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ABOUT COVER

Associate editor of *World Journal of Hepatology*, Dr. Yong-Ping Yang is a Distinguished Professor at Peking University Health Science Center in Beijing, China. Having received his Bachelor's degree from Yanbian University in 1985, Dr. Yang undertook his postgraduate training at PLA Medical College, receiving his Master's degree in 1992. He rose to Chief Physician in the Hepatology Division of the Fifth Medical Center of the Chinese PLA General Hospital in 2003 and has held the position since. His ongoing research interests involve liver fibrosis, cirrhosis and hepatocellular carcinoma, with a particular focus on cryoablation and cryo-immunotherapy for hepatocellular carcinoma. Currently, he serves as Chairman of the Department of Liver Disease of the Chinese PLA General Hospital and as President of the Chinese Research Hospital Association for the Study of the Liver Disease. (L-Editor: Filipodia)

AIMS AND SCOPE

The primary aim of *World Journal of Hepatology* (*WJH*, *World J Hepatol*) is to provide scholars and readers from various fields of hepatology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

   WJH mainly publishes articles reporting research results and findings obtained in the field of hepatology and covering a wide range of topics including chronic cholestatic liver diseases, cirrhosis and its complications, clinical alcoholic liver disease, drug induced liver disease autoimmune, fatty liver disease, genetic and pediatric liver diseases, hepatocellular carcinoma, hepatic stellate cells and fibrosis, liver immunology, liver regeneration, hepatic surgery, liver transplantation, biliary tract pathophysiology, non-invasive markers of liver fibrosis, viral hepatitis.

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ORIGINAL ARTICLE

Basic Study

Aceclofenac-induced hepatotoxicity: An ameliorative effect of *Terminalia bellirica* fruit and ellagic acid

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Abstract

BACKGROUND

Aceclofenac (ACF), a widely used nonsteroidal anti-inflammatory drug, has been associated with a number of severe cases of clinical hepatotoxicity. *Terminalia bellirica,* an evergreen tree, is known to have several ethnomedicinal uses including antioxidant and hepatoprotective effects. Hence *T. bellirica* fruit extracts and its phytoconstituent ellagic acid (EA) are expected to provide protection against oxidative stress and liver damage produced by long-term use of ACF.

AIM

To evaluate the antioxidant and hepatoprotective activities of *T. bellirica* fruit extracts and EA against ACF-induced toxicity in albino Wistar rats.

METHODS

The *in vitro* antioxidant activities of *T. bellirica* fruit ethyl acetate and aqueous extracts were measured by metal ion chelation and nitric oxide radical scavenging assays. The *in vivo* antioxidant and hepatoprotective effects of *T. bellirica* extracts (200 mg/kg) and EA (40 mg/kg) in ACF-induced hepatotoxic rats were assessed in serum and liver tissue after oral administration for 21 d. Silymarin (40 mg/kg) was used as a standard control. Oxidative stress markers in the blood (ferric reducing ability of plasma and lipid peroxidation inhibition) and liver tissues (superoxide dismutase, catalase and malondialdehyde) were analyzed using standard protocols. Liver function markers such as alkaline phosphatase, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, lactate dehydrogenase, γ-glutamyl transferase, creatinine, total protein, and uric acid were evaluated in rat serum.

RESULTS

The *T. bellirica* fruit ethyl acetate extract exhibited superior metal ion chelating and nitric oxide radical scavenging abilities during *in vitro* antioxidant assays as compared to aqueous extracts. Oral administration of ACF in rats (15 mg/kg) for

21 d produced oxidative stress and adversely affected liver function suggesting liver injury. Treatment with extracts (ethyl acetate and aqueous), EA and silymarin accounted for a significant reduction in the adverse effects of ACF on oxidative stress and liver function markers in serum and hepatic tissue in rats. Histopathological evaluation of the liver indicated that the extracts and EA significantly decreased the degree of liver damage. The *in vivo* efficacy of EA was higher than *T. bellirica* fruit extracts. Of these extracts, ethyl acetate extract revealed comparatively better antioxidant and hepatoprotective activity.

CONCLUSION

Ellagic acid and *T. bellirica* fruit extracts exhibited considerable hepatoprotective and antioxidant activities in long-term ACF-treated rats.

Key Words: *Terminalia bellirica*; Ellagic acid; Aceclofenac; Hepatotoxicity; Antioxidant; Histopathology

Core Tip: Hepatotoxicity is the most serious adverse effects of aceclofenac (ACF). In this study, ACF-induced hepatic damage in rats was investigated. ACF administration (15 mg/kg/d) for 21 d produced severe hepatotoxicity and oxidative stress as demonstrated by abnormal elevations in serum and tissue markers. Co-administration of *Terminalia bellirica* fruit extracts (200 mg/kg) and ellagic acid (40 mg/kg) significantly attenuated ACF-induced hepatotoxicity. These results showed that supplementation with the test compounds led to restoration of serum liver function markers (SGOT, GPT, GGT, LDH, ALP, total protein, urea, uric acid, creatinine) and hepatic antioxidant status (superoxide dismutase, catalase, TBARS). Hence *T. bellirica* fruit extracts and ellagic acid have the potential to act as a hepatoprotectant and antioxidant in the treatment of drug-induced hepatotoxicity and oxidative stress. To the best of our knowledge, this is the first study to evaluate the therapeutic efficacy of *T. bellirica* fruit extracts and ellagic acid as hepatoprotective agents against ACF-induced hepatotoxicity.

INTRODUCTION

Oxidative stress is characterized as disparity between the free radical generation and antioxidant defense mechanisms. As a consequence, free radicals attack biomolecules including lipids, proteins and DNA, thus leading to the development of various ailments at cellular and organ levels which ultimately precipitate in a disease etiology viz., hepatotoxicity, inflammation, cancer, diabetes, cardiovascular, and neurodegenerative disorders *etc.*[1,2]. Oxidative stress not only causes DNA damage, lipid peroxidation, and protein oxidation but also produces interference in the physiologic adaptation phenomenon and regulation of intracellular signal transduction mechanisms[3]. Antioxidants (enzymatic and non-enzymatic) existing in the living system are typically effective in neutralizing the adverse effects of free radicals. Numerous synthetic antioxidants are presently used in several food and pharmaceutical sectors although they are reported to produce toxicity. Hence, there is a growing demand from consumers for the utilization of natural antioxidants due to their virtuous efficacy and fewer side effects on health[4,5].

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently recommended for the management of pain and inflammatory conditions. They obstruct the activity of cyclooxygenase-1 and -2 enzymes[6]. Aceclofenac (ACF), an established NSAID, is a phenylacetic acid derivative and chemically termed 2-[(2´,6´-dichlorophenyl) amino] phenylacetoxyacetic acid. In humans, ACF is metabolized to 4'-hydroxyaceclofenac *via*

cytochrome P450 2C9b (CYP2C9)[7]. ACF has been shown to arouse selective inhibition of COX-2 as a consequence of limited and continued biotransformation to diclofenac[8]. Prolonged administration of ACF harms gastrointestinal mucosa by irritant action, affecting mucosal permeability and/or prevention of prostaglandin synthesis. Moreover, it exhibits analgesic, antipyretic, and anti-inflammatory activities, and inhibits the arachidonic acid pathway. In addition, prolonged consumption of ACF is associated with upper gastrointestinal complications, mainly perforated and bleeding peptic ulcer[9]. Previous studies on NSAIDs documented that they have adverse effects on the liver; but the incidence of these side effects is inconclusive. However, studies related to ACF-induced liver injuries are very limited.

Silymarin (SLM) is a polyphenolic flavonoid extracted from the fruit and seed of *Silybum marianum* (milk thistle). It is a well-recognized therapeutic agent for hepatic injury and has been used in the treatment of liver cirrhosis and severe hepatitis. SLM is also useful in the mitigation of damage inflicted by toxic compounds[10]. SLM has been shown to provide protection against hepatic, renal, neuronal, and gastric injury[11]. The hepatoprotective potential of SLM is related to its stabilizing action on cytoplasmic membranes[12]. Studies on different animal models have unveiled the notable therapeutic action of SLM on hepatic injury of diverse etiology[13].

Terminalia bellirica Roxb. (Combretaceae) is a perennial plant widely found in tropical regions and frequently observed in South-East Asia[14]. Its fruit has been used in various ailments in the indigenous medical system to cure cough, asthma, diarrhea, dyspepsia, anemia, cancer, fever and inflammation, and to promote rejuvenation[15,16]. It is one of the ingredients in "Triphala", an ayurvedic formulation rich in antioxidants which is believed to promote health, immunity and longevity[17], and is used for the treatment of various disorders including fever, constipation, chronic ulcers, anemia, asthma and jaundice[18]. Chemical profiling of *T. bellirica* fruit revealed that gallic acid was one of the major active components of the fruit (2.6 mg/g of total polyphenols). However, other phytochemicals such as ellagic acid, ethyl gallate, chebulagic acid and β-sitosterol have also been reported to be present in noteworthy concentrations[19,20]. *T. bellirica* fruit has been scientifically proven to possess antibacterial, antifungal, antioxidant, antidiabetic and hepatoprotective effects[21,22]. The combination of three lignans and a flavan from *T. bellirica* fruit extract showed significant anti-HIV, anti-malarial and antifungal activity *in vitro*[23]. Ellagic acid (EA) is a polyphenolic compound present in *T. bellirica* fruit in a considerable amount (1.3-2.2 mg/g of total polyphenols), and has been studied extensively due to its medicinal attributes[24,25]. The antioxidant effect of EA is attributed to its free radical scavenging and metal ion chelating abilities, along with enhancement of cellular antioxidant defense. EA also protects cells from free radical-mediated DNA damage[26,27]. However, there are no reports on the therapeutic potential of *T. bellirica* fruit extracts and EA as a hepatoprotectant against ACF-induced toxicity. The present study reports the antioxidant and hepatoprotective activities of *T. bellirica* fruit ethyl acetate (Eth) and aqueous (AQ) extracts as well as EA against ACF-induced oxidative stress and hepatotoxicity. To the best of our knowledge, this is the first study to assess the antioxidant and hepatoprotective effects of *T. bellirica* fruit extracts (Eth and AQ) and EA against ACF-induced liver injury in albino Wistar rats.

MATERIALS AND METHODS

Chemicals
Silymarin (Sigma), ellagic acid (Himedia Laboratories Pvt Ltd.), aceclofenac (Ipca Laboratory), ferrozine, ferric chloride, sodium nitroprusside, sulfanilamide, napthyldiamine, phosphoric acid, potassium chloride, and ferrous sulfate (Sisco Research Laboratory (SRL) Pvt. Ltd.) were procured from scientific suppliers. Biochemical kits for estimation of creatinine, uric acid, total protein, and serum enzymes (GPT, ALP, GOT, LDH, GGT) were purchased from Erba Transasia Bio-medicals Ltd. All other laboratory chemicals such as dimethyl sulfoxide, trichloroacetic acid, thiobarbituric acid, bovine serum albumin, butylated hydroxyanisole, sodium hydroxide, and hydrogen peroxide were also procured from SRL, India.

Collection of plant fruits and their extraction
Fruits of *T. bellirica* were bought from Prayagraj local market and ground into a fine powder. A total of 100 g fruit powder was sequentially extracted with ethyl acetate and water in Soxhlet apparatus[28]. The extract was dried under reduced pressure.

Experimental animals

Wistar rats (either sex) of a similar age group (weight 150-200 g) were used in the experiments. They were maintained in a temperature (24 ± 2°C) and humidity (40% ± 5%) controlled environment with a 12 h light/dark cycle. Rats were provided with a standard rodent diet and water *ad libitum*. The study was carried out according to the Guidelines of Institutional Animal Ethics Committee, University of Allahabad, India in agreement with the Committee for the Purpose of Control and Supervision of Experiments on Animals.

Experimental design and treatment schedule

The rats were distributed into six groups with five rats in each group. These groups were as follows: Group-I - normal rats; Group-II - ACF treated (15 mg/kg); Group-III - ACF treated rats administered with the standard drug (Silymarin 40 mg/kg); Group-IV- ACF treated rats administered with ellagic acid (40 mg/kg); Group-V- ACF treated rats co-administered with the aqueous extract (200 mg/kg) and Group-VI- ACF treated rats co-administered with the ethyl acetate extract (200 mg/kg) of *T. bellirica* fruit. During the experimental period, all the rats received a single oral dose of the drugs or extracts or the combinations thereof for 21 d.

Measurement of body weight and relative weight

The weight of the rats was measured before administration of the drugs and test compounds every day until sacrifice. The relative liver weight was also determined after sacrifice by the formula: Relative liver weight = [liver weight/body weight] × 100.

Blood collection

4 mL blood was drawn by puncturing the rat's heart. Of which, 2.5 mL blood was used for clot formation and serum was separated at 2500 rpm for 10 min. The remaining blood was placed into anticoagulant ampoules and kept in a cold environment before processing. The plasma was separated after centrifugation at 3000 rpm for 10 min. Both the serum and plasma were stored at -70 °C for further analysis.

Assessment of in vitro antioxidant activity

Metal ion chelating activity: Ferrous ion chelation by the *T. bellirica* fruit AQ and Eth extracts was assessed using the method described by Dinis *et al*[29] with minor modifications[30]. Extracts were dissolved in distilled water instead of methanol. A small amount (200 μL) of extract samples was combined with ferric chloride (50 μL, 2 mmol/L). Ferrozine (200 μL, 5 mmol/L) was added to start the reaction followed by vigorous shaking, and then left for 10 min at room temperature. Butylated hydroxytoluene was used as a positive control. Absorbance was recorded at 562 nm. Chelating activity was estimated by the following formula:

% Metal ion chelating ability = $[(A_0-A_1)/A_1] \times 100$.

Where A_0 and A_1 are absorbance of the control and test samples, respectively.

Nitric oxide radical scavenging activity: Nitric oxide (NO) radical scavenging activity was determined by the method of Green *et al*[31]. To test extracts (0.5 mL), 1.0 mL of sodium nitroprusside (0.01 mol/L in PBS) was added and incubated at 25°C for 3 h followed by the addition of an equal volume of Griess reagent and left for 30 min at room temperature. The concentration of test compounds ranged between 10-100 μg/mL in the final reaction mixture. Ascorbic acid was used as a standard and absorbance was measured at 546 nm. The NO radical scavenging activity was determined using the formula:

% NO scavenging = $[(A_c-A_s)/A_c] \times 100$.

A_c and A_s designate absorbance values of the control and test samples, respectively.

Lipid peroxidation inhibition assay: Lipid peroxidation inhibition (LPOI) by the *T. bellirica* fruit extracts was measured by the method of Halliwell *et al*[32] in 10% rat liver homogenate. BHA was used as a control. The % LPOI was determined using the following formula:

% Lipid peroxidation inhibition = $[(A_0-A_1)/A_0] \times 100$.

Where A_0 and A_1 are the absorbance of the control and test samples, respectively at 532 nm.

Estimation of total antioxidant activity by the ferric reducing antioxidant power assay: The ferric reducing antioxidant power (FRAP) assay[33] is a method for measuring total antioxidant potential of test compounds. To 0.05 mL plasma, FRAP

reagent (4.5 mL) was added and absorbance was recorded at 593 nm after 5 min. The final concentration of *T. bellirica* fruit extracts in the reaction mixture was 44.44 µg/mL, while the concentration of EA and SLM was 8.89 µg/mL. Ferrous sulfate (100-1000 µmol/mL) was used to create a calibration curve and the result was expressed as the FRAP value (µM FeSO$_4$·7H$_2$O equivalent/L plasma).

Biochemical analysis

Assessment of liver function markers in serum: The biochemical parameters including SGPT, SGOT, GGT, LDH, ALP, total protein, uric acid, and creatinine were assayed using commercially available kits (Erba Diagnostics Kits).

Preparation of liver tissue homogenate: The liver tissue homogenate (10% w/v) was prepared in phosphate buffer (0.1 mol/L, pH-7.4 with 0.15 mol/L KCl). Crude homogenate was centrifuged (1000 × *g* for 30 min, 4°C) and the supernatant was used for estimation of antioxidant enzymes and other biochemical analytes.

Assessment of antioxidant status in tissue homogenate

Estimation of malondialdehyde (MDA) in liver homogenate: Lipid peroxidation was assayed in tissue homogenate using the method of Niehaus and Samuelsson[34]. Thiobarbituric acid reagent (2 mL) was added to tissue homogenate (100 µL) and the content was boiled for 1 h followed by measurement of absorbance at 532 nm. The peroxidation product was represented as nM MDA/mg protein using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Determination of total protein in liver tissue homogenate: The total protein present in the liver homogenate was measured by the method of Lowry *et al*[35].

Superoxide dismutase activity: The superoxide dismutase (SOD) activity was assayed by the method of Marklund and Marklund[36]. One unit of enzyme activity represents 50% inhibition of pyrogallol autooxidation per min.

Catalase activity: The catalase (CAT) activity was measured by assessing the reduction in the absorbance of H$_2$O$_2$ at 240 nm for 3 min at the interval of 30 s[37]. One unit of CAT activity is defined as micromoles of H$_2$O$_2$ disintegrated per min using the molar absorbance of H$_2$O$_2$ (43.6 M^{-1} cm^{-1}).

Histological analysis of liver: The liver biopsies from rats were fixed in 10% formalin, dehydrated in graded alcohol, and then embedded in paraffin wax blocks. The paraffin-block was sliced (5 µm) successively using a rotary microtome. The liver slices were stained with hematoxylin and eosin (H and E) on albumin-coated sterilized glass slides[38]. After mounting in DPX, the sections were studied for histological changes under a light microscope (× 40 magnification).

Statistical analysis

All the experiments were performed in triplicate. Results are represented as mean ± SD. GraphPad Prism software was used to create the graphs. *P* values (< 0.05) were considered significant.

RESULTS

Assessment of in vitro antioxidant activity

Metal ion chelation activity: *T. bellirica* fruit extracts (AQ and Eth) exhibited marked concentration-dependent metal ion chelating activity (13%-85%) (Figure 1). The degree of discoloration showed the chelating efficacy of the fruit extracts. Highest chelation potential was observed for the Eth extract (85.38%, IC$_{50}$ 168 µg/mL) followed by the AQ extract (56.42%, IC$_{50}$ 220 µg/mL). Butylated hydroxytoluene showed 90% metal ion chelation activity at a concentration of 100 µg/mL.

Nitric oxide radical scavenging activity: NO radical scavenging activity of *T. bellirica* fruit extracts was evaluated at different concentrations (10-100 µg/mL) and the results were expressed in terms of % NO radical scavenging activity (Figure 2). Considerable radical scavenging activity was observed in the test compounds during *in vitro* assay. The AQ extract exhibited comparatively lower NO radical scavenging activity (16%-66%, IC$_{50}$ 70 µg/mL) than the Eth extract (26%-83%, IC$_{50}$ 48 µg/mL) at all test
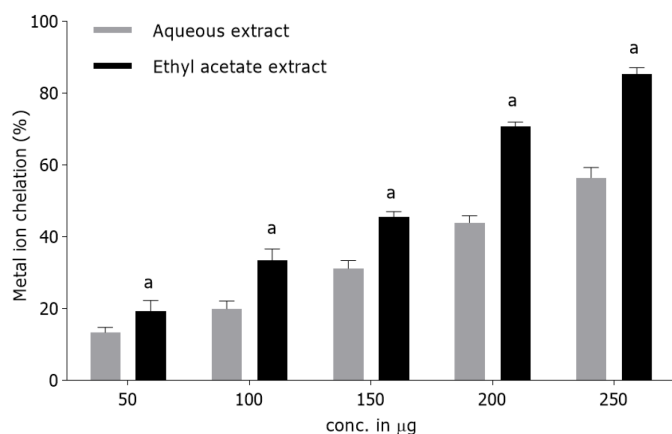
Figure 1 *In vitro* metal ion chelating activity of *Terminalia bellirica* fruit aqueous and ethyl acetate extracts. The chelating activity was measured at different concentrations (50-250 μg/ml). Butylated hydroxytoluene was included for comparison and absorbance was measured at 562 nm. Results are presented as mean ± SD of triplicates. [a]$P < 0.05$ as compared to butylated hydroxytoluene.
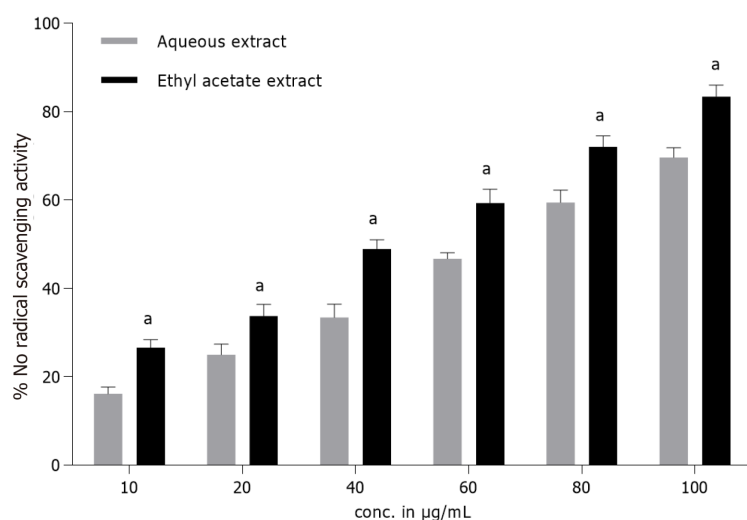
Figure 2 *In vitro* nitric oxide radical scavenging activity of *Terminalia bellirica* fruit aqueous and ethyl acetate extracts at different concentrations. Butylated hydroxytoluene was included as a standard and absorbance was measured at 546 nm. Results are presented as mean ± SD of triplicates. [a]$P < 0.05$ as compared to butylated hydroxytoluene.

concentrations. BHA (0.33-3.3 μg/mL) accounted for 37%-84% activity.

Lipid peroxidation inhibition activity: Fruit extracts of *T. bellirica* displayed dose-dependent anti-lipid peroxidative activity during *in vitro* assay. The Eth extract exhibited comparatively higher inhibitory response against Fe^{2+}-triggered lipid peroxidation in liver homogenate signifying its lipo-protective efficacy. The LPOI values for the Eth and AQ extracts at a concentration of 5 mg/mL were 75% and 63%, respectively (Figure 3). However at lower concentration (1 mg/mL), the LPOI values for the Eth and AQ extracts were about 23% and 15%, respectively. Standard antioxidant BHA (2 mg/mL) under similar experimental conditions produced about 85% inhibition of lipid peroxidation.

Ameliorative effect of fruit extracts and ellagic acid on aceclofenac toxicity in vivo
Assessment of change in body weight and relative liver weight: A noteworthy decline in body weight was observed in ACF-treated rats (group II) in comparison with untreated rats (group I) (Table 1). Group I rats showed approximately 18.83% gain in body weight during the same time period. The percentage loss in body weight in group II rats (20.50%) was markedly higher than that in group I ($P < 0.0001$) and groups III-V ($P < 0.005$). Co-administration of EA and *T. bellirica* fruit extracts (AQ and

Table 1 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on body weight and liver weight of aceclofenac treated rats			
Groups	Body weight, change (%)	Absolute liver, weight (g)	Relative liver, weight (%)
Group I	18.83 ± 1.15	4.62 ± 0.16	2.85 ± 0.29
Group II	-20.50 ± 6.87[a]	6.56 ± 0.41[a]	3.95 ± 1.29[a]
Group III	-09.17 ± 3.78[b]	4.82 ± 0.19[b]	3.05 ± 0.35[b]
Group IV	-11.06 ± 3.77[b]	5.17 ± 0.87[b]	3.13 ± 0.46[b]
Group V	-18.50 ± 6.26[b]	5.70 ± 1.09[b]	3.44 ± 0.58[b]
Group VI	-14.83 ± 3.21[b]	5.03 ± 0.95[b]	3.21 ± 0.64[b]

Group I: Control rats; Group II: Aceclofenac (ACF) treated rats; Group III: ACF + Silymarin treated rats; Group IV: ACF + Ellagic acid treated rats; Group V: ACF + Aqueous extract treated rats; Group VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD (n = 5). Silymarin was used as a positive control.
[a]Represents a significant difference compared with the control ([a]$P < 0.0001$).
[b]Represents a significant difference compared with group II ([b]$P < 0.005$).
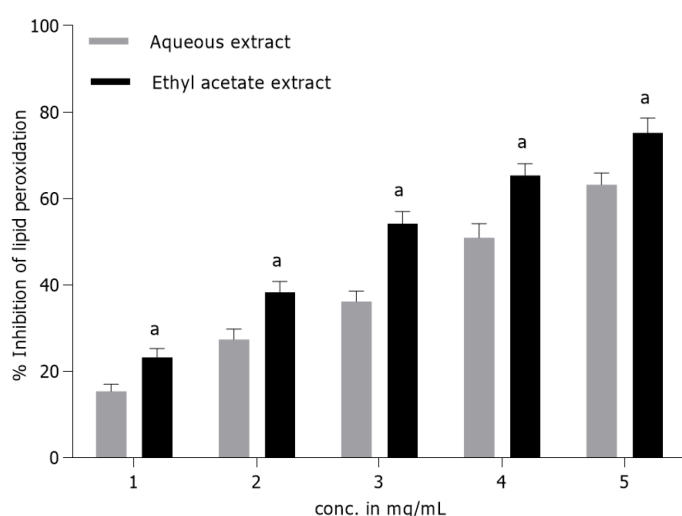
Figure 3 Percentage inhibition of lipid peroxidation in rat liver homogenate by *Terminalia bellirica* fruit extracts at different concentrations. Butylated hydroxytoluene (2 mg/mL) was included for comparison and accounted for approximately 85% lipid peroxidation inhibition. The results are shown as mean ± SD of triplicates ([a]$P < 0.05$).

Eth) with ACF exhibited a restorative effect (81%-89% recovery) on body weight. Standard drug SLM showed maximum recovery potential (90.83%) followed by EA (88.94%), Eth (85.17%) and AQ (81.50%). Furthermore, an inverse correlation was observed between body weight and relative liver weight. Relative liver weight increased from 2.85% in the control to 3.95% in ACF treated rats ($P < 0.0001$), while treatment with EA and *T. bellirica* fruit extracts (Eth and AQ) showed a restorative effect on the liver weight of rats. In comparison to group II rats, the recovery following administration of EA and *T. bellirica* fruit extracts was statistically significant ($P < 0.005$) (Table 1).

Assessment of total antioxidant activity by FRAP Assay: The therapeutic effect of *T. bellirica* fruit extracts (Eth and AQ) and EA on plasma FRAP are shown in Figure 4. In group II rats, the administration of ACF resulted in a marked decrease ($P < 0.05$) in plasma FRAP (5.76 μmol/L) as compared to group I. This indicated a reduction in the antioxidant potential of plasma with a simultaneous rise in oxidative stress. Co-administration of EA (group IV), AQ (group V) and Eth (group VI) with ACF caused a significant improvement ($P < 0.05$) in plasma antioxidant capacity.

Assessment of change in serum markers: The measurement of various markers of hepatic function is used in the diagnosis and treatment of a variety of diseases. The effects of ACF and test compound combination (*T. bellirica* fruit extracts and EA) on serum biomarkers including total protein, creatinine, urea, SGOT, SGPT, LDH, GGT,

Figure 4 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on the ferric reducing ability of plasma in aceclofenac-treated rats.
Group I-normal control; Group II-Aceclofenac (ACF) treated rats; Group III-ACF + Silymarin treated rats; Group IV-ACF + Ellagic acid treated rats; Group V- ACF + Aqueous extract treated rats; Group VI: ACF + Ethyl acetate extract treated rats. Silymarin was used as a positive control. The data are represented as mean ± SD (n = 5, ^aP < 0.01; ^bP < 0.05 as compared to group II).
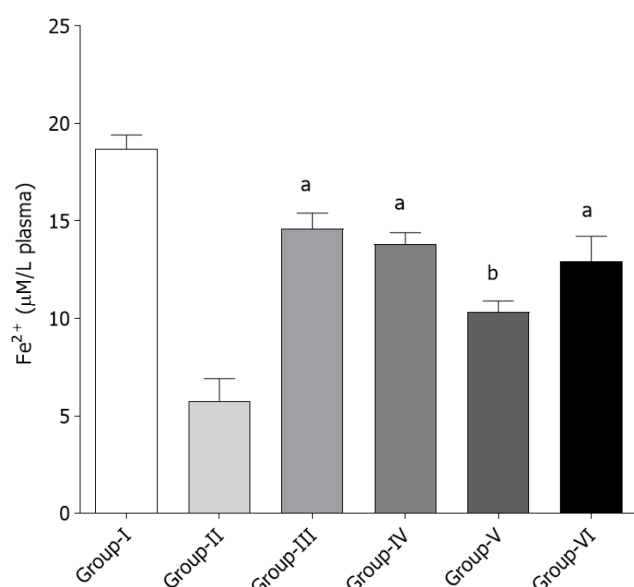
ALP and uric acid are shown in Table 2. Oral administration of ACF led to an elevation in serum creatinine, uric acid, SGOT, SGPT, ALP, LDH, and GGT (group II) (P < 0.05). Co-administration of extracts/EA/SLM with ACF resulted in marked restoration of these biochemical indices.

Assessment of MDA in liver tissue: ACF administration for three weeks caused an approximate seven-fold rise in MDA level (18.63 nmol/mg protein) in liver tissues as compared to the control (2.68 nmol/mg protein) (Table 2). SLM treatment reduced the level of MDA by up to 1.7-fold (4.5 nmol/mg protein) in group III rats. *T. bellirica* fruit AQ and Eth extract-treated groups also accounted for a noteworthy reduction in hepatic tissue MDA level (12.28 and 9.46 nmol/mg protein, respectively). Furthermore, co-administration of EA with ACF resulted in a comparatively better recovery in hepatic MDA level (6.74 nmol/mg protein) as compared to *T. bellirica* fruit extracts.

Assessment of antioxidant enzyme in liver tissue homogenate: ACF treatment in group II rats caused a noteworthy reduction (P < 0.05) in hepatic antioxidant enzyme activity *i.e.*, SOD (12.04 U/mg protein) and catalase (3.21 U/mg protein) as compared to the control group (SOD-31.09 U/mg protein and catalase-8.45 U/mg protein). Appreciable restoration (P < 0.05) in hepatic tissue catalase activity was observed in the EA-treated groups (7.19 U/mg protein) followed by the Eth (6.23 U/mg protein) and AQ (5.77 U/mg protein) groups as compared with group II. Moreover, co-administration of EA and Eth and AQ extracts with ACF also caused appreciable enhancement (P < 0.05) in SOD enzyme activity (27.24, 21.15, 19.80 U/mg protein, respectively). It was observed that EA showed comparatively similar enzymatic activity to SLM treatment (29.11 U/mg protein) (Table 2).

Histopathological changes in liver: Histological sections of the normal rat liver slices showed intact hepatocytes with sinusoidal spaces and evenly distributed cytoplasm (Figure 5A). Oral administration of ACF resulted in severe hepatic damage as confirmed by immense hepatocellular deterioration, necrosis, sinusoidal dilatation, infiltration of inflammatory cells and cytoplasmic vacuolation (Figure 5B). However, treatment with EA and *T. bellirica* fruit extracts in ACF treated rats reduced hepatic damage and associated alterations and thereby improved liver structure and function (Figure 5D-F). Administration of SLM displayed relatively higher hepatoprotective efficacy (Figure 5C). The histopathological improvement observed in liver sections with EA, *T. bellirica* extracts and SLM in ACF treated rats had a direct correlation with liver weight, body weight and serum liver function markers along with tissue

Table 2 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on serum hepatic function markers in aceclofenac treated rats

Groups	Total protein (g/dL)	ALP (IU/L)	SGOT (IU/L)	SGPT (IU/L)	Uric acid (mg/dL)	Creatinine (mg/dL)	LDH (IU/L)	GGT (IU/L)
Group I	6.05 ± 0.45	87.16 ± 14.19	69.37 ± 10.24	58.19 ± 09.13	1.71 ± 0.37	0.80 ± 0.33	304.76 ± 10.23	4.27 ±1.08
Group II	3.64 ± 0.14[a]	157.32 ± 26.16[a1]	165.40 ± 12.51[a1]	158.43 ± 8.24[a1]	6.87 ± 0.22[a]	3.12 ± 1.31[a]	689.34 ± 11.21[a1]	9.29 ± 2.15[a1]
Group III	5.72 ± 0.17[b1]	93.49 ± 9.08[b1]	85.21 ± 16.34[b1]	69.09 ± 4.71[b1]	2.32 ± 0.13[b1]	1.37 ± 0.18[b1]	401.19 ± 3.89[b1]	5.87 ± 0.24[b]
Group IV	5.52 ± 0.31[b1]	96.65 ± 11.25[b1]	89.56 ± 9.13[b1]	74.98 ± 11.38[b1]	2.34 ± 0.24[b1]	1.49 ± 0.17[b1]	449.54 ± 15.09[b1]	6.18 ± 0.51[b1]
Group V	4.75 ± 0.38[b2]	121.49 ± 16.13[b2]	140.17 ± 4.75[b1]	132.11 ± 14.17[b1]	3.38 ± 0.52[b2]	2.72 ± 0.18[b2]	587.37 ± 7.94[b2]	7.69 ± 0.27[b2]
Group VI	5.60 ± 0.11[b2]	107.17 ± 12.37[b1]	113.6 ± 4.89[b1]	109.25 ± 04.85[b1]	2.53 ± 0.19[b2]	2.16 ± 0.06[b1]	505.18 ± 11.45[b1]	6.68 ± 0.39[b2]

Group-I: Control rats; Group-II: Aceclofenac (ACF) treated rats; Group-III: ACF + Silymarin treated rats; Group-IV: ACF + Ellagic acid treated rats; Group-V: ACF + Aqueous extract treated rats; Group-VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD (*n* = 5). Silymarin was used as a positive control. ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; GGT: Glutamyl transferase.
[a]Represents a significant difference compared with the control.
[a1]*P* < 0.0001.
[b]Represents a significant difference compared with group II.
[b1]*P* < 0.0005.
[b2]*P* < 0.005.
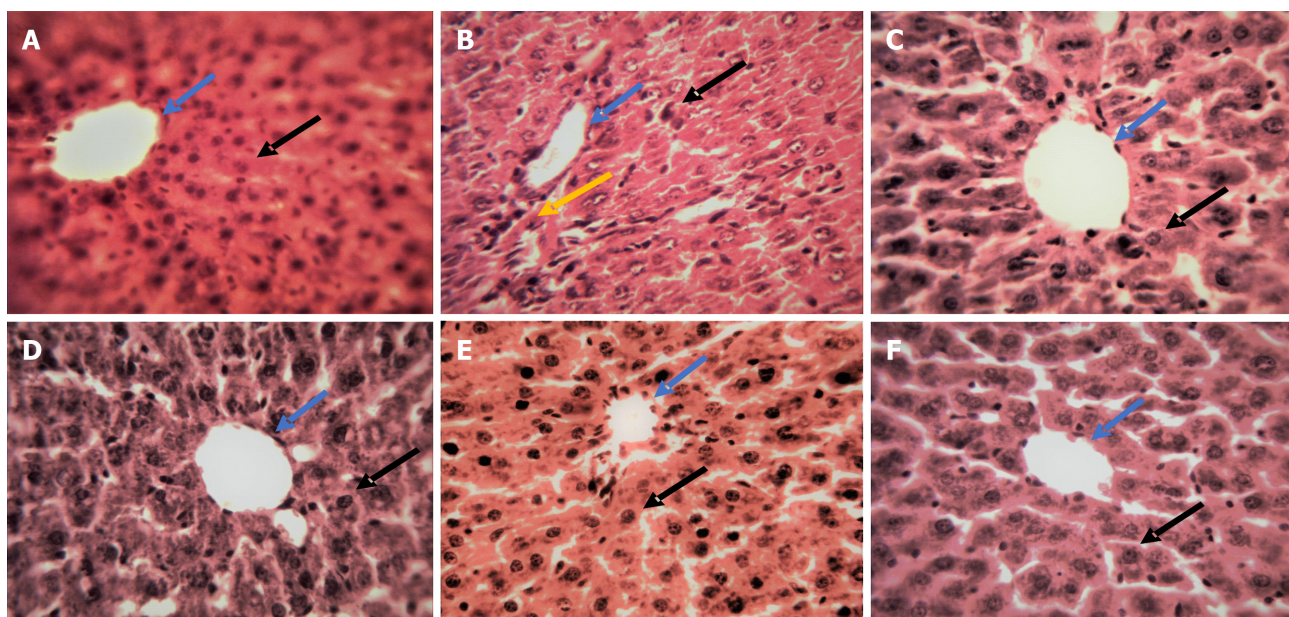
Figure 5 Histopathological changes in liver slices after oral administration of *Terminalia bellirica* fruit extracts, ellagic acid and Silymarin in Aceclofenac-treated rats. A: Control; B: Aceclofenac (ACF) treated rats; C: ACF + Silymarin treated rats; D: ACF + Ellagic acid treated rats; E: ACF + Aqueous extract treated rats; and F: ACF + Ethyl acetate extract treated rats. The blue color arrow represents central vain, black arrow represents a nucleus and the yellow arrow represents inflammatory cells.

antioxidants.

DISCUSSION

The antagonistic properties of drugs and synthetic antioxidants have drawn the attention of scientists to explore new sources of natural antioxidants and hepatoprotectants which are more potent in mitigating oxidative stress and averting

the initiation of disease[4,39]. Antioxidants hinder the oxidation of critical biomolecules by preventing the cascade of oxidizing chain reactions[5]. Interestingly, few studies on the antioxidant properties of *T. bellirica* fruit have been carried out[16,22,40]. However, this is the first study to assess the antioxidant and hepatoprotective attributes of *T. bellirica* fruit extracts and its constituent EA against ACF-induced liver injury in albino Wistar rats.

Antioxidant evaluation of *T. bellirica* fruit Eth and AQ extracts showed significant radical scavenging activities during *in vitro* metal ion chelation and NO radical scavenging assays. It was measured by a pink color complex formed due to ferrozine and Fe^{2+} interaction. Metal chelating ability showed the potential of the test compounds to protect lipids from oxidative damage[2]. In the present study, *T. bellirica* fruit Eth extract (IC_{50} 168 µg/mL) showed higher ion chelating ability as compared to the AQ extract (IC_{50} 220 µg/mL) (Figure 1). The chelation process promotes the lipophilicity of the metal ion and thereby favoring its penetration through the lipoid membrane. This action diminishes the production of $OH^{\cdot}$ radical and thus averts the beginning of lipid peroxidation[41]. Previous studies have also confirmed the positive association between metal chelation and lipoprotective activities[42]. Moreover, it has been recognized that chelating agents act as secondary antioxidants by forming bonds with metals thus lowering their redox potential and stabilizing the oxidized state of the metal ion[43].

Nitric oxide is required during inflammatory processes but higher concentrations are toxic to tissues including vascular damage and other ailments. Sodium nitroprusside in the presence of oxygen generates nitrite ions at physiological pH, which is analyzed by a specific method[44]. The nitrite radical undergoes diazotization reaction with sulfanilamide and subsequent coupling with naphthyl ethylene diamine generates pink chromophore. The radical scavenging activity of fruit extracts may be attributed to its competition with oxygen to react with nitric oxide[45]. In the experiment, the Eth extract (83%, IC_{50} 48 µg/mL) showed comparatively better NO scavenging activity than the AQ extract (74%, IC_{50} 57 µg/mL) (Figure 2). The occurrence of p-hydroxyl groups in the aromatic ring structure and conjugated double bonds that make the electrons more delocalized are structural prerequisites for potent radical scavenging action by the extracts and BHA. The p-hydroxy system possesses electron-donating properties and is a radical target. The number, positions of OH-groups and the type of group replacements are mainly accountable for phenylpropanoids functioning as effective antioxidant[46], anti-inflammatory, enzyme modulator or antiproliferative agents[47].

Lipid peroxidation is a free radical-triggered redox process associated with inflammation and biochemical changes in the lipids[42,48]. It rapidly starts with the action of hydroxyl radicals generated during the Fenton reaction in the presence of iron (II)[49]. In this study, the Eth extract (5 mg/mL) exhibited a 75% decrease in peroxidation product suggesting its lipoprotective ability, while comparatively less activity was observed with the AQ extract (63%) (Figure 3). The protection accorded by the test extracts could be ascribed to the metal ion (Fe^{3+}) chelation which is crucial for the production of hydroxyl radicals[41]. The antioxidants break the oxidation chain reaction initiated by free radicals through transfer of reducing equivalents (H^+) from the phenolic hydroxyl groups, thus producing a stable end product that does not promote further lipid oxidation. *T. bellirica* fruit has been shown to possess potent chelating ability and therefore it may exhibit appreciable inhibitory action on lipid peroxidation[40]. These results are corroborated by a recently published study from our laboratory, which advocated that *T. bellirica* fruit Eth extract had comparatively higher antioxidant activity than the AQ extract during *in vitro* analysis[16].

FRAP provides a direct assessment of the antioxidant or reducing capacity of the samples. Reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex by the test compounds is the basis for measurement of this ability producing a blue color which is measured at 593 nm[33]. The absorbance of the reaction mixture is directly correlated with the reducing ability of the sample. The FRAP value is an indicator of the hydrogen or electron-donating ability of test samples[50]. The FRAP value shown by the EA treated rat group was significantly higher ($P < 0.05$) than that in the *T. bellirica* fruit extract treated groups (Figure 4).

Phytoconstituents isolated from *T. bellirica* fruit were previously reported to be antioxidant, anti-inflammatory and hepatoprotective agents. Triterpenoidal compounds (*e.g.*, oleanolic and ursolic acids) are extensively found in food and therapeutic herbs[51]. The hepatoprotective efficacy of oleanolic acid against carbon tetrachloride (CCl_4) and ursolic acid against ethanol-induced liver injury has been confirmed[52,53]. Both compounds individually exhibited significant *in vitro* antioxidant and anti-inflammatory activities in PC12 cell lines exposed to 1-methyl-4-

phenylpyridinium ion or H_2O_2[54]. Moreover, oleanoic acid triggered expression of phase II response genes and stimulated the antioxidant enzymes and transcription factor (Nrf2)[55]. It also blocked the NF-κB pathway which was further substantiated by a high binding affinity towards NF-κB subunits (p50 and p52), TNF-α and COX-2 during *in silico* experiments[55,56].

The pharmacological activity of *T. bellirica* fruit extracts evaluated in this study can be accredited mainly to the higher amount of phenolic compounds and flavonoids. Tannins are phenolic compounds responsible for the bitter taste of foods and beverages[57]. A significant correlation between tannin content and total antioxidant activity has been established previously[58]. Additionally, tannins extracted from the acetone extract of natural products showed potent antioxidant properties compared with the low molecular-weight phenolic compounds derived from the same plant samples[57]. Recently, researchers evaluated the hepatoprotective and antioxidant attributes of gallic acid and EA against CCl_4-induced hepatic injury in mice[59,60]. Gallic acid produced superior DPPH scavenging activity than EA[59] and methyl gallate[61]. This activity could be ascribed to the availability of a free carboxyl group.

Administration of ACF for 21 d led to a fall in body weight demonstrating the adverse effect of long-term treatment in Wistar rats. It has been reported that AFC caused gastric ulcers in an animal model leading to difficulty in food intake that culminated in malnourishment[62]. The unusual rise in liver weight in ACF treated rats seems to be due to its toxic behavior. Previous studies suggested that a decline in body weight during hepatic injury is associated with the interplay of adiponutrin and abdominal fat[63]. However, treatment with *T. bellirica* fruit extracts (Eth and AQ) and EA suppressed the toxic effect of ACF in rats and improved body weight. This result was more pronounced in SLM treated rats.

The activity of reactive oxygen species (ROS), mitochondrial stress, immune response, and idiosyncratic reactions are the prime causes of hepatic injury resulting from prolonged use of ACF. However, the precise mechanism of its toxic behavior is still unclear[7]. Altered serum and tissue biomarkers along with histological changes are clinical indicators of liver damage. Abnormally high levels of SGOT, SGPT, ALP, uric acid, creatinine, LDH and GGT in the circulation are directly correlated with severe liver injury[6,11]. In the current study, prolonged ACF intake caused hepatic damage in rats, as evidenced by a marked rise in serum and tissue markers as compared to the control rats. These study results are in line with previous studies[7,64]. The findings suggested that *T. bellirica* fruit Eth and AQ extract (200 mg/kg) and EA (40 mg/kg) treatment reduced ACF-induced elevations in the levels of these parameters towards the normal range revealing improvement in hepatic function (Table 2). Similar results have been observed in SLM treated rats.

The intracellular antioxidant enzymes such as SOD and CAT act as the first line of defense against oxidative damage in hepatic tissue. SOD catalyzes the dismutation of superoxide (O_2^-) radical into H_2O_2 and oxygen, which is one of the chief cellular defense mechanisms[3]. Reduced SOD and CAT activities in ACF administered rats indicated the diminished potential of ROS scavenging action. In ACF fed rats, treatment with *T. bellirica* fruit extracts and EA enhanced the level of SOD in hepatic tissue (Table 3). A similar trend was also observed in the SLM treated group. Additionally, CAT also oozes into the extracellular fluid as a result of tissue damage. Inside the cell it has the potential to act as a strong antioxidative agent, and thus increases cell survival[1]. Decreased CAT activity in ACF-fed rats revealed lowered tissue protection ability. Co-administration of *T. bellirica* fruit extracts and EA in ACF-treated rats significantly enhanced the activity of CAT (Table 3). Previous studies on EA suggested that the hepatoprotective activity may be associated with its antioxidant and radical scavenging properties. EA might be responsible for increasing enzymatic protein synthesis or reducing the ROS-mediated loss of CAT activity[65]. It seems possible that CAT might act as an essential autocrine antioxidant and survival element. Furthermore, ROS produced as an offshoot of ACF treatment, also destroy liver tissues by stimulating lipid peroxidation. Increased level of MDA confirmed the higher rate of lipid peroxidation causing tissue damage and breakdown of cellular antioxidant defense systems[66-68]. Co-administration of *T. bellirica* fruit extract (200 mg/kg) and EA (40 mg/kg) with ACF appreciably reduced lipid peroxidation in rats.

Histological analysis of ACF-treated rat liver slices also corroborated abnormal alterations observed in serum markers and tissue antioxidants. ACF produced notable impairment in the anatomical features of liver tissue encompassing pathological irregularities such as inflammatory infiltration, the formation of vacuoles and focal necrosis. *T. bellirica* fruit extracts (Eth and AQ) and EA treatment significantly lowered the number of damaged hepatocytes. Furthermore, the hepatoprotective potential of extracts and EA was also confirmed by the dearth of necrotic and inflammatory lesions

Table 3 Effect of *Terminalia bellirica* fruit extracts and ellagic acid on antioxidant markers in liver tissue homogenate of aceclofenac treated rats

Groups	MDA (nmol/mg protein)	SOD (U/mg protein)	Catalase (U/mg protein)
Group I	2.68 ± 0.08	31.09 ± 0.71	8.45 ± 0.17
Group II	18.63 ± 1.43[a]	12.04 ± 1.49[a]	3.21 ± 0.84[a]
Group III	4.50 ± 0.18[b1]	29.11 ± 0.35[b1]	7.59 ± 0.41[b1]
Group IV	6.74 ± 0.14[b1]	27.24 ± 0.57[b1]	7.19 ± 0.37[b1]
Group V	12.28 ± 0.12[b2]	19.80 ± 0.62[b2]	5.77 ± 1.19[b2]
Group VI	9.46 ± 0.19[b1]	21.15 ± 0.49[b2]	6.23 ± 0.92[b2]

Group-I: Control rats; Group-II: Aceclofenac (ACF) treated rats; Group-III: ACF + Silymarin treated rats; Group-IV: ACF + Ellagic acid treated rats; Group-V: ACF + Aqueous extract treated rats; Group-VI: ACF + Ethyl acetate extract treated rats. Each value is expressed as mean ± SD (n = 5). Silymarin was used as a positive control. SOD: Superoxide dismutase; MDA: Malondialdehyde.

[a]Represents a significant difference compared with the control ([1]$P < 0.0001$).

[b]Represents a significant difference compared with group II.

[b1]$P < 0.0001$.

[b2]$P < 0.005$.

in rat tissue sections. Comparatively better hepatoprotective efficacy was observed in SLM and EA treated rats.

CONCLUSION

The results of the current study suggest that the administration of *T. bellirica* fruit extracts and EA have considerable hepatoprotective efficacy against ACF-induced oxidative stress and hepatic damage in Wistar rats. ACF adversely altered the levels of serum liver function markers and tissue antioxidants. Abnormal levels of biomarkers might be the result of peroxidation reactions and biotransformation of ACF in liver. These reactions are accountable for oxidative damage to cellular components. *T. bellirica* fruit extracts and EA treatment significantly ameliorated the hepatic injury inflicted by ACF. However, further evaluation is warranted to reveal the complete mechanism of action of different phytoconstituents present in *T. bellirica* fruit which might be helpful in the development of a new therapeutic agent of natural origin.

ARTICLE HIGHLIGHTS

Research background

Hepatotoxicity is one of the common side effects of nonsteroidal anti-inflammatory drugs (NSAIDs). Aceclofenac , a prodrug in the aryl-acetic acid class, is an oral NSAID effective in the treatment of painful inflammatory diseases. Chronic use of aceclofenac damages gastrointestinal mucosa by irritant action, causing an alteration in mucosal permeability and/or suppression of prostaglandin synthesis.

Research motivation

Previous studies on *Terminalia bellirica* fruit revealed that it possesses a wide range of bioactive compounds that are accountable for its antioxidant and radical scavenging potential against various types of experimental models. Moreover, its fruit has a positive impact on NSAIDs-induced liver injury. Therefore, in this study we explored the therapeutic attributes of *T. bellirica* fruit and ellagic acid against aceclofenac-induced hepatotoxicity and oxidative stress.

Research objectives

The major objectives were to evaluate the antioxidant and hepatoprotective activities of *T. bellirica* fruit ethyl acetate (Eth) and aqueous (AQ) extracts and its constituent ellagic acid against aceclofenac-induced hepatotoxicity in albino Wistar rats.

Research methods

The antioxidant activities of *T. bellirica* fruit extracts were measured in *vitro* by metal ion chelation and nitric oxide radical scavenging assays. The *in vivo* antioxidant and hepatoprotective effects of *T. bellirica* extracts (200 mg/kg) and ellagic acid (40 mg/kg) in aceclofenac-induced hepatotoxic rats were evaluated in serum and liver tissue. The ferric reducing ability of plasma and lipid peroxidation inhibition were measured in blood. Liver function markers such as ALP, GPT, GOT, LDH, γ-glutamyl transferase, creatinine, total protein, and uric acid were evaluated in rat serum and superoxide dismutase, catalase and malondialdehyde were analyzed in liver tissues using standard protocols.

Research results

T. bellirica fruit Eth extract (IC_{50} 168 μg/mL) showed higher ion chelating ability than the AQ extract (IC_{50} 220 μg/mL). Similarly, the Eth extract (IC_{50} 48 μg/mL) showed comparatively better NO scavenging activity than the AQ extract (IC_{50} 57 μg/mL). The Eth extract also decreased lipid peroxidation by 75% indicating its lipoprotective ability. The ferric reducing ability of plasma value in the EA treated rat group was significantly higher ($P < 0.05$) than that in the *T. bellirica* fruit extract treated groups *in vivo*. *T. bellirica* fruit extracts and ellagic acid treatment suppressed the toxic effect of aceclofenac in rats and improved the body weight coupled with restoration of serum liver function markers and tissue specific antioxidants.

Research conclusions

The results of the current study suggest that the administration of *T. bellirica* fruit extracts and ellagic acid exhibited considerable hepatoprotective efficacy against aceclofenac-induced oxidative stress and hepatic damage in Wistar rats. Abnormal levels of biomarkers may have occurred due to peroxidation reactions and biotransformation of aceclofenac in liver. These reactions were responsible for oxidative damage to cellular components. *T. bellirica* fruit extracts and ellagic acid treatment significantly ameliorated the hepatic injury induced by aceclofenac.

Research perspectives

T. bellirica fruit extracts and its phytoconstituent ellagic acid exhibited appreciable radical scavenging, antioxidant and hepatoprotective activity in aceclofenac-induced liver injury. However, further evaluation is warranted to reveal the complete mechanism of action of different phytoconstituents present in *T. bellirica* fruit which might be helpful in the development of a new therapeutic agent of natural origin.

ACKNOWLEDGEMENTS

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