

Hepatoprotective action of celery (*Apium graveolens*) leaves in acetaminophen-fed freshwater fish (*Pangasius sutchi*)

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Abstract Acetaminophen (APAP)-induced liver damage is one of the most common problems among the population. Therefore, the study was aimed to investigate the hepatoprotective effect of celery leaves on APAP-induced toxicity in a freshwater fish, *Pangasius sutchi*. Fish were divided into four experimental groups of 6 fish each. Group 1 served as control. Group 2 fish were exposed to APAP (500 mg/kg) for 24 h. Groups 3 and 4 fish were exposed to APAP + celery leaf powder (CE) (500 mg/kg) and CE for 24 h, respectively. The severity of liver damage, hepatic lipid, glycogen, ions status and histological alterations was examined. The characterization of CE extract was also performed. APAP-exposed fish showed elevated levels of both circulating and tissue hepatotoxic markers (AST, ALT and ALP), reduced hepatic glycogen and lipid contents (TG and cholesterol), increased tissue lipid peroxidation markers (TBARS, LHP and PCO), altered tissue levels of enzymatic (SOD, CAT, GPx and GST) and non-enzymatic (GSH) antioxidants and cellular thiol levels (T-SH, P-SH and NP-SH), and reduced hepatic ions (Na^+ , K^+ and Ca^{2+}) and abnormal liver histology. The abnormalities associated with APAP exposure were reversed on treatment with CE. The TLC separation and HPLC quantification of petroleum

ether/acetone extract of CE showed the peaks for highly efficient flavonoids such as rutein, quercetin and luteolin. The observed hepatoprotective effect of CE might be due to its rich flavonoids.

Keywords Acetaminophen · Hepatotoxicity · Lipid Peroxidation · Celery leaves

Introduction

Liver injury or liver dysfunction induced by acetaminophen, chemotherapeutic agents, carbon tetrachloride, alcohol, dantrolene sodium, valproic acid and isonicotinic acid hydrazide, etc., is a major health problem. Recently, liver injury has become one of the major problems in aquaculture; many farms have been suffering from the “liver and gall syndrome”, with the symptom of liver enlargement (up to twofold of original size) and colour change. The causes of this disease are not clear; pathogenic bacteria or viruses have not been identified. Xenobiotic challenge due to drug abuse and environmental pollution may be one of the most important causes of the disease. To prevent and control fish diseases, a large quantity of antibiotics and chemicals have been added into the water environment and feeds, which may, in turn, cause problems to fish.

It has been reported that, among a variety of drugs, acetaminophen (APAP, *N*-acetyl-4-aminophenol) is the most common cause of drug-induced liver injury

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(Ostapowicz et al. 2002). APAP is a non-steroidal anti-inflammatory drug (NSAID) commonly known as paracetamol, which has been widely used for over 50 years for the effective treatment as an analgesic and antipyretic at therapeutic doses (Xu et al. 2008). According to FDA; currently, 235 approved prescriptions and drug products contain APAP as an active ingredient. Although APAP is a well-known analgesic and antipyretic, its overdose (3 h following 500 mg APAP/kg body weight) causes acute liver failure (Hinson et al. 1998).

Acetaminophen metabolism generates the toxic metabolite, *N*-acetyl-*p*-benzoquinone imine (NAP-QI), and it is efficiently detoxified by glutathione (GSH), which is an important cellular antioxidant for detoxification of drugs and foreign chemicals. Therefore, an overdose will saturate the conjugation pathways of GSH and cause depletion of cellular GSH. This subsequently leads to a reduced capacity of GSH to detoxify NAPQI. It has been noted that APAP overdose depletes intracellular glutathione within 1–4 h (Al-Turk and Stohs 1981; Lores Arnaiz et al. 1995), resulting in accumulation of intracellular reactive oxygen and nitrogen species (ROS/RNS), causing oxidative/nitrosative stress and thus enhancing cellular injuries and organ dysfunction including renal damage (Hart et al. 1994). The overproduced NAPQI can covalently bind to the cysteinyl thiol groups of cellular proteins and form protein-(cystein-S-yl)-APAP adducts (Hoffmann et al. 1985), which may impair protein functioning. An increased NAPQI also induces/mediates the production of reactive oxygen species. Experimental studies have shown that a toxic dose of APAP produces hepatic necrosis in both humans (Davidson and Eastham 1966) and animals (Mudge et al. 1978) and also causes oxidative stress by depleting GSH and resulting in mitochondrial damage and cellular death (Agarwal et al. 2011).

N-acetylcysteine (NAC) is a standard drug used along with APAP to reduce APAP-linked problems. Even though NAC has anti-inflammatory, antioxidant and vasodilatory effects, (Lauterburg et al. 1984) it causes side effects including nausea, vomiting, flatus, diarrhoea, gastroesophageal reflux and even anaphylactic shock (Goldfrank and Flomenbaum 2006). Therefore, the identification of plant-based effective and safe drug(s) is essential to treat APAP-mediated toxicity.

Herbal medicines have recently gained much attention as alternative medicine to treat or prevent human diseases. Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease (Chattopadhyay 2003). Plant secondary metabolites (flavonoids) have shown antioxidative, anti-inflammatory, anti-proliferative, radical-scavenging activity and pro-apoptotic effects in various cell types (Ross and Kasum 2002). These biological compounds with various pharmacological functions contribute to the protection of hepatic cells and tissue against the deleterious effects of ROS and other free radicals (Simic and Jovanovic 1990; Sugihara et al. 1999). This suggests that flavonoid-based drug could be useful to alleviate APAP toxicity without side effects.

Celery (*Apium graveolens*; family, *Apiaceae*) is used in Indian system of medicine owing to its richness in flavonoids and antioxidant property for the treatment for liver ailments. Studies have indicated that celery lowers blood pressure, regulates heart function and reduces complications of diabetes (Mimica-Dukić and Popović 2007). The healing property of celery is due to its bioactive compounds like rutein, quercetin, luteolin, kaempferol, apigenin and myricetin. (Mimica-Dukić and Popović 2007). However, no reports have been found on the protective effect of *A. graveolens* on APAP-induced hepatotoxicity. Thus, we hypothesized that flavonoids rich in celery would prevent APAP-linked abnormalities. The present study examined the protective action of celery in acetaminophen-induced hepatotoxicity in *P. sutchi* in terms of hepatotoxic markers, oxidative stress, antioxidant and hepatic ions status, glycogen and lipid levels, and H & E staining. In addition, the constituents of celery were identified and quantitated by TLC and HPLC, respectively.

Materials and methods

Chemicals and drugs

The chemicals, solvents and drugs were of analytical grade and were purchased from Hi-Media Laboratories Pvt. Ltd. Mumbai, India, and Merck Chemicals Company, MO, USA. The biochemical and enzyme assay kits were purchased from Qualigen Diagnostics, Mumbai, India.

Plant collection and processing

The medicinal plant celery (*A. graveolens*) was collected from the local market, Chennai, Tamil Nadu, India. Matured celery plant leaves were used in the study. Pesticides and other contaminants were removed according to National Institute of Nutrition guidelines (85 % of contaminants, specifically pesticides, were removed by washing with water and subsequent treatment like blanching to facilitate complete removal of pesticides and contaminants). The aerial parts of the plants were washed with tap water, rinsed with distilled water and air-dried under shade with good ventilation at room temperature (37 °C) for a week. The leaves were cut into small pieces and crushed into fine powder using ball mill, and the powder (500 mg/kg body weight) was used for the experimental study (Ruepp et al. 2002).

Animals and maintenance

Fish of uniform size of length (15.3 ± 2 cm) and weight (28.46 ± 1.24 g) were segregated from the stock and acclimatized to laboratory conditions for 10 days in aquariums ($60 \times 30 \times 35$ cm) with 45 L of dechlorinated tap water (6–8 individuals per aquarium); the total mortality of fish was less than 1 %. During the experiment, water temperature was 30 ± 2 °C, pH was 7.5 ± 0.25 , dissolved oxygen was 8.6 ± 0.5 mg/L, and the photoperiod was 12:12 h light/dark cycle with total hardness 129.7 ± 8.3 mg as CaCO_3/L . Water was always aerated during acclimatization. The fish were fed throughout experimental period, but feeding was stopped 2 days prior to exposure. The fish were divided into four groups of six fish each.

Group 1 Control fish (CON) were fed with commercial pellet feed (protein 40 %, calcium 4 %, crude fat 5 %, fibre 3 %, ash 10 % and moisture 8 %) throughout experimental period.

Group 2 Fish were treated with APAP for 24 h in static tank containing 5 L of water. (APAP; 500 mg/kg was dissolved in 50 mL water at 70 °C and cooled to room temperature and added to the static tank).

Group 3 Fish were treated with APAP + CE for 24 h in static tank containing 5 L of water. (APAP; 500 mg/kg was dissolved in 50 mL of water at

70 °C and cooled to room temperature and mixed with CE (500 mg/kg body weight) and added to the static tank)

Group 4 Fish were treated with CE (500 mg/kg) alone for 24 h in static tank.

At the end of the experimental period, the fish were killed by decapitation. Blood was collected, and liver tissue was excised immediately and processed for analysis.

The liver tissue was homogenized in 0.01 M Tris-HCl (pH 7.2) and centrifuged at $24,000 \times g$ at 4 °C for 15 min. The supernatant obtained was used as an enzyme extract for the determination of enzymes. The protein content of tissue homogenate was estimated by the method of Lowry et al. (1951).

Biochemical analysis

Alanine transaminase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined spectrophotometrically using commercial kits (Qualigen Diagnostics, Mumbai, India). Glycogen content was estimated by the method of Morales et al. (1973). Cholesterol was estimated by the method of Natio (1984). Triglycerides were estimated by the method of Buccolo (1973). Levels of ions (Ca^{2+} , Na^+ and K^+) in the liver tissue were estimated using certified standards (USP1086334, USP1613407 and USP1549807, respectively) by the method of AOAC (1980) using an Atomic Absorption Spectrophotometer (Varian SpectrAA 10/20, Varian, Inc, India). The detection limits for the assay of Ca^{2+} , Na^+ and K^+ tend to range between 0.06, 0.01, 0.03 and 100 ± 10 mg/L, respectively.

Oxidative stress markers and antioxidants

The content of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxide (LHP) was measured by the methods described elsewhere (Rajasekar and Anuradha 2006). For TBARS measurement, tissue homogenate was deproteinized with 10 % trichloroacetic acid (TCA) and the precipitate was treated with thiobarbituric acid (TBA) at 90 °C for 1 h. The pink colour was measured at 535 nm, which gave a measure of TBARS. 1,1',3,3'-tetra methoxy propane was used as the standard, and the concentration was expressed as nanomol/mg protein. LHP content was measured in

methanol extract of tissue homogenate. A 0.2 mL aliquot of lipid sample was mixed with 1.8 mL of the reagent, which contained 90 mL of methanol, 10 mL of 250 mM sulphuric acid, 88 mg of butylated hydroxytoluene, 7.6 mg of xylenol orange and 9.8 mg of ferrous ammonium sulphate. The chromophore developed was read at 560 nm. The amount of hydroperoxide produced was calculated by using the molar extinction co-efficient of 4.6×10^4 (moles/L)/cm, and the concentration was expressed as $\mu\text{mol}/\text{mg}$ protein. The level of protein carbonyl was measured by the method of Levine et al. (1990). The liver tissue was homogenized in 10 mM HEPES buffer containing 137 mM NaCl, 4.6 mM potassium chloride, 1.0 mM potassium dihydrogen phosphate and 0.6 mM magnesium sulphate. The homogenate was centrifuged at $40,000 \times g$ for 20 min. The supernatant was mixed with dinitrophenyl hydrazine (DNPH) in 2 N hydrochloric acid and allowed to stand at room temperature (37 °C) for 1 h. The protein-hydrazone derivative was precipitated with TCA, and the precipitate was washed thrice with ethanol–ethylacetate (1:1). The precipitate was dissolved in guanidine HCl (6 M) solution and was read at 390 nm. Bovine serum albumin was used as a standard, and the concentration was expressed as $\mu\text{mol}/\text{mg}$ protein.

Activities of superoxide dismutase (SOD) (E.C.1.15.1.1), catalase (CAT) (E.C.1.11.1.6), glutathione peroxidase (GPx) (E.C.1.15.1.9) and glutathione-S-transferase (GST) (E.C.2.5.1.14) were measured in liver tissue by the methods described elsewhere (Rajasekar and Anuradha 2006). Briefly, SOD was assayed by the inhibition of the formation of NADH-phenazine methosulphate nitroblue tetrazolium formazan. CAT and GPx activities were assayed by measuring the amount of substrate consumed (hydrogen peroxide and glutathione, respectively) after carrying out the reactions for a specified period of time. GST was assayed using 1-chloro, 2,4-dinitrobenzene as a substrate. Total (T-SH), non-protein (NP-SH) and protein bound (P-SH) sulphhydryl groups were determined by the method of Sedlak and Lindsay (1968). For T-SH measurement, the liver homogenate in Tris buffer was treated with dithionitrobenzoic acid (DTNB, 99 mg/25 mL methanol) and made up to 10 mL with absolute methanol. The mixture was centrifuged at $3,000 \times g$ for 15 min. The absorbance of the clear supernatant was read at 412 nm. For NP-SH, liver tissue homogenate was treated with 50 % TCA. The thiol content was determined in the

supernatant by the reaction with DTNB using glutathione as the standard. P-SH value was obtained by subtracting NP-SH from TSH.

Histology

For histopathological analysis, small pieces of liver were immediately removed and fixed in 10 % formalin solution. It was then dipped in different concentrations of alcohol in ascending order and finally in absolute alcohol (10 min each) for removing water. It was kept in methyl benzoate until it sank and dipped in benzene for removing alcohol. The tissue was then infiltrated with molten paraffin (60–70 °C) for 1 h and 15 min. A boat was filled with molten paraffin, and the tissue was placed in it. The paraffin was then cooled until it hardened, enclosing the tissue. Using a rotary hand microtome, sections of 4- to 5- μ -thick paraffin-infiltrated tissue were made. The tissues were de-paraffinized with xylene and treated with 100, 90 and 70 % alcohol (10 min each) for removing undesirable pigment and other materials. The sections were then stained with haematoxylin and counter stained with eosin and dehydrated with 70 and 100 % alcohol for 10 min each. The sections were mounted using dibutylphthalate in xylene and examined under microscope.

Sample preparation for TLC and HPLC

The finely powdered plant material (1 g) was extracted with 10 mL of petroleum ether-acetone (8:2) solvents for 15 min. The sample was centrifuged at $671 \times g$ for 10 min. The supernatant was collected and filtered through Whatman No. 1 filter paper. The filtrate was used for chromatographic separation.

TLC separation of celery extract

About 1 mg/mL of the sample was spotted on the TLC plates, and the chromatogram was developed using petroleum ether-acetone solvent in ratio of 8:2. The spots were identified, and their R_f values were calculated and compared with standard R_f values of bioactive flavonoids.

HPLC analysis of celery extract

The prepared extract was also quantified by using reversed phase HPLC. A liquid chromatography

(LCGC—Agilent, injection volume 20 μ L) equipped with Nova-Pak C—18 (Waters associates, Milford, MA) column (4.6 mm \times 24 cm) using methanol, water and phosphoric acid (100:100:1) mixture as mobile phase and UV detection (270 nm) at flow rate of 1.5 mL/min was used. Chromatogram was compared with the chromatogram of standards. All the determinations were carried out in duplicates.

Statistical analysis

Values are expressed as mean \pm SD. Data within the groups are analysed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). Value of $p < 0.05$ was considered statistically significant.

Results

Effect of CE on tissue gross morphology

The gross morphology of the liver of control and experimental fish was observed at the end of the experimental period. A change in normal appearance of liver was observed in APAP-treated fish, (i.e.) liver appeared in dark black colour, whereas the colour change was reduced in APAP + CE-treated fish. CE-treated control fish showed normal morphology of liver as that of control fish.

Effect of CE on hepatotoxic markers

Figure 1 represents the levels of hepatotoxic markers (ALT, AST and ALP) in blood (Fig. 1a) and liver tissue (Fig. 1b) of control and experimental fish. An increase in both circulating and tissue levels of ALT, AST and ALP in APAP-exposed fish indicates the severity of liver damage. The levels of hepatotoxic markers, ALT, AST and ALP, were reduced to near-normal levels in the APAP + CE-treated fish. CE-treated control fish did not show any effect on these parameters.

Effect of CE on oxidative stress markers

Oxidative stress parameters (LHP, TBARS and protein carbonyl) in liver are presented in Table 1. APAP-treated fish showed an increase in lipid peroxidation

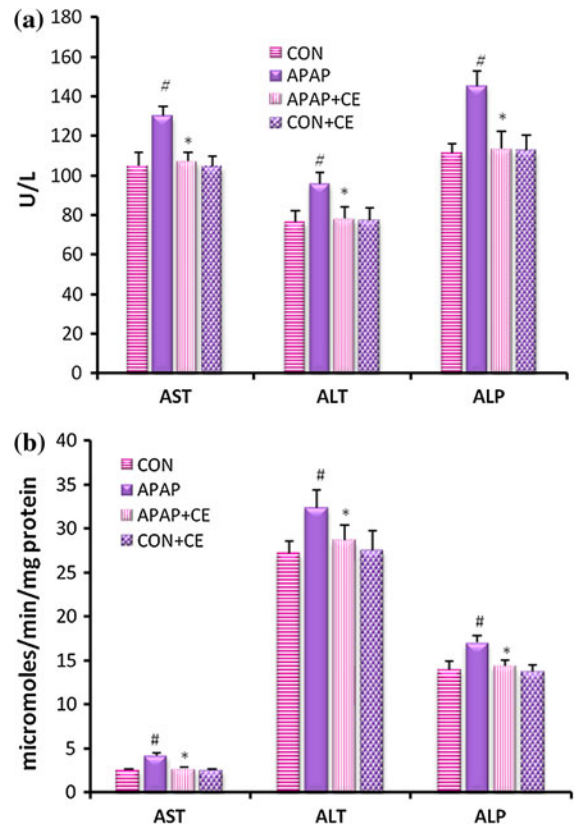


Fig. 1 Effect of CE on hepatotoxic markers in blood (a) and liver (b) of control and experimental fish. Values are mean \pm SD ($n = 6$ in each group). [#]($p < 0.05$) as compared to CON. ^{*}($p < 0.05$) as compared to APAP. CON control, APAP acetaminophen, CE celery

markers such as TBARS, LHP and protein carbonyl in liver tissue. This indicates susceptibility of liver damage under APAP exposure, whereas in APAP + CE-treated fish, the levels of LPO markers were reduced significantly ($p < 0.05$) to values close to those in control fish. CE-treated control fish did not show any changes in the levels of lipid peroxidation markers.

Effect of CE on enzymatic and non-enzymatic antioxidants

The activities of enzymatic (SOD, CAT, GPx and GST) and non-enzymatic (GSH) antioxidants and cellular thiols level (T-SH, NP-SH and P-SH) in the liver are shown in Table 2. APAP-exposed fish showed a decrease in the levels of SOD, GPx, GSH, T-SH, NP-SH and P-SH and an increase in the levels

Table 1 Effect of celery leaves on oxidative stress markers in liver of control and experimental fish

Parameters	CON	APAP	APAP + CE	CON + CE
LHP ^c	0.22 ± 0.01	0.34 ± 0.02 ^a	0.24 ± 0.01 ^b	0.21 ± 0.01
TBARS ^d	5.26 ± 0.36	7.45 ± 0.43 ^a	5.32 ± 0.12 ^b	5.24 ± 0.18
Protein carbonyl ^c	3.77 ± 0.11	8.58 ± 0.64 ^a	4.15 ± 0.22 ^b	3.75 ± 0.17

Values are mean ± SD ($n = 6$ in each group)

CON control, APAP acetaminophen, CE celery

^a ($p < 0.05$) as compared to CON

^b ($p < 0.05$) as compared to APAP. Data within the groups were analysed using one-way ANOVA followed by Duncan's multiple range test (DMRT)

^c μmoles/mg protein, ^d nanomoles/mg protein

Table 2 Effect of celery leaves on enzymatic and non-enzymatic antioxidants and thiols in liver of control and experimental fish

Parameters	CON	APAP	APAP + CE	CON + CE
<i>Enzymatic antioxidants</i>				
SOD ^d	3.82 ± 0.21	2.76 ± 0.20 ^a	3.65 ± 0.28 ^b	3.87 ± 0.12
CAT ^e	10.19 ± 0.74	36.99 ± 1.06 ^a	12.58 ± 0.96 ^b	10.25 ± 0.94 ^c
GPX ^f	1.89 ± 0.07	0.57 ± 0.03 ^a	1.79 ± 0.09 ^b	1.91 ± 0.12 ^c
GST ^g	0.71 ± 0.05	1.33 ± 0.14 ^a	0.76 ± 0.05 ^b	0.73 ± 0.06
<i>Non-enzymatic antioxidants</i>				
GSH ^d	0.38 ± 0.02	0.18 ± 0.01 ^a	0.36 ± 0.03 ^b	0.38 ± 0.02
<i>Thiols</i>				
NP-SH ^d	0.75 ± 0.03	0.22 ± 0.02 ^a	0.71 ± 0.02 ^b	0.76 ± 0.03 ^c
T-SH ^d	2.51 ± 0.07	1.72 ± 0.04 ^a	2.46 ± 0.03 ^b	2.52 ± 0.04 ^c
P-SH ^d	1.77 ± 0.05	1.50 ± 0.05 ^a	1.74 ± 0.03 ^b	1.76 ± 0.07

Values are mean ± SD ($n = 6$ in each group)

CON control, APAP acetaminophen, CE celery

^a ($p < 0.05$) as compared to CON

^b ($p < 0.05$) as compared to APAP

^c ($p < 0.05$) as compared to APAP and APAP + CE. Data within the groups were analysed using one-way ANOVA followed by Duncan's multiple range test (DMRT)

^d μmoles/min/mg protein

^e μmoles of H₂O₂ consumed/min/mg protein

^f μmoles of CDNB-GSH conjugate formed/min/mg protein

^g μg of GSH formed/min/mg protein

of CAT and GST, whereas the enzymatic and non-enzymatic antioxidants alterations linked with APAP exposure were ameliorated in APAP + CE-treated fish. CE-treated control fish did not show any alteration in the normal enzymatic and non-enzymatic antioxidants status, whereas the hepatic thiol content was increased, suggesting thiol-promoting action of CE.

Effect of CE on glycogen and lipids

Table 3 gives the concentrations of glycogen and lipids (cholesterol and triglyceride) in liver of control and experimental fish. The levels of glycogen, cholesterol and triglyceride were reduced in the APAP-treated fish compared to those of the control fish. The metabolic alterations linked with APAP

Table 3 Effect of celery leaves on hepatic glycogen and lipid levels of control and experimental fish

Parameters	CON	APAP	APAP + CE	CON + CE
Glycogen ^d	2.13 ± 0.10	1.24 ± 0.05 ^a	1.98 ± 0.12 ^b	2.14 ± 0.12 ^c
Cholesterol ^d	10.70 ± 0.68	7.27 ± 0.47 ^a	10.18 ± 0.54 ^b	10.61 ± 0.97
Triglyceride ^d	4.14 ± 0.21	2.02 ± 0.10 ^a	3.73 ± 0.27 ^b	4.08 ± 0.11 ^c

Values are mean ± SD ($n = 6$ in each group)

CON control, APAP acetaminophen, CE celery

^a ($p < 0.05$) as compared to CON

^b ($p < 0.05$) as compared to APAP

^c ($p < 0.05$) as compared to APAP and APAP + CE. Data within the groups were analysed using one-way ANOVA followed by Duncan's multiple range test (DMRT)

^d mg/g tissue

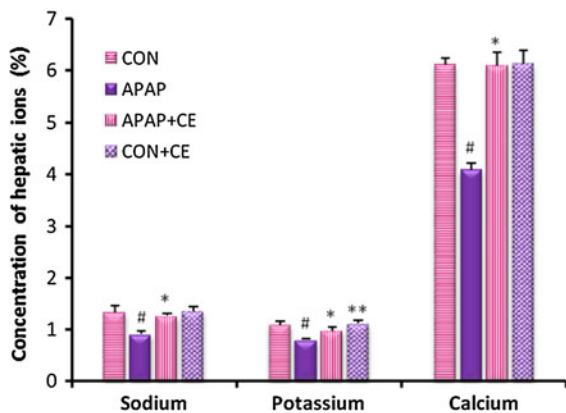


Fig. 2 Effect of CE on the hepatic ions status of control and experimental fish. Values are mean ± SD ($n = 6$ in each group). # ($p < 0.05$) as compared to CON. * ($p < 0.05$) as compared to APAP. ** ($p < 0.05$) as compared to APAP and APAP + CE. CON control, APAP acetaminophen, CE celery

exposure were brought back to near-normal levels in APAP + CE-treated fish. CE-treated control fish did not show any alterations in glycogen and lipid levels.

Effect of CE on hepatic ions

Figure 2 indicates the levels of hepatic ions in control and experimental fish. The hepatic levels of sodium (Na^+), potassium (K^+) and calcium (Ca^{2+}) were significantly lowered in APAP-exposed fish, whereas the altered hepatic ions were restored to near-normal ($p < 0.05$) in the APAP + CE-treated fish. CE-treated control fish did not show any alterations in the hepatic mineral levels.

Effect of CE on tissue histology

Figure 3 represents the liver histology of control and experimental fish. The H&E staining of liver clearly showed vacuolar degeneration (Fig. 3b-A*), hepatocellular damage (Fig. 3b-B*) and congestion (Fig. 3b-C*) in APAP-treated fish; these changes were reduced (i.e. mild vacuolar degeneration, reduced hepatocellular damage and absence of congestion) in the simultaneous APAP + CE-treated fish. Control (Fig. 3a) and CE-received control fish (Fig. 3d) showed normal morphology of liver.

Identification of flavonoids by TLC

Figure 4 reveals the TLC separation of CE. The chromatogram showed 7 clear bands (Fig. 4). The R_f values of the separated bands were compared with standard R_f values of flavonoids. The results indicated the existence of plant pigments and flavonoids (xanthophylls, rutein, quercetin, chlorophyll b, luteolin, chlorophyll a and myricetin) in the extract of CE.

High-performance liquid chromatography (HPLC) analysis

Figure 5 gives the HPLC chromatogram of standard and CE extract. The flavonoids content of CE extract was quantified by HPLC using standard flavonoids: rutein, quercetin, luteolin and kaempferol (Fig. 5a). Sharp peaks for flavonoids such as rutein, quercetin and luteolin were obtained in the petroleum ether/acetone extract of CE (Fig. 5b), and the concentration of observed flavonoids was found to be 1.99, 15.89 and 27.20 mg/dL, respectively.

Fig. 3 H&E staining of the liver tissue in control and experimental fish.

(a) Normal liver architecture of control fish. The APAP-treated fish (b) showed vacuolar degeneration (A^*), hepatocellular damage (B^*) and congestion (C^*) which was found to be absent in the control fish, whereas simultaneously APAP + CE-treated fish (c) showed reduced hepatocellular damage, mild vacuolar degeneration and absence of congestion. CE-received control fish (d) showed normal morphology of liver

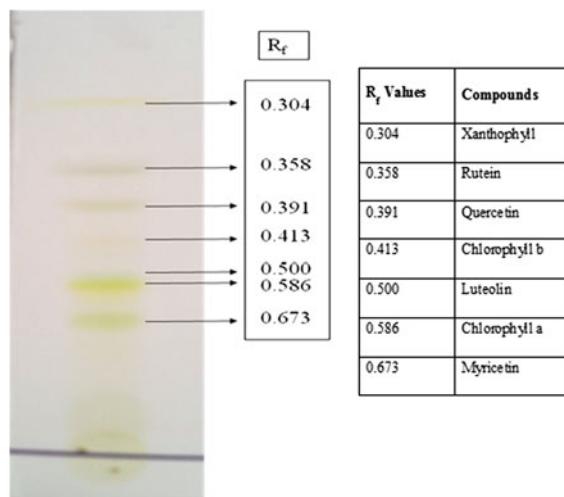
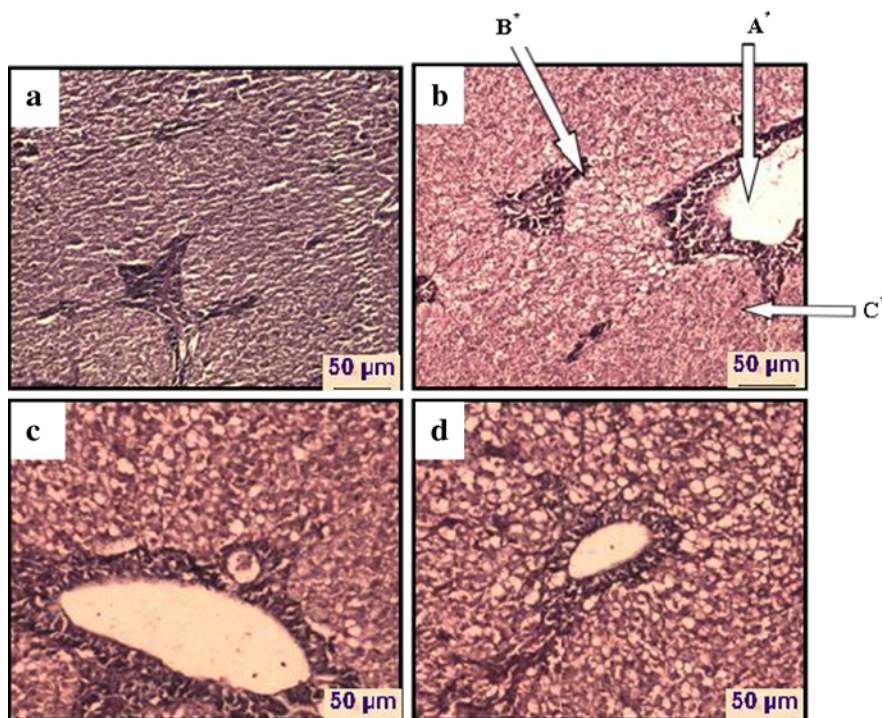


Fig. 4 Thin-layer chromatogram of CE extract. Seven clear bands pertaining to xanthophylls, rutein, quercetin, chlorophyll b, luteolin, chlorophyll a and myricetin were separated from the petroleum ether/acetone mixture extract of CE

Discussion

The present study investigated the effect of CE on APAP-induced liver damage, biochemical alterations and liver histology in a freshwater fish *P. sutchi*. The appearance of dark black liver of APAP-exposed fish

confirmed the state of liver toxicity. In the simultaneously APAP + CE-treated fish, colour of liver appeared normal, suggesting the highest hepatoprotective effect of CE; and this could possibly be attributed to the high level of flavonoids (Wegner and Fintelmann 1999) content of celery which may preserve normal liver architecture from APAP-mediated stress and damage.

The precise mechanism of APAP-induced liver toxicity is not clear; however, it is known that the reactive metabolite, NAPQI, plays a key role (Jaeschke and Bajt 2006). It is reported that toxic exposure of APAP in fish results in rare local hepatocyte necrosis without zonal pattern (Blair et al. 1990). But other studies have suggested that the less hepatotoxic effect of APAP in fish may be due to a relative inability to convert APAP to its reactive intermediate (Thomas and Wofford 1984; Hinton et al. 2001). Concurrently, another report has shown that APAP itself independently forms protein adducts in mitochondrial liver samples within a short period (60 min) of exposure and develops acute toxicity with liver histological alterations in rats (Ruepp et al. 2002). It has also been suggested that ROS-induced, lipid peroxidation is the most invoked mechanism for tissue injury, including due to drug-induced toxicity

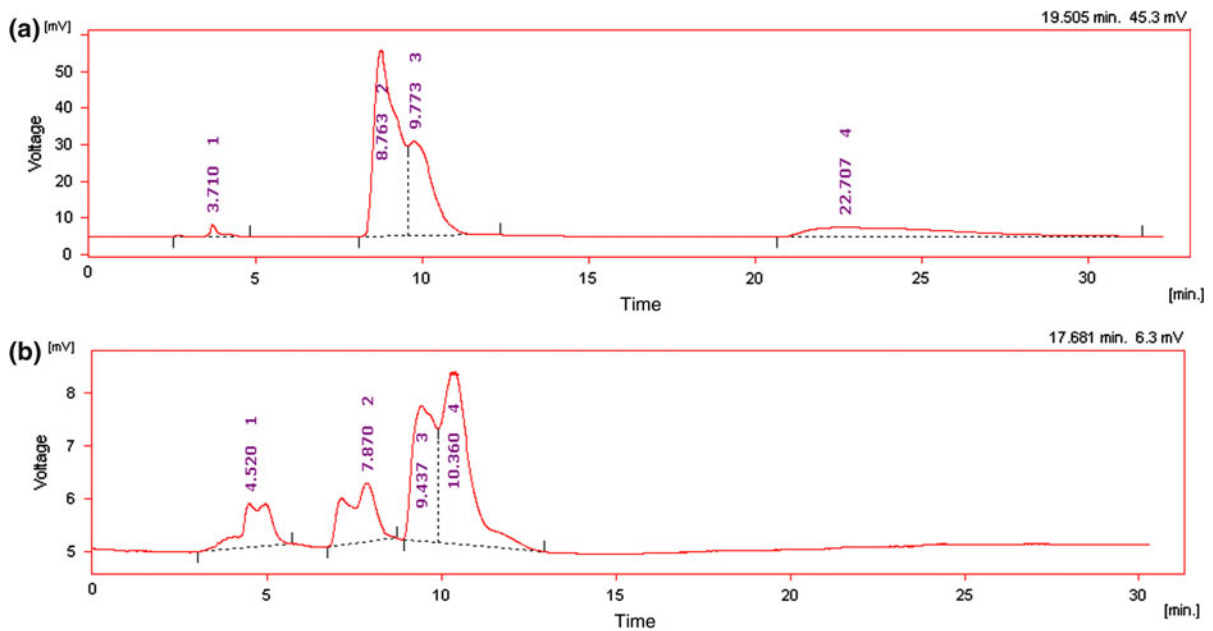


Fig. 5 HPLC chromatogram of standard flavonoids (a) and CE extract (b). The retention time for standard flavonoids such as rutein (3.710), quercetin (8.763), luteolin (9.773) and

kaempferol (22.707) was compared with the chromatogram of CE extract which showed *sharp peaks* for rutein (4.520), quercetin (7.870) and luteolin (9.437)

(Negre-Salvayre et al. 2010). Our study suggests that apart from NAPQI, APAP and its other reactive metabolites and metabolites of oxidative stress may directly react with APAP molecular targets like GST and GPx (Qiu et al. 1998) in 24-h exposure and this might develop a specific acute toxicity in *P. sutchi*.

Increase in AST, ALT and ALP activity in blood and liver tissue of APAP-exposed fish reveals APAP-induced liver tissue damage. ALT and AST are two mitochondrial enzymes (Gharaei et al. 2011) and are also found in the cell cytoplasm in higher concentrations particularly ALT. Higher levels of these enzymes in blood might be a result of leakage from cells due to its high concentration and cell membrane damage. It has been reported that paracetamol metabolism triggers lipid peroxidation and thereby causes liver injury in fish and mice (Gharaei et al. 2011; Wendel et al. 1982). On the other hand, these enzymes are involved in stress-induced gluconeogenesis, suggesting that the rise in aminotransferases may be due to a need to process amino acids from proteins as a result of extensive tissue damage (Masola et al. 2008).

Excess ROS promotes protein oxidation, forming protein carbonyls and advanced oxidation protein product (AOPP), which have been described as reliable

markers for protein damage (Alderman et al. 2002; Chevion et al. 2000). It also promotes lipid peroxidation, forming lipid peroxidation end products (TBARS and MDA). The increased levels of TBARS, LHP and protein carbonyl in the liver tissue of the freshwater fish indicate tissue protein and lipid damage exerted by APAP, and this could be due to APAP-mediated massive ROS generation and increased peroxidation. Our finding is supported by other researchers, who have observed that oral exposure of APAP (500 mg/kg) in a freshwater fish (*Oreochromis mossambicus*) for 24-h duration induces tissue damage and alters the antioxidants (Kavitha et al. 2011). An over dose of APAP (750 mg/kg) causes severe liver damage and increases lipid peroxidation to minor extent in normal animals (Knight et al. 2003). Abdul Hamid et al. (2012) have also shown the significant elevation of plasma protein carbonyls and renal homogenate AOPP levels in the paracetamol-treated rats, indicating the existence of oxidative stress and upregulation of NAPQI production (due to reduced capacity of GSH) (Hart et al. 1994).

APAP + CE-treated *P. sutchi* showed reduced hepatic lipid peroxidation, suggesting antioxidant activity of CE; and this could possibly neutralize or

inhibit the NAPQI-induced free radical generation under APAP exposure. Studies have shown anti-colon cancer activity, antioxidant activity and good healing property of CE due to its bioactive compounds like rutin, quercetin, luteolin, kaempferol, apigenin and myricetin (Mimica-Dukic and Popovic 2007). In our study, the identified flavonoids content of celery leaf extract might attenuate APAP-induced lipid peroxidation.

SOD converts the superoxide anion radical to O_2 and H_2O_2 , which is detoxified by both CAT and GPx through reduction of H_2O_2 to H_2O and O_2 (Vutukuru et al. 2006). Superoxide can react with nitric oxide to form a potent oxidant and nitrating species called peroxynitrite ($ONOO^-$) (Radi et al. 2001). In the present study, APAP exposure for 24 h reduced the SOD activity in the liver of freshwater fish, indicating the interaction of SOD with peroxynitrate. It has been reported that APAP forms peroxynitrate in cultured hepatocytes (Yan et al. 2010). The nitration of mitochondrial SOD by nitrating species and the oxidation of the cysteine group in SOD by excess superoxide radicals or their transformation to H_2O_2 might reduce SOD activity (Agarwal et al. 2011).

The increased CAT activity in liver of APAP-treated fish reveals protective mechanism against oxidative stress. It has been demonstrated that CAT is so efficient that it cannot be saturated by H_2O_2 at any concentration (Mate's and Sa'nchez-Jime'nez 1999). In the present study, the elevated tissue CAT activity could possibly be due to acquisition of tolerance to APAP-mediated increased accumulation of toxic H_2O_2 and other oxyradicals.

GPx additionally detoxifies hydrogen and organic hydroperoxides, utilizing glutathione (GSH) as co-factor, hence having an important role in protecting cells from lipid peroxidation. The reduced tissue GPx activity in *P. sutchi* under APAP exposure reveals the damage of GPx protein. Qiu et al. (1998) identified 30 proteins modified by APAP at a dose of 350 mg/kg for 2 h. Of these, six were mitochondrial proteins, including GPx and the adenosine triphosphate (ATP) synthase α -subunit. The observed reduction in GPx activity could be due to the structural alteration of GPx protein by APAP or its metabolic intermediate(s). Additionally, APAP-mediated GSH depletion could also be one of the reasons for the downregulated activity of GPx in the liver tissue of APAP-treated fish.

Glutathione-S-transferases are enzymes of the phase II biotransformation pathway, which detoxify a variety of chemical compounds by conjugating toxic electrophilic compounds with GSH. The upregulated GST activity and decreased GSH in liver of APAP-exposed freshwater fish suggest the efficient neutralization of ROS by GST in association with GSH. Our finding is supported by Sun et al. (1988) who explained the decline in GSH level at lower concentrations and inductions of GST activity were observed in *Carassius auratus* liver, exposed to pyrene.

The non-enzymatic biological antioxidant GSH acts as a cellular reducing agent which protects against toxic substance-mediated cellular redox changes. From the study, it was noted that lowered GSH and cellular thiol status (TSH, P-SH and NP-SH) in the liver of APAP-treated fish infers severe oxidative damage exerted by APAP. Two reasons could contribute to the cellular GSH and thiol depletion under APAP exposure: one is conjugation of NAPQI or metabolite of APAP with GSH, and another is transformation from GSH to GSSG under severe oxidative stress.

The altered tissues enzymatic (SOD, CAT, GPx, and GST) and non-enzymatic antioxidants (GSH) and cellular thiol levels (T-SH, P-SH and NP-SH) were brought to near-normal levels in APAP + CE-treated fish, suggesting the antioxidant-promoting activity of CE. Generally, it has been shown that flavonoids chelate both iron and copper by their carbonyl and hydroxyl groups and also function as terminators of free radicals by donation of electrons to form stable products. Flavonoids are very effective scavengers of hydroxyl and peroxy radicals as well as quenching superoxide radicals and singlet oxygen (Simic and Jovanovic 1990).

High dosage of APAP depleted the hepatic levels of glycogen, triglyceride and cholesterol in the *P. sutchi*, indicating impairment in the carbohydrate and lipid metabolism. Loss of glycogen or lipid can occur as a direct effect of intoxication, or it may occur secondary to decreased body condition caused by inanition, stress or concurrent disease (Wolf and Wolfe 2005). Recently, it has been shown that both subtoxic and toxic doses of APAP downregulate genes of energy-consuming biochemical pathways including gluconeogenesis (glucose-6-phosphatase), fatty acid synthesis (fatty acid synthase; sterol-C4-methyl oxidase-like) and cholesterol synthesis (3-hydroxy-3-

methylglutaryl-coenzyme A synthase 1) and upregulates genes of energy-producing biochemical pathways, such as glycolysis/gluconeogenesis (6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1) (Yuxia and Richard 2010).

In the simultaneously APAP + CE-treated fish, APAP-mediated hepatic glycogen and lipid depletion were restored to near-normal, suggesting metabolic regulatory action of CE and this could be due to its high content of antioxidant flavonoids (apigenin, luteolin, quercetin, rutein, etc.), in addition to their vitamins and mineral contents (Wada and Ou 2002).

The liver of APAP-treated fish showed reduced Na^+ , K^+ and Ca^{2+} ions when compared with control fish, suggesting that APAP-treated group experienced ionic disturbances. In freshwater fish, membrane-bound ATPases (Mg^{2+} and Na^+/K^+ -ATPases) play a significant role in ionic regulation of cellular components and maintenance of tissue osmolarity (Sancho et al. 2003) against concentration gradients and across membranes (Kirschner 2004). ATPases are integral membrane proteins which require thiol groups and phospholipids to maintain their structure and function (Hazarika and Sarkar, 2001). Several xenobiotics and oxidative stress can adversely affect Na^+/K^+ -ATPases activity (De la Torre et al. 2007). Bironaite and Ollinger (1997) have also reported that lipid peroxidation can influence the function of Ca^{2+} and Mg^{2+} -ATPases and the activity of membrane Ca^{2+} translocase. This suggests that peroxidation not only alters the lipid milieu but also affects the structural as well as functional integrity of cell membrane-bound enzymes including Mg^{2+} ATPases, Ca^{2+} ATPases and Na^+ , K^+ ATPases. Our study did not measure membrane-bound ATPases activity in the liver of APAP-treated fish; however, APAP and its toxic metabolite(s)-mediated cellular thiols depletion was observed and this could possibly affect the structure and function of membrane-bound ATPases and thereby altered the hepatic minerals in the APAP-treated freshwater fish.

Simultaneously, APAP + CE-treated fish showed near-normal hepatic mineral levels, which reveals the membrane-stabilizing action of CE against APAP and this could possibly restore the hepatic ions in *P. sutchi*.

Histopathological examination of APAP-exposed fish liver tissue showed hepatocellular vacuolar degeneration and blood vessel congestion. These histological alterations linked with APAP exposure were effectively mitigated in the simultaneously

APAP + CE-treated fish. This suggests that the short-term treatment of efficient flavonoids [rutein (1.99 mg/dL), quercetin (15.89 mg/dL) and luteolin (27.20 mg/dL)]-rich CE could act in multiple ways (i.e. chelating or scavenging the APAP-generated oxyradicals, protecting antioxidant enzymes, preserving hepatocyte structure, promoting antioxidants levels, maintaining normal carbohydrate and lipid metabolism and hepatic ions) to preserve the normal liver function in *P. sutchi* from the toxic effects of APAP. In relation to public health, when there is a possibility of APAP toxicity, the inclusion of such flavonoid-rich celery in the diet is strongly suggested.

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References

- Abdul Hamid Z, Budin SB, Wen Jie N, Hamid A, Husain K, Mohamed J (2012) Nephroprotective effects of *Zingiber zerumbet* Smith ethyl acetate extract against paracetamol-induced nephrotoxicity and oxidative stress in rats. *J Zhejiang Univ-Sci B* 13:176–185
- AC AO (1980) Official methods of analysis, 13th edn. Association of Official Analytic Chemists, Washington DC, pp 376–384
- Agarwal R, Macmillan-Crow LA, Rafferty TM, Saba H, Roberts DW, Fifer EK (2011) Acetaminophen-induced hepatotoxicity in mice occurs with inhibition of activity and nitration of mitochondrial manganese superoxide dismutase. *J Pharmacol Exp Ther* 337:110–116
- Alderman CJ, Shah S, Foreman JC, Chain BM, Katz DR (2002) The role of advanced oxidation protein products in regulation of dendritic cell function. *Free Radic Biol Med* 32:377–385
- Al-Turk WA, Stohs SJ (1981) Hepatic glutathione content and aryl hydrocarbon hydroxylase activity of acetaminophen-treated mice as a function of age. *Drug Chem Toxicol* 4:37–48
- Bironaite D, Ollinger K (1997) The hepatotoxicity of rhein involves impairment of mitochondrial functions. *Chem Biol Interact* 103:35–50
- Blair JB, Hinton DE, Miller MR (1990) Morphological changes in trout hepatocytes exposed to acetaminophen. *Mar Environ Res* 28:1–4
- Buccolo G (1973) Quantitative determination of serum triglycerides by use of enzymes. *Clin Chem* 19:476–482
- Chattopadhyay RR (2003) Possible mechanism of hepatoprotective activity of *Azadirachta indica* leaf extract: part II. *J Ethnopharmacol* 89:217–219

- Chevion M, Berenshtein E, Stadtman ER (2000) Human studies related to protein oxidation: protein carbonyl content as a marker of damage. *Free Radic Res* 33:S99–S108
- Davidson DG, Eastham WN (1966) Acute liver necrosis following overdose of paracetamol. *Br Med J* 5512:497–499
- De la Torre FR, Salibia'n A, Ferrari L (2007) Assessment of the pollution impact on biomarkers of effect of a freshwater fish. *Chemosphere* 68:1582–1590
- Gharaei A, Ghaffari M, Keyvanshokoo S, Akrami R (2011) Changes in metabolic enzymes, cortisol and glucose concentrations of Beluga (*Huso huso*) exposed to dietary methyl mercury. *Fish Physiol Biochem* 37:485–493
- Goldfrank LR, Flomenbaum N (2006) Goldfrank's toxicologic emergencies, 8th edn. McGraw Hill Professional, New York, pp 1623–1628
- Hart SG, Beierschmitt WP, Wyand DS, Khairallah EA, Cohen SD (1994) Acetaminophen nephrotoxicity in CD-1 mice. I. Evidence of a role for in situ activation in selective covalent binding and toxicity. *Toxicol Appl Pharmacol* 23:23–54
- Hazarika A, Sarkar SN (2001) Effect of isoproterenol pretreatment on the biochemical toxicodynamics of anilofos in male rats. *Toxicol* 165:87–95
- Hinson JA, Pike SL, Pumford NR, Mayeux PR (1998) Nitrotyrosine-protein adducts in hepatic centrilobular areas following toxic doses of acetaminophen in mice. *Chem Res Toxicol* 11:604–607
- Hinton DE, Segner H, Braunbeck T (2001) Toxic responses of the liver. In: Schlenk D, Benson WH (eds) Target organ toxicity in marine and freshwater teleosts. Taylor & Francis, London, pp 224–268
- Hoffmann KJ, Streeter AJ, Axworthy DB, Baillie TA (1985) Identification of the major covalent adduct formed in vitro and in vivo between acetaminophen and mouse liver proteins. *Mol Pharmacol* 27:566–573
- Jaeschke H, Bajt ML (2006) Intracellular signaling mechanisms of acetaminophen-induced liver cell death. *Toxicol Sci* 89:31–41
- Kavitha P, Ramesh R, Bupesh G, Stalin A, Subramanian P (2011) Hepatoprotective activity of Tribulus terrestris extract against acetaminophen-induced toxicity in a freshwater fish (*Oreochromis mossambicus*). *In Vitro Cell Dev Biol—Animal* 47:698–706
- Kirschner LB (2004) The mechanisms of sodium chloride uptake in hyper regulating aquatic animals. *J Exper Biol* 2004:1439–1452
- Knight TR, Fariss MW, Farhood A, Jaeschke H (2003) Role of lipid peroxidation as a mechanism of liver injury after acetaminophen overdose in mice. *Toxicol Sci* 76:229–236
- Lauterburg BH, Smith CV, Hughes H, Mitchell JR (1984) Biliary excretion of glutathione and glutathione disulfide in the rat. Regulation and response to oxidative stress. *J Clin Invest* 73:124–133
- Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shallittiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. *Meth Enzymol* 186:464–478
- Lores Arnaiz S, Llesuy S, Cutrin JC, Boveris A (1995) Oxidative stress by acute acetaminophen administration in mouse liver. *Free Radic Biol Med* 19:303–310
- Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Masola B, Chibi M, Kandare E, Naik YS, Zaranyika MF (2008) Potential marker enzymes and metal-metal interactions in *Helisoma duryi* and *Lymnaea natalensis* exposed to cadmium. *Ecotoxicol Environ Saf* 70:79–87
- Mate's JM, Sa'nchez-Jime'nez F (1999) Antioxidant enzymes and their implications in pathology process. *Front Biosci* 4:339–345
- Mimica-Dukić N, Popović M (2007) Apiaceae Species. A promising sources of pharmacologically active compounds: *Petroselinum crispum*, *Apium graveolens* and *Pastinaca sativa*. *Rec Prog Med Plant Spec* 21:132–133
- Morales MA, Jobbagy AJ, Terenzi HF (1973) Mutations affecting accumulation of glycogen. *Neurospora News Lett* 20:24–25
- Mudge GH, Gemborys MW, Duggin GG (1978) Covalent binding of metabolites of acetaminophen to kidney protein and depletion of renal glutathione. *J Pharmacol Exp Ther* 206:218–226
- Natio HK (1984) Cholesterol. In: Kaplan A et al (eds) Clinical Chemistry. The C.V.Mosby Co. St Louis, Toronto, Princeton, pp 1194–11206, 437
- Negre-Salvayre A, Auge N, Ayala V, Basaga H, Boada J, Brenke R (2010) Pathological aspects of lipid peroxidation. *Free Radic Res* 44:1125–1171
- Ostapowicz G, Fontana RJ, Schiodt FV, Larson A et al (2002) Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. *Ann Intern Med* 137:947–954
- Qiu Y, Benet LZ, Burlingame AL (1998) Identification of the hepatic protein targets of reactive metabolites of acetaminophen in vivo in mice using two-dimensional gel electrophoresis and mass spectrometry. *J Biol Chem* 273:17940–17953
- Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A (2001) Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* 30:463–488
- Rajasekar P, Anuradha CV (2006) Fructose-induced hepatic gluconeogenesis: effect of L-carnitine. *Life Sci* 80:1176–1183
- Ross JA, Kasum CM (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu Rev Nutr* 22:19–34
- Ruepp SU, Tonge RP, Shaw J, Wallis N, Pognan F (2002) Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. *Toxicol Sci* 65:135–150
- Sancho E, Ferná'ndez-Vega C, Ferrando MD, Andreu-Molinari E (2003) Eel ATPase activity as biomarker of thiobencarb exposure. *Ecotoxicol Environ Saf* 56:434–441
- Sedlak J, Lindsay RH (1968) Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellmans reagent. *Anal Biochem* 25:192–205
- Simic MG, Jovanovic SV (1990) Mechanisms of inactivation of oxygen radicals by dietary antioxidants and their models. *Basic Life Sci* 52:127–137
- Sugihara N, Arakawa T, Ohnishi M, Furuno K (1999) Anti and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. *Free Radic Biol Med* 27:1313–1323

- Sun Y, Oberley LW, Li Y (1988) A simple method for clinical assay of superoxide dismutase. *Clin Chem* 34:497–500
- Thomas P, Wofford HW (1984) Effects of metals and organic compounds on hepatic glutathione, cysteine, and acid-soluble thiol levels in mullet (*Mugil cephalus* L.). *Toxicol Appl Pharmacol* 76:172–182
- Vutukuru SS, Chintada S, Radha MKR, Venkateswara J, Anjaneyulu Y (2006) Acute effects of copper on superoxide dismutase, catalase and lipid peroxidation in the freshwater teleost fish, *Esomus danricus*. *Fish Physiol Biochem* 32:221–229
- Wada L, Ou B (2002) Antioxidant activity and phenolic content of Oregon cranberries. *J Agric Food Chem* 50:3495–3500
- Wegner T, Fintelmann V (1999) Flavonoids and bioactivity. *Wein Med Wochem Sihr* 149:241–247
- Wendel A, Jaeschke H, Gloger M (1982) Drug-induced lipid peroxidation in mice—II. Protection against paracetamol-induced liver necrosis by intravenous liposomally entrapped glutathione. *Biochem Pharmacol* 33:3601–3605
- Wolf JC, Wolfe MJ (2005) A brief overview of nonneoplastic hepatic toxicity in fish. *Toxicol Pathol* 33:75–85
- Xu QJ, Chen WM, Gao G (2008) Seasonal variations in microcystin concentrations in Lake Taihu, China. *Environ Monit Assess* 145:75–79
- Yan HM, Ramachandran A, Bajt ML, Lemasters JJ, Jaeschke H (2010) The oxygen tension modulates acetaminophen induced mitochondrial oxidant stress and cell injury in cultured hepatocytes. *Toxicol Sci* 117:515–523
- Yuxia C, Richard SP (2010) Use of transcriptomics in understanding mechanisms of drug-induced toxicity. *Pharmacogenomics* 11:573–585