Hepatoprotective and Antioxidant Activity of *Sphaeranthus indicus* L. against Paracetamol-induced Hepatic Damage in Mice

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**ABSTRACT**

*Sphaeranthus indicus* L., belonging to the family Asteraceae, is used in traditional medicine for the treatment of hepatopathy, indigestion, bronchitis, anaemia, epilepsy, convulsions, asthma, etc. The petroleum ether, chloroform, ethyl acetate and residual fractions of hydromethanol extract of the whole plant of *S. indicus* at a dose of 200 mg/kg bw was administered orally for 7 days. Silymarin (25 mg/kg, orally) was used as the standard. On 7th day, paracetamol (2 g/kg) was administered orally, 1 h before drug administration to induce hepatotoxicity. After 24 h of final drug administration, blood samples were collected and activities of serum marker enzymes (aspartate and alanine aminotransferase, alkaline and acid phosphatase) uric acid and bilirubin levels were determined. The levels of tissue protein, enzymatic antioxidants like superoxide dismutase, catalase, glutathione peroxidase, peroxidase, glutathione reductase, and non-enzymatic antioxidant reduced glutathione and end products of lipid peroxidation namely, lipid hydroperoxides and malondialdehyde (MDA) were measured in the liver homogenate. Oral administration of the fractions resulted in a significant reduction in serum marker enzymes, uric acid and bilirubin levels and liver MDA level and a significant improvement in antioxidant enzymes when compared with paracetamol damaged mice. Profound fatty degeneration, fibrosis and necrosis observed in the hepatic architecture of paracetamol-treated mice were found to capture near-normalcy in drug co-administered mice. The hepatoprotective activity of the chloroform fraction was found to be comparable with silymarin. The aerial parts of *S. indicus* protected the liver against paracetamol damage. The probable mechanism of action may be due to the protection against oxidative damage produced by paracetamol.

**Keywords:** antioxidant enzymes, hepatic necrosis, lipid peroxidation, oxidative stress, silymarin

**Abbreviations:** ACP, acid phosphatase; ALP, alkaline phosphatase; ALT, alanine amino transferase; AST, aspartate amino transferase; CF, chloroform fraction; DTNB, dithio-bis-nitro benzoi acid; EAF, ethyl acetate fraction; GSH, glutathione; LH, lipid hydroperoxides; NAPQI, N-acetyl-p-benzo-quinoneimine; PEF, petroleum ether fraction; RF, residual fraction; TBARS, thiobarbituric acid reactive substances

**INTRODUCTION**

Paracetamol (also known as acetaminophen) is a widely used analgesic and antipyretic agent available as an over-the-counter medication. Although considered safe at therapeutic doses, in overdose, paracetamol causes acute centrilobular hepatic necrosis that can be fatal (Burke et al. 2006). Paracetamol is metabolized primarily in the liver, where 60-90% is converted to inactive compounds by conjugation with sulfate and glucuronide, and then excreted by the kidney. Only a small portion (5-10%) of the therapeutic dose is metabolized via the hepatic cytochrome P450 enzyme system (specifically CYP2E1 and CYP1A2). The toxic effects are due to alkylating metabolite, N-acetyl-p-benzo-quinoneimine (NAPQI) which initiates a sequence of events that eventually leads to hepatic cell death. After over dose of paracetamol, the gluturonidation and sulfation routes become saturated and as a consequence, paracetamol is increasingly metabolized into NAPQI. It can alkylate and oxidize intracellular glutathione and protein thiol groups, subsequently leading to lipid peroxidation and liver damage (Akah and Odo 2010; D’Mello and Rana 2010). Besides this mechanism, paracetamol is also shown to directly inhibit cellular proliferation, induce oxidative stress, alter calcium homeostasis and deplete ATP levels. Thus, all of these changes are considered potentially fatal to the cell (Biswas et al. 2010).

Historically plants have been used in folk medicine to treat various diseases and are rich natural sources of antioxidants. Many researchers have examined the effect of plants used traditionally by indigenous people to support liver function and treat diseases of the liver (Olateye and Pocha 2008). In most cases, research has confirmed traditional experience by discovering the mechanism and mode of action of these plants. *Sphaeranthus indicus* L. belonging to the family Asteraceae is widely distributed in India. *S. indicus* is used in indigenous medicine for the treatment of hepatopathy, indigestion, bronchitis, anaemia, pain in the uterus and vagina, piles, epileptic convulsions, asthma, etc. (Nadkarni 1982). The whole plant contain significant amounts of antioxidants such as methyl chavicol, α-ionone, δ-cadinene, p-methoxy cinnamaldehyde as major constituents of essential oil (Warrier et al. 1995). The plant has been reported for its antibacterial and antifungal (Kumar et al. 2006), anxiolytic (Ambavade et al. 2006), in vitro antioxidant (Shirwaikar et al. 2006) wound healing (Sadafa et al. 2006), and antimicrobial activities (Duraipandiyan et al. 2009). To our knowledge, there are no available reports on the hepatoprotective activity of the plant. Hence, the present study was carried out to evaluate the hepatoprotective and antioxidant activities of the various fractions of *S. indicus* hydromethanol extract against paracetamol-induced hepatic damage in mice.

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MATERIALS AND METHODS

Experimental animals

Swiss albino mice of either sex weighing between 20-25 g were used for the study. The animals were housed in polypropylene cages inside a well-ventilated room. The room temperature was maintained at 23 ± 2°C with a 12 h light/dark cycle. The animals were fed with commercial rat feed pellets (Hindustan Lever Ltd., India) and provided with drinking water ad libitum. All animal procedures were reviewed and approved by the institutional animal ethical committee.

Plant material

The plant material consists of dried powdered leaves of S. indicus. The plant was approximately six months old and was in vegetative stage. The plants were collected from Coimbatore district, Tamil Nadu, during May 2007. The plant was identified and authenticated by Dr. G.V.S. Murthy, Joint Director, Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore bearing the reference number BS/SC/5/23/07-08/Tech.396.

Preparation of extract and fractionation

Fresh leaves of the plant were dried in shade under room temperature, powdered mechanically and sieved through No. 20 mesh sieve and extracted with methanol: water (7: 3) and the hydro-methanol extract was partitioned separately against petroleum ether, chloroform and ethyl acetate separately in the order of increasing polarity. The PEF, CF, EAF and RF of S. indicus were used for the in vivo study.

Drugs and chemicals

Paracetamol was obtained as a gift sample from Eurochem Laboratories Ltd, Alathur, Tamil Nadu, India. Silymarin was purchased from Microlabs, Bangalore, India. Thiobarbituric acid, trichloro acetic acid, butylated hydroxytoluene, oxidized glutathione, epinephrine and diithio-bis-nitro benzoic acid (DTNB) were obtained from Sisco Research Laboratories Pvt., Ltd., Mumbai, India. 2,2'-dipyridyl and O-dianisidine were obtained from Hi Media Laboratories Ltd., Mumbai, India. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Acute toxicity studies

Swiss albino mice of either sex (20-25 g) maintained under standard laboratory conditions was used. Animals were divided into four groups consisting of five animals each which received a single oral dose (2000 mg/kg, body weight (bw)) of PEF, CF, EAF and RF. Animals were kept overnight fasting prior to drug administration. After the administration of the fraction, food was withheld for further 3-4 h. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h (with special attention during the first 4 h) and daily thereafter for a period of 14 days. Once daily cage side observations included changes in skin and fur, eyes and mucous membrane (nasal), and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, lacrimation, perspiration, piloerection, urinary incontinence and defecation) and CNS (ptosis, drowsiness, gait, tremors and convulsions) changes (OECD 2000).

Selection of dose of the fraction

LD50 was done as per OECD guidelines for fixing the dose for biological evaluation. The LD10 of the fraction as per OECD guidelines falls under category 4 values with no signs of acute toxicity at doses of 2000 mg/kg. The biological evaluation of the fractions was carried out at a dose of 200 mg/kg bw (OECD 2000).

Hepatoprotective activity

Mice were divided into 6 groups consisting of six animals each. Group I received 0.5% w/v carboxymethyl cellulose (10 ml/kg, orally) and served as solvent control. Group II received paracetamol (2 g/kg, orally) and served as the paracetamol-treated control. Groups III–VI received the PEF, CF, EAF and RF of S. indicus respectively at a dose of 200 mg/kg orally. Group VII received silymarin (Microlabs, Bangalore, India) (25 mg/kg, orally), which served as the positive control (Asha et al. 2007). All the fractions and the standard drug were suspended in 0.5% w/v carboxymethyl cellulose administered for 7 consecutive days. Hepatotoxicity was induced by a single administration of paracetamol at a dose of 2 g/kg body weight orally (Emmanuel et al. 2001; Olaleye et al. 2006).

On the 7th day, animals belonging to groups II–VII were administered paracetamol at a dose of 2 g/kg orally, 1 h before the final drug administration to induce liver damage. After 24 h of drug administration, blood was collected by retro orbital puncture under mild ether anesthesia. The serum was used for the assay of marker enzymes like aspartate amino transferase (AST; EC number 2.6.1.1), alanine amino transferase (ALT; EC number 2.6.1.2), acid phosphatase (ACP; EC number 6.2.1.15), alkaline phosphatase (ALP; EC number 3.1.3.1), uric acid and bilirubin. The enzyme levels were assayed using standard kits from Agappe Diagnostics Ltd, Kerala, India. The results were expressed as units/l. Animals were sacrificed by cervical dislocation and the liver samples were dissected out and washed immediately with ice-cold saline to remove as much blood as possible. Liver homogenate (5% w/v) were prepared in cold 50 mM potassium phosphate buffer (pH 7.4) using a Remi homogenizer. The unbroken cells and cell debris were removed by centrifugation at 5000 rpm for 10 min and the resulting supernatant was used for the assay of lipid peroxidation and antioxidant enzymes. The amount of total protein present in the tissue was estimated by the method of Lowry et al. (1951). Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LH) were measured by the method of Nichols and Samuelson (1986). Superoxide dismutase (SOD; EC number 1.15.1.1) activity was determined by the inhibition of auto catalyzed adrenochrome formation in the presence of the homogenate at 480 nm (Kakkar et al. 1984). Catalase (CAT; EC number 1.11.1.6) activity was estimated by the catalysis of H2O2 to H2O in an incubation mixture adjusted to pH 7.0 was recorded at 254 nm (Sinha 1972). Glutathione reductase (GSSH; EC number 1.8.1.7) activity was determined spectrophotometrically by the decrease in absorbance at 340 nm (Racker 1955). Glutathione peroxidase (GPx; EC number 1.11.1.9) activity was measured by the procedure of Paglia and Valentine (1967). Peroxidase (Px; EC number 1.11.1.1) activity was measured spectrophotometrically by following the change in absorbance at 460 nm due to O-dianisidine oxidation in the presence of H2O2 and enzyme (Lobarzewski and Ginalska 1995). The estimation of non-enzymatic antioxidant reduced glutathione (GSH) was based on the reaction of reduced glutathione with DTNB and the absorbance was measured at 412 nm (Ellman 1959).

Histopathological studies

A portion of liver tissue in each group was fixed in 10% formalin, dehydrated in gradually increasing concentrations of ethanol (50-100%), cleared in xylene and embedded in paraffin for histopathological studies. Liver sections of 5 μm thickness were prepared. Hematoxylin and eosin were used for staining and later the microscopic slides of liver cells were photographed (haematoxylin and eosin X 100).

Statistical analysis

Statistical analysis of the results was carried out using GraphPad software by one-way ANOVA followed by Dunnett’s test. Results are expressed as mean ± SEM from six rats in each group. P < 0.05 was considered significant.
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RESULTS

In acute toxicity studies, it was found that the animals were safe up to a maximum dose of 2000 mg/kg bw. There were no changes in normal behavioral pattern and no signs and symptoms of toxicity and mortality were observed.

Animals treated with paracetamol at a dose of 2 g/kg exhibited a significant (\( P < 0.01 \)) rise in serum marker enzymes (viz. AST, ALT, ACP and ALP), uric acid and bilirubin levels when compared to the control group. Pretreatment with the petroleum ether, chloroform and ethyl acetate fractions of S. indicus at a dose of 200 mg/kg for 7 days significantly (\( P < 0.05 \)) reduced the serum enzymes, uric acid and bilirubin levels when compared to the paracetamol control. The reduction in all the above biochemical parameters exhibited by the chloroform fraction is similar when compared with that of the silymarin (25 mg/kg)-treated group.

The activity produced by the residual fraction was insignificantly (\( P > 0.05 \)) compared to the paracetamol control (Table 1).

Total protein level was significantly (\( P < 0.01 \)) reduced in the paracetamol-treated group when compared to the control. Pretreatment with the petroleum ether, chloroform and ethyl acetate fractions significantly elevated the protein level. The activity produced by the fractions was comparable to that of silymarin-treated group. But there was no significant increase in tissue protein in the residual fraction-treated group (Table 2).

The end products of tissue lipid peroxidation namely, malondialdehyde (MDA) and LH was found to be significantly (\( P < 0.01 \)) higher in the paracetamol-treated group than measured in the control group. Pretreatment with the petroleum ether, chloroform and ethyl acetate fractions of S. indicus significantly (\( P < 0.01 \)) decreased the elevated MDA and LH levels. The reduction in MDA and LH levels produced by the residual fraction was found to be insignificant (\( P > 0.05 \)). Treatment with silymarin also significantly decreased the levels of lipid peroxidation (Table 2).

The level of tissue enzymatic and non-enzymatic antioxidants in paracetamol control group was found to be significantly (\( P < 0.01 \)) lower than in normal control. Pre-treatment with the PEF, CF and EAF of S. indicus significantly increased the enzyme levels and brought them to near normalcy. The effect of the CF was comparable with silymarin treated group. There was no significant increase in the enzyme levels in RF treated group compared to paracetamol control (Table 3).

The results of the histopathological studies of control and experimental animals are presented in Fig. 1A–G. Section of control mouse liver shows normal liver parenchyma, central vein and steatosis. Section of mouse liver treated with paracetamol shows foci of extensive haemorrhagic necrosis, more around the central vein (Fig. 1B). Liver section of mice treated with the PEF shows few hepatocytes with microvesicular steatosis and congestion of sinusoids (Fig. 1C). Liver section of mice treated with the CF shows hepatic parenchyma with congestion of central vein and microvesicular steatosis (Fig. 1D). Liver section of mice treated with the EAF shows normal liver parenchyma with the periphery showing haemorrhagic necrosis (Fig. 1E). Liver section of mice treated with the RF shows few hepatocytes with microvesicular steatosis and the periporal area shows focal inflammatory cell collection (Fig. 1F). Liver section of mice treated with the standard drug silymarin shows normal hepatic parenchyma with mild congestion of sinusoids (Fig. 1G).
species (Murugesh et al. 2004). Treatment of mice with the fractions of S. indicus inhibited the deleterious effect due to an accumulation of superoxide radical and H$_2$O$_2$ (Raghavendra et al. 2004).

Paracetamol on overdose will elevate MDA, a byproduct of lipid peroxidation. Therefore an increase in the MDA level indicates an increase in the depth of lipid peroxidation, a well-known mechanism of hepatic damage (James et al. 2003). Studies have demonstrated that either superoxide anion or its dismutation product H$_2$O$_2$, which have been shown to be generated by the CYP 450 system, are the species responsible for the peroxidative attack which reaches its target after the GPx defense mechanism is paralyzed by lack of substrate.

Lipid peroxidation has been discussed as a basic deteriorative mechanism in the chain of events and it leads to liver dysfunction. The prevention of lipid peroxidation is connected with the GSH metabolism. Hepatic GPx can remove H$_2$O$_2$ and organic peroxides in the cell compartments were CAT is absent, or can reduce in a second line of defense already formed lipid hydroperoxides to the hydroxyl compounds. In both cases, reduced glutathione is consumed (Gupta et al. 2004).

A decrease in the level of non-enzymatic antioxidant like glutathione in paracetamol-treated mice was due to the inhibition of its protective effect against lipid peroxidation. Glutathione is a very important tripeptide, which can remove the free radical species such as H$_2$O$_2$, superoxide anion radical, alkoxyl radical and maintains the membrane protein thiols and acts as a substrate for GPx and glutathione transferase. Administration of the fractions of S. indicus minimized the effect of paracetamol against tissue non-enzymatic antioxidant activities (Table 3).

Silymarin, by its stabilizing action on the plasma membrane, has been shown to normalize paracetamol-induced elevated biochemical parameters in the serum and liver. It has protective effect on paracetamol-induced lipid peroxidation and glutathione depletion. Studies showed that silymarin prevented the hepatic cell necrosis induced by paracetamol in 87.5% of the animals. Silymarin exhibits its hepatoprotective action either by preventing hepatic cell necrosis or by inducing hepatic cell regeneration (Dixit et al. 2007).

CONCLUSION

In conclusion, the results of this study clearly demonstrated that the fractions of Sphaeranthus indicus exhibited potent hepatoprotective activity against paracetamol-induced hepatic damage in mice. This may be due to their antioxidant and free radical scavenging properties. Further studies are needed to isolate and purify the active principles involved in the hepatoprotective efficacy of the whole plant of S. indicus.

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