–200 g), 6–8 weeks old, were obtained from the Central Animal House Facility of Hamdard University. Rats were housed in an animal care facility under room temperature ($25 \pm 1°C$) with 12 h light/dark cycles and were given free access to standard pellet diet and tap water. Before the treatment, rats were left for 7 days to acclimatize. Animals received humane care in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and prior permission was sought from the Institutional Animal Ethics Committee (IAEC No: 173/CPCSEA, 28 January 2000).

Experimental procedure

In the present study, we have evaluated the preventive efficacy of chrysin, against ethanol-induced hepatic and renal toxicity. Thirty female Wistar rats were divided into five groups, each with six animals. Group I as control received vehicle (distilled water), whereas Group II, III and IV were treated orally with ethanol (5, 8, 10 and 12 g/kg body weight (b. wt) per week respectively) for 4 weeks. Groups III and IV were administered with chrysin orally at 20 mg/kg b. wt (D1) and 40 mg/

kg b. wt (D2), respectively, 1 h prior to ethanol treatment, Group V was given only chrysin (40 mg/kg b. wt;D2).

After 28 days of ethanol administration, rats were sacrificed by cervical dislocation under mild anesthesia, and blood was taken by cardiac puncture for various serological parameters. Liver and kidney samples were taken at the same time for various biochemical parameters.

Preparation of post-mitochondrial supernatant, cytosolic and microsomal fractions

Liver and kidneys were removed and cleaned with ice-cold saline (0.85% sodium chloride). homogenates (10%) of liver and kidney tissues were obtained in a buffer solution containing 10 mM Tris–HCl, 250 mM, sucrose pH 7.4, using a Potter Elvehjen homogenizer and were centrifuged at 3000 r.p.m. for 10 min by the Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot so obtained was centrifuged at 12,000 r.p.m. for 20 min. to obtain PMS, which was used as a source of various enzymes. The supernatant obtained was further ultra-centrifuged at 34,000 r.p.m. for 1 h to obtain cytosolic fraction for ADH activity. The precipitate obtained was washed with homogenizing buffer to obtain the microsomal fraction for cytochrome P450 2E1 (CYP 2E1) activity. All the experimental manipulations were carried out at 4°C.

CYP 2E1 activity

The catalytic activity of CYP 2E1 was analyzed by measuring p-nitrophenol hydroxylation as described by Reinke and Moyer (1985). The reaction mixtures contained a 100 mM potassium phosphate buffer (pH 6.8), 1.0 mM ascorbic acid, 1 mM NADPH, 1 mg of hepatic microsomes and 100 mM p-nitrophenol in a total volume of 1.0 ml. The 4-nitrocatechol that was formed was determined spectrophotometrically at 511 nm. Data were expressed as nmol/mg/min.

ADH activity

ADH activity was determined by the method of Bonnichsen and Brink (1955). Briefly, ADH activity was measured in 50 mM glycine (pH 9.6), 0.8 mM NAD, 3 mM ethanol and 50 µl of cytosolic fraction in a final volume of 1 ml. Enzyme activity was measured at 340 nm and the activity was calculated as nmol NADH formed/min/mg protein using a molar extinction co-efficient of $6.22 \times 10^6 \, M^{-1} \, cm^{-1}$.

Assay for xanthine oxidase activity

The activity of xanthine oxidase (XO) was assayed by the method of Athar *et al.* (1996). The reaction mixture consisted of 0.2 ml PMS which was incubated for 5 min at 37°C with 0.8 ml of phosphate buffer (0.1 M, pH 7.4). The reaction was started by adding 0.1 ml of xanthine (9 mM) and kept at 37°C for 20 min. The reaction was terminated by the addition of 0.5 ml of ice-cold PCA (10% v/v). After 10 min, 2.4 ml of distilled water was added and centrifuged at 4000 r.p.m. for 10 min and µg uric acid formed/min/mg protein was recorded at 290 nm.

Assay for catalase activity

The catalase activity was assessed by the method of Claiborne (1985). In short, the reaction mixture was comprised of 0.05 ml of PMS, 1.0 ml of hydrogen peroxide (0.019 M), 1.95 ml of phosphate buffer (0.1 M, pH 7.4), in a total volume of 3 ml. Changes in absorbance were recorded at 240 nm, and the change in absorbance was calculated as nmol H_2O_2 consumed per min per mg of protein.

Estimation of lipid peroxidation

The assay of lipid peroxidation (LPO) was done according to the method of Wright et al. (1981). The reaction mixture consisted of 0.58 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml microsome, 0.2 ml of ascorbic acid (100 mM) and 0.02 ml of ferric chloride (100 mM) in a total of 1 ml. This reaction mixture was then incubated at 37°C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1 ml of TCA (10%). Following addition of 1.0 ml of TBA (0.67%), all the tubes were placed in a boiling water bath for a period of 20 min. The tubes were shifted to an ice bath and then centrifuged at $2500 \times g$ for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm. The results were expressed as the nmol MDA formed/h/g tissue at 37°C by using a molar extinction coefficient of $1.56 \times 10^5 M^{-1} cm^{-1}$.

Estimation of GSH

GSH was assessed by the method of Jollow et al. (1974). A quantity of 1.0 ml of 10% PMS mixed with 1.0 ml of (4%) sulphosalicylic acid was taken and then incubated at 4°C for a minimum time period of 1 h and then centrifuged at 4°C at $1200 \times g$ for 15 min. The reaction mixture of 3.0 ml was composed of 0.4 ml of supernatant, 2.2 ml of phosphate buffer (0.1 M, pH 7.4) and 0.4 ml of DTNB (4 mg/ml). The yellow color developed was read immediately at 412 nm on the spectrophotometer (Perkin Elmer, lambda EZ201). The GSH concentration was calculated as nmol GSH conjugates/g tissue.

Assay for glutathione peroxidase activity

The activity of glutathione peroxidase (GPx) was calculated by the method of Mohandas et al. (1984). The total volume of 2 ml was composed of 0.1 ml of EDTA (1 mM), 0.1 ml of sodium azide (1 mM), 1.44 ml of phosphate buffer (0.1 M, pH 7.4), 0.05 ml of GR (1 IU/ml), 0.05 ml of GSH (1 mM), 0.1 ml of NADPH (0.2 mM) and 0.01 ml of H_2O_2 (0.25 mM) and 0.1 ml of 10% PMS. The depletion of NADPH at 340 nm was recorded at 25°C. Activity of the enzyme was calculated as nmol NADPH oxidized/min/mg protein with the molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Assay for GR activity

GR activity was measured by the method of Carlberg and Mannervik (1975). The reaction mixture was composed of 1.65 ml of phosphate buffer (0.1 M, pH 7.6), 0.1 ml of NADPH (0.1 mM), 0.05 ml of GSSG (1 mM), 0.1 ml of EDTA (0.5 mM) and 0.1 ml of 10% PMS in a total volume of 2 ml. Enzyme activity was assessed at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min mg protein using the molar extinction coefficient of $6.22 \times 10^3 M^{-1} cm^{-1}$.

Assay for serum aspartate aminotransferase and alanine aminotransferase activity

Alanine aminotransferase (AST) and aspartate aminotransferase (ALT) activity were determined by the method of Reitman and Frankel (1957). Each substrate (0.5 ml; 2 mM α-ketoglutarate and either 200 mM L-alanine or L-aspartate) was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1.0 ml with 0.1 M (pH 7.4) phosphate buffer. The reaction mixture was incubated for exactly 30 and 60 min at 37°C for ALT and AST, respectively. Then 0.5 ml of 1 mM dinitrophenyl hydrazine (DNPH) was added to the reaction mixture; after another 30 min at room temperature, the color was developed by the addition of 5.0 ml of NaOH (0.4 N) and the product read at 505 nm.

Assay for lactate dehydrogenase activity

Lactate dehydrogenase (LDH) activity was estimated in serum by the method of Korenberg (1955). The assay mixture consisted of 0.2 ml of serum, 0.1 ml of 0.02 M NADH, 0.1 ml of 0.01 M sodium pyruvate, 1.1 ml of 0.1 M (pH 7.4) phosphate buffer and distilled water in a total volume of 3 ml. Enzyme activity was recorded at 340 nm, and activity was calculated as nmol NADH oxidized/min/mg protein.

Blood urea nitrogen

Estimation of blood urea nitrogen (BUN) was done by the method of Kanter (1975). Protein-free filtrate was prepared by adding serum and an equal amount of 10% TCA; then the mixture was centrifuged at 2000 r.p.m. and the supernatant was obtained. To 0.5 ml of the protein-free filtrate were added 3.5 ml of distilled water, 0.8 ml of diacetylmonoxime (2%) and 3.2 ml of sulfuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml of 85% phosphoric acid with 140 ml of water and 50 ml of concentrated sulfuric acid). The reaction mixture was placed in a boiling water bath for 30 min and then cooled. The absorbance was read at 480 nm.

Serum creatinine level

Creatinine was estimated by the method of Hare (1950). Protein-free filtrate was prepared. To 1.0 ml of serum were added 1.0 ml of sodium tungstate (5%), 1.0 ml of sulfuric acid (0.6 N) and 1.0 ml of distilled water. After mixing thoroughly, the mixture was centrifuged at $800 \, g$ for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05%) and 1.0 ml of sodium hydroxide (0.75 N). The absorbance at 520 nm was read exactly after 20 min.

Estimation of protein

The protein concentration in all samples was determined by the method of Lowry et al. (1951), using BSA as standard.

Statistical analysis

Differences between groups were analyzed using analysis of variance (ANOVA) followed by Dunnet's multiple comparisons test. All data points are presented as the treatment groups' mean ± standard error of the mean (SE).

RESULTS

Effect of chrysin on body weight and relative organ weight

Figure 1 shows per week average body weight of the animals treated with different treatment regimes for 4 weeks. After 4 weeks of study, rats treated with ethanol only showed a slight decrease in body weight (0 day = 202 ± 1.07; 28th day = 200.8 ± 2.1). However, the body weights of rats of Group III (0 day = 190 ± 2.3; 28th day = 198 ± 2.08) and Group IV (0 day = 186 ± 2.8; 28th day = 194.4 ± 2.18) indicate an increase of 8 g in both groups; this elevation in the body weight is almost similar to the increase in body weight of control animals (0 day = 202 ± 2.6; 28th day = 211.2 ± 2.8).

It has also been found that there is a slight decrease in relative liver and kidney weights, observed in the rats treated with ethanol only (liver = 2.15 ± 0.05; kidney = 0.24 ± 0.007) when compared with the control group (liver = 2.41 ± 0.09;

kidney = 0.27 ± 0.008). This slight decrease in relative organ weight has been restored by the chrysin administration (liver: D1 = 2.37 ± 0.17; D2 = 2.41 ± 0.11 kidney: D1 = 0.26 ± 0.017; D2 = 0.27 ± 0.014). However, there is no significant change in the relative organ weights in only chrysin-treated group (Fig. 2).

Effect of chrysin on ethanol-metabolizing enzymes

In ethanol-treated groups (Fig. 3), both livers and kidneys showed significant ($P < 0.001$) enhancement in ADH activities (8.4 ± 0.54 and 6.4 ± 0.35) when compared with the control group (6.24 ± 0.44 and 4.25 ± 0.24), respectively. The enhancement in ADH levels is 12 and 34% at dose D1 (7.64 ± 0.34) and D2 (6.92 ± 0.21) in liver and 21% in kidney tissues at dose D2 (5.47 ± 0.42) when compared with the ethanol-treated groups. Chrysin at a dose of 20 mg/kg b. wt shows a non-significant elevation in ADH activity in the kidney but there is 21% increase in ADH activity at higher dose (40 mg/kg b. wt) of chrysin administration ($P < 0.05$), whereas no changes in ADH activity were observed in only D2 groups.

Ethanol caused significant induction of CYP 2E1 ($P < 0.001$) in both hepatic (22.3 ± 0.74; 83%) and renal (16.26 ± 0.83; 108%) tissues when compared with the control group (12.18 ± 0.50). Treatment with chrysin brought back the level
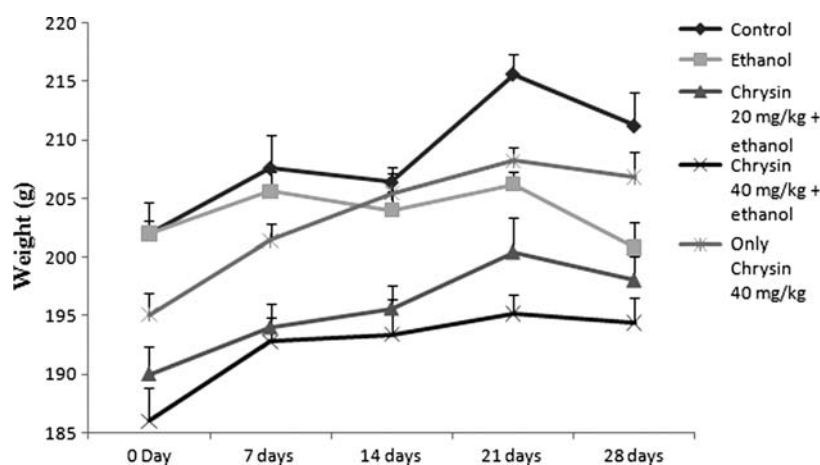

Fig. 1. Average increase in body weight (g) per week.
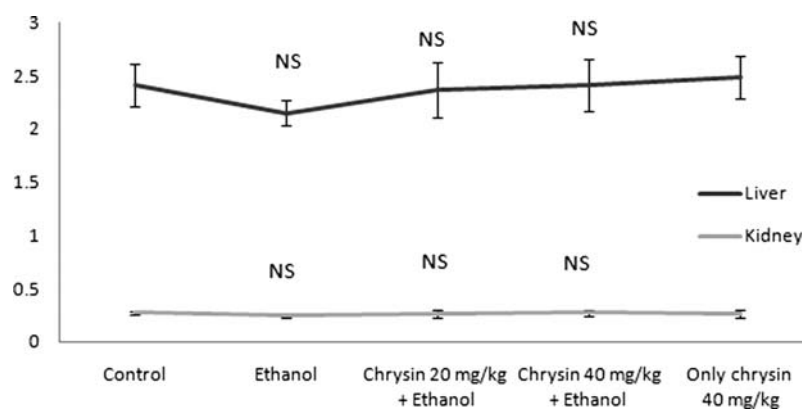

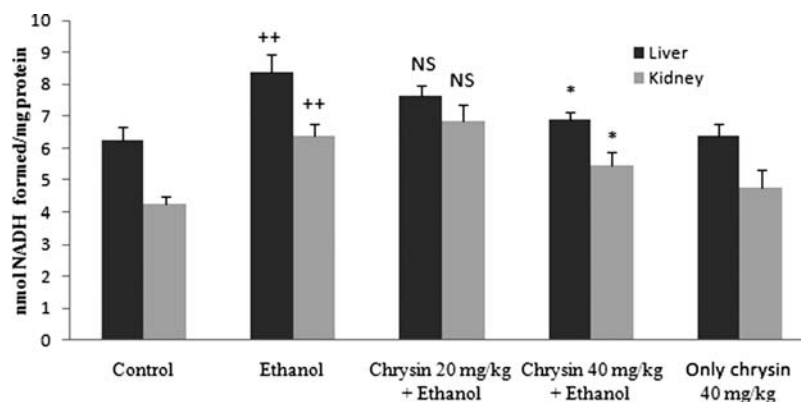Fig. 2. Relative organ weight.

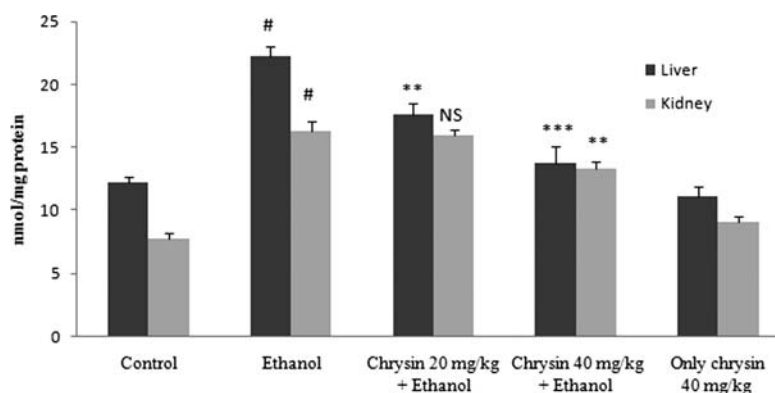Fig. 3. ADH activity in liver and kidney tissues.

Fig. 4. CYP 2E1 activity in liver and kidney.

Table 1. Effect of chrysin administration on ethanol-mediated depletion of hepatic GSH, GPx, GR, Catalase and elevation in XO activity in rats.

Groups	Reduced glutathione (μmol GSH conjugate/g tissue)	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/min/mg protein)	Catalase (nmol H_2O_2 consumed/min/mg protein)	Xanthine oxidase (μg of uric acid formed/min/mg protein)
Control	0.651 ± 0.01	449.6 ± 20.53	452.2 ± 17.8	54.2 ± 0.08	0.66 ± 0.06
Ethanol	$0.323 \pm 0.14\#$	$281.7 \pm 24.5\#$	$270.2 \pm 7.1\#$	$35.53 \pm 3.22\#$	$1.36 \pm 0.17\#$
Chrysin D1 + ethanol	$0.424 \pm 0.06**$	$366.8 \pm 9.02**$	303.4 ± 4.96^{NS}	41.33 ± 1.6^{NS}	0.92 ± 0.05^{NS}
Chrysin D2 + ethanol	$0.57 \pm 0.03***$	$376.1 \pm 8.3**$	$370.2 \pm 25.5***$	$45.54 \pm 1.04**$	$0.75 \pm 0.1**$
Only chrysin D2	0.660 ± 0.04	480.6 ± 8.36	473.5 ± 17.7	48.44 ± 0.90	0.67 ± 0.10

Each value represents mean $\pm$ S.E., $n = 6$.
NS, non-significant.
$\#P < 0.001$ compared with the corresponding value for control group.
$*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the corresponding value for ethanol-treated group.

of CYP 2E1 to normal in both hepatic (D1 = 38%, 17.31 ± 0.3, $P < 0.01$; D2 = 70%, 13.77 ± 1.34, $P < 0.001$) and renal tissue (D1 = NS, D2 = 13.26 ± 0.57, 38%, $P < 0.01$; Fig. 4).

Effect of chrysin on XO activity

XO reflected significant increase ($P < 0.001$) in the enzyme activity in hepatic (Table 1) and renal tissue (Table 2) of the ethanol-treated groups when compared with control. Chrysin significantly restores the level of XO activity by 66 and 92% in liver tissue and 51 and 80% in renal tissues at dose D1 and D2, respectively. Only D2 group showed no significant change when compared with the control group.

Effect of chrysin on hepatic and renal membrane damage (LPO)

MDA formation was measured to demonstrate the oxidative damage in ethanol-induced liver and renal injury of Wistar rats. A significant ($P < 0.001$) amplification of the MDA formation was found in the ethanol-treated groups in both hepatic (21.26 ± 1.001) and renal (8.72 ± 0.48) tissue when compared with respective controls (8.26 ± 0.48 and 4.38 ± 0.6). We have observed that treatment with chrysin at D1 and D2 leads to the significant restoration ($P < 0.01$ and $P < 0.001$, respectively) of membrane integrity in liver (D1 = 16.14 ± 0.44; D2 = 10.9 ± 1.6) and kidneys (D1 = 6.03 ± 0.47; D2 = 6.03 ± 0.43) when compared with ethanol-treated

Table 2. Effect of chrysin administration on ethanol-mediated depletion of renal GSH, GPx, GR, Catalase and elevation in XO activity in rats.

Groups	Reduced glutathione (μmol GSH conjugate/g tissue)	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)	Glutathione reductase (nmol NADPH oxidized/min/mg protein)	Catalase (nmol H_2O_2 consumed/min/mg protein)	Xanthine oxidase (μg of uric acid formed/min/mg protein)
Control	0.256 ± 0.015	251.2 ± 8.04	245.51 ± 2.41	25.32 ± 1.16	0.11 ± 0.01
Ethanol	$0.168 \pm 0.012^{\#}$	$115.5 \pm 13.2^{\#}$	$114.4 \pm 3.44^{\#}$	$13.52 \pm 0.58^{\#}$	$0.21 \pm 0.02^{\#}$
Chrysin D1 + ethanol	$0.199 \pm 0.015*$	$162.4 \pm 3.48**$	122.8 ± 0.21^{NS}	$18.57 \pm 0.56**$	0.15 ± 0.015^{NS}
Chrysin D2 + ethanol	$0.248 \pm 0.02**$	$218.4 \pm 0.15***$	$194.04 \pm 1.88**$	$25.5 \pm 1.18***$	$0.12 \pm 0.012**$
Only chrysin D2	0.252 ± 0.001	238.4 ± 8.19	218.4 ± 4.35	27.09 ± 0.72	0.119 ± 0.003

Each value represents mean $\pm$ SE, $n = 6$.
NS, non-significant.
$\#P < 0.001$ compared with the corresponding value for control group.
$*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared with the corresponding value for ethanol-treated group.
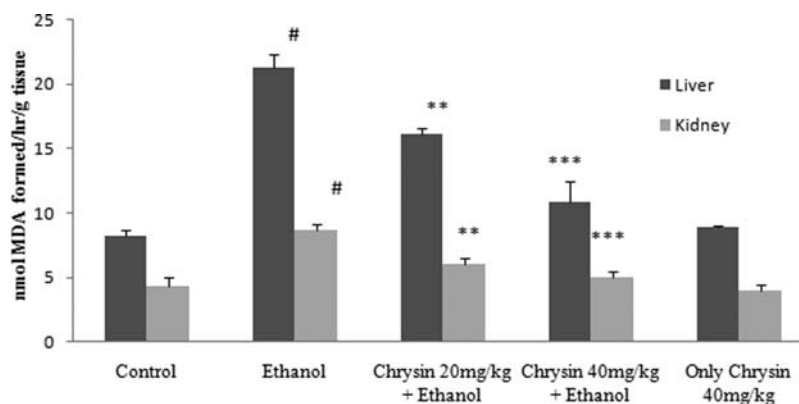

Fig. 5. Inhibition of LPO by chrysin in liver and kidney.

groups (Fig. 5). Chrysin alone did not show any significant difference when compared with control.

Chrysin treatment restores the activities of hepatic and renal anti-oxidants

Ethanol treatment was found to diminish hepatic and renal anti-oxidants GSH (50 and 34%) GPx (37 and 54%), GR (40 and 53%) and catalase (34 and 46%) when compared with the corresponding control group ($P < 0.001$). Treatment of chrysin significantly increases the level of GSH, GPx, GR and catalase in liver at dose D1 and D2 by 16 and 38%, 19 and 21%, 7 and 22% and 10 and 18% and in kidney by 12 and 31%, 19 and 41%, 4 and 32% and 20 and 46%, respectively (Tables 1 and 2), which indicates anti-oxidant property of chrysin against ethanol-induced oxidative stress.

Chrysin attenuates ethanol-induced hepatotoxicity

Ethanol-treated groups showed 112% ($P < 0.001$), 98% ($P < 0.001$) and 45% ($P < 0.001$) increase in serum AST, ALT and LDH levels, respectively, when compared with the control group. Chrysin administration was found significantly effective in the normalization of these serum toxicity markers by 38% ($P < 0.001$), 45% ($P < 0.001$) and 27% ($P < 0.01$) at D1 and 67% ($P < 0.001$), 67% ($P < 0.001$) and 43% ($P < 0.001$) at D2 when compared with ethanol-treated groups (Figs 6 and 7).

Chrysin treatment inhibits renal damage

The effect of chrysin administration on ethanol-mediated leakage of kidney toxicity markers (BUN and creatinine)

were shown in Figs 8 and 9. Figures 8 and 9 showed that rats treated with ethanol showed a significant increase in BUN (42.42 ± 1.76; $P < 0.01$) and creatinine (3.18 ± 0.1; $P < 0.001$) levels when compared with control (BUN $= 30.62 \pm 0.45$; creatinine $= 1.99 \pm 0.35$). Marked inhibition was observed in BUN at D2 (34.17 ± 2.03; $P < 0.01$) and in the creatinine level at D2 (2.02 ± 0.16; $P < 0.01$). No significant difference was found in the only D2 group compared with control.

Histopathology of liver tissue

Analysis of tissue sections of animals from different treatment groups under microscope ($\times 10$ and $\times 40$ enlargement) revealed marked changes when compared with control group animals (Fig. 10A and B). In the ethanol-treated animals, there was an apparent inflammatory response around the central vein in terms of infiltration of inflammatory cells. Moreover, ethanol also caused vacuolar degeneration and pronounced necrosis around the central vein (Fig. 10C) in liver tissue. In contrast, chrysin administration at both the doses (20 and 40 mg/kg b. wt) protected the liver histology against ethanol-induced alterations (Fig. 10D and E). Only chrysin administration (40 mg/kg b. wt) does not show any alterations from normal liver histology.

DISCUSSION

Alcohol consumption has been related to several alcohol-related illnesses, including cancer, liver pathology,
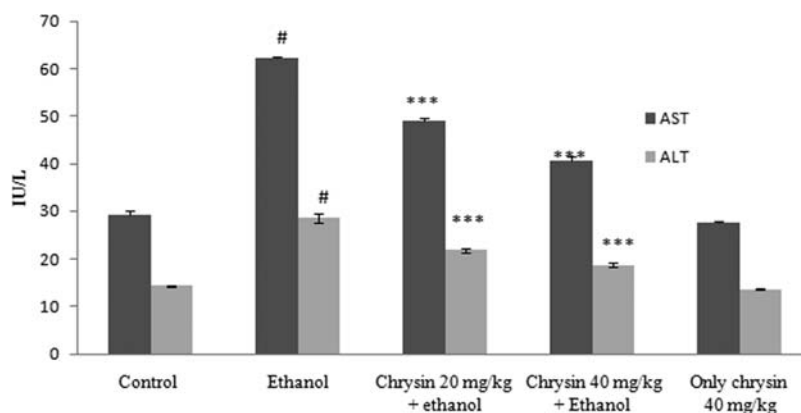
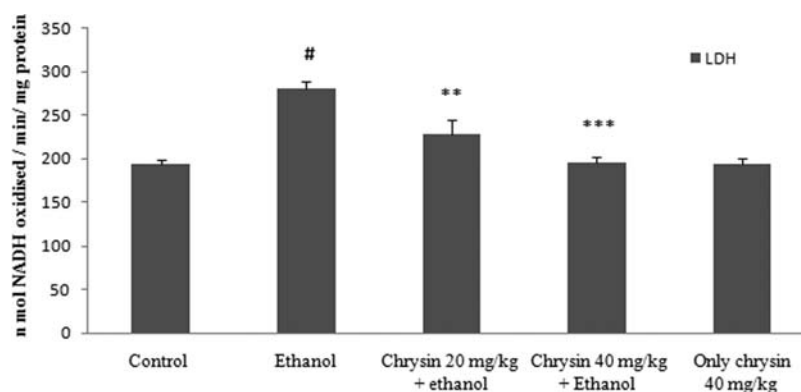Fig. 6. Serum markers of liver toxicity AST and ALT.

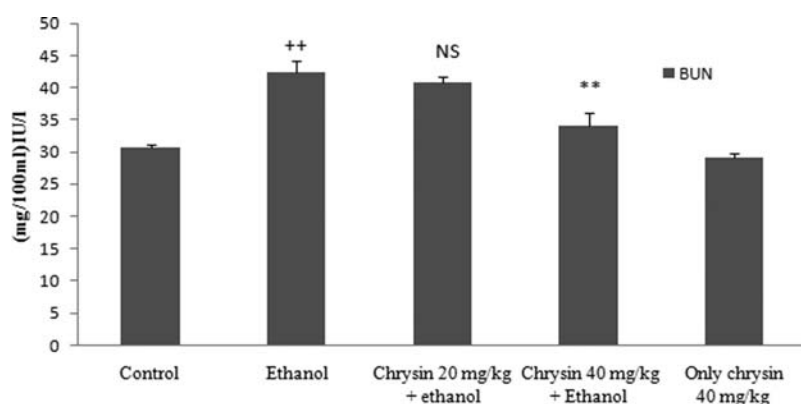Fig. 7. Effect of administration of chrysin on cytotoxicity marker LDH.

Fig. 8. Effect of chrysin administration on serum BUN level.

myopathy, cerebellar atrophy, testicular injury and immune suppression (Ishii *et al*., 1997). In the present study, an attempt has been made to prevent ethanol-induced liver and renal toxicity by suppressing ethanol-metabolizing enzymes by chrysin. Per week sequential increased dose of ethanol from 5 to 12 g/kg b.wt was used to induce maximum tissue damage and to overcome the tolerance produced by the ethanol consumption at the same dose. Rats treated with ethanol revealed lower growth in body and relative liver weight (Figs 1 and 2). Chronic consumption of alcohol does not produce a gain in body weight (Lieber, 1991). This poor

growth in body weight might be due to the reduction in adipose tissue content. Similar observations have been placed on record by Aruna *et al.* (2005) and Das and Vasudevan (2005). Contrarily, chrysin exhibited a potential ability to counteract the ethanol-induced changes in the body weight and relative organ body weight.

ADH is the key enzyme involved in the catabolism of ethanol to cytotoxic acetaldehyde giving rise to ROS (Gonthier *et al.,* 1991). Increased activity of ADH is associated with ROS formation, despite the fact the oxygen is not directly involved (Mattia *et al*., 1993). Moreover, ROS
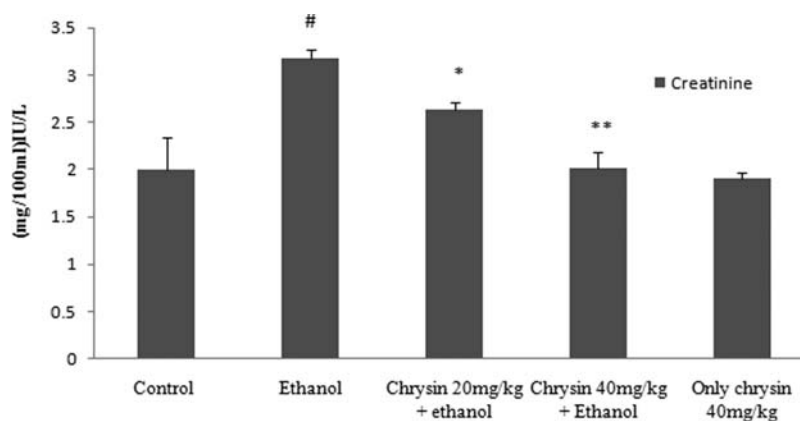
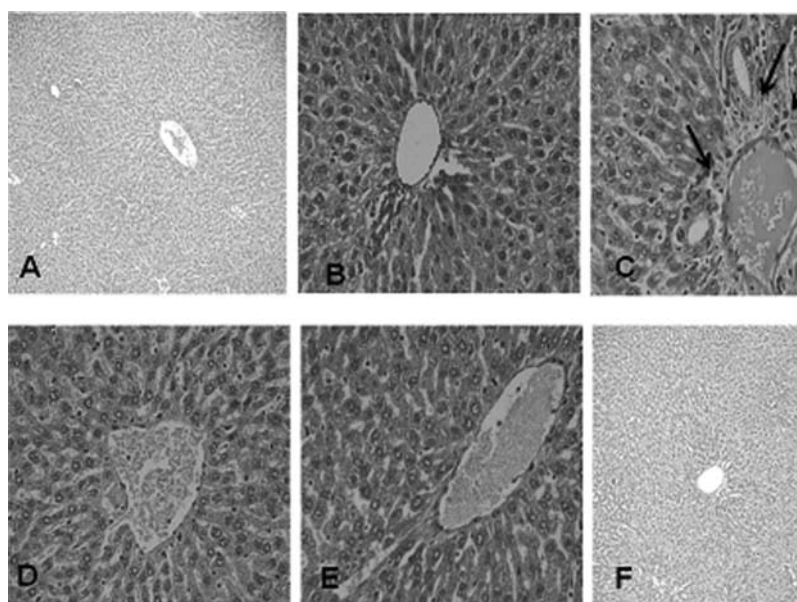Fig. 9. Effect of chrysin treatment on serum creatinine levels.

Fig. 10. Histopathology of liver.

formation from ethanol metabolism is associated with CYP 2E1 activity. Oxidation of ethanol to acetaldehyde by CYP 2E1 is coupled with the reduction of dioxygen to a variety of ROS, including $O_2^{\bullet-}$ (Mira *et al.*, 1995; Lieber, 1997). Our results are in complete conformity with the above findings that increased ethanol ingestion is linked with the enhancement of $O_2^{\bullet-}$ and other ROS, leading to an increase in the activities of alcohol-catabolizing enzymes and lipid peroxidation of cell membranes, causing oxidative stress and disruption in membrane integrity. Administration of chrysin significantly suppressed the activities of enzymes (CYP 2E1, ADH and XO) involved in the catabolism of ethanol in the liver and kidney when compared with the ethanol-administered rats, thereby reducing ROS-mediated tissue injuries during ethanol administration.

The cellular damage exhibits a good correlation with the enzyme leakage (Sehrawat and Sultana, 2006). Serum AST, ALT, LDH, BUN and creatinine are the most sensitive markers employed in the diagnosis of hepatic and renal damage (Sallie *et al.*, 1991). The present study entirely collaborates with the above findings that chronic ethanol administration leads to elevated levels of serum toxicity markers of the liver (ALT, AST and LDH) and the kidneys (BUN and creatinine), that is, indices of hepatic and renal dysfunction. The increase in the activities of these enzymes in the serum and subsequent fall in the tissue might be due to the leakage of these cytosolic enzymes into the circulatory system resulting from liver and kidney damage during ethanol administration. This is indicative of the onset of renal and hepato-cellular damage due to kidney and liver dysfunction and disturbance in the biosynthesis of these enzymes, with alteration in the membrane permeability. Administration with chrysin prevented ethanol-induced renal toxicity and hepatotoxicity, as indicated by a precipitous drop in serum ALT, AST, BUN, creatinine levels and LDH activity, possibly by maintaining the renal cellular and hepato-cellular membrane integrity. This is an indicator of possible nephro- and hepato-protective efficacy offered by chrysin compared with the untreated and ethanol-intoxicated groups.

Generation of ROS from acetaldehyde during ethanol metabolism may contribute to the oxidative stress in kidney

and liver tissues (Fernandez-Checha *et al.,* 1997; Rodrigo *et al.,* 1998) which is evident by a significant decrease in the activities of catalase and glutathione and dependent enzymes GPx and GR, in ethanol-treated rats. The decreased hepatic GSH in ethanol-intoxicated rats could be the result of hexose monophosphate shunt impairment and thereby altering oxidant: anti-oxidant status in the cells (Lu, 1999). During ethanol ingestion, the activities of some intracellular anti-oxidants have been reported to decrease with the increase in lipid peroxidation levels (Diplock *et al.,* 1994), and this fact is concomitant with the results of the present study, which was also in agreement with reports of Fernandez and Videla (1981) and Jaya *et al.* (1993). The analysis of the anti-oxidant status in our study indicates that both non-enzymatic and enzymatic anti-oxidants were decreased due to alcohol administration. Administration of chrysin with alcohol significantly modulates the anti-oxidant status in the liver and kidney of rats, suggesting the modulating effect of chrysin on cellular anti-oxidant defence.

On the basis of literature data (Neuman *et al*., 2002) available, our results permit us to conclude that the protective efficacy of chrysin against ethanol-induced liver and kidney injury must be attributed to its anti-oxidant properties enhancing natural anti-oxidant enzymes, cell membrane stabilizing property by inhibiting lipid peroxidation, suppression of microsomal CYP 2E1 activity, being a source for generation of free radicals in case of ethanol toxicity (Nordmann *et al.,* 1992) and inhibition of the ADH enzyme involved in the catabolism of ethanol to cytotoxic acetaldehyde. So it can be concluded that chrysin can be used as a potent hepato-protective and nephro-protective modulator against ethanol-induced hepatic and renal injuries.

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