Effect of Centella asiatica against anti-tuberculosis drugs-induced hepatotoxicity: Involvement of mitochondria and oxidative stress

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Abstract

The liver is an integral organ in the body and plays a vital role for the metabolism of endogenous and exogenous agents. Drug-induced liver toxicity is one of the most common causes of liver injury. It accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease. Hepatotoxicity is associated with the first line antituberculosis drugs such as isoniazid and rifampicin. Therefore, there is a need for pharmacological interventions for the treatment of hepatotoxicity. The present study was designed to evaluate the hepatoprotective effect of the Centella asiatica extract on anti-TB drugs-induced hepatotoxicity. Hepatotoxicity was induced by administration of anti-TB drugs (isoniazid and rifampicin). Hepatotoxicity was assessed by significant elevation in oxidative stress, mitochondrial complex alterations and elevated levels of liver marker enzymes. Treatment with Centella asiatica (20, 40 mg/kg p.o.) attenuated the anti-TB drugs induced oxidative stress, mitochondrial complex alterations and elevated levels of liver marker enzymes (viz. SGOT, SGPT, ALP). Histopathological studies also show the promising effect. Therefore, the present study shows the hepatoprotective effect of Centella asiatica. Therefore, Centella asiatica could be a new pharmacological intervention in the treatment of hepatotoxicity.

Keywords: Antioxidant, Centella asiatica, Anti-TB drugs, Mitochondrial complex.

Introduction

Liver is one of the most important organs in the human body performing excretion & metabolism. Acute & chronic hepatic disease has been recently increased but efficient drugs without side effects have not been developed.\(^1\) Drug-induced hepatotoxicity is a potential serious adverse effect of anti-tuberculosis (Anti-TB) regimens containing isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZA).\(^2\) A high risk of hepatotoxicity has been reported in Indian patients (up to 11.5%) than in their Western counterparts (up to 4.3%). A meta-analysis of studies involving several anti-tuberculosis drug regimens estimates the incidence of liver toxicity is 2.6% with co-administered isoniazid and rifampicin, 1.6% with isoniazid alone, and 1.1% with rifampicin alone.\(^3\) Oxidative stress may either due to the overproduction of reactive oxygen species (ROS) or to the decrease of cellular antioxidant levels. Oxidative stress can lead to severe adverse effects on cells and tissues by causing lipid peroxidation and DNA damage. The role of oxidative-stress as a mechanism of hepatotoxicity caused by combination of isoniazid (INH) and rifampicin (RIF) is no more investigational topic perhaps this could be a useful experimental animal model.\(^4,5\) Mitochondria is an important organelle for cell survival and functions. Mitochondrial dysfunctions have been observed in INH hepatotoxicity, with altered mitochondrial permeability and increased apoptosis of the hepatocytes.\(^6\) Management of liver disease is still a challenge to the modern medicine. Only a few modern drugs are available for treating liver diseases. However, these modern medical treatments are still far from satisfactory. In the absence of reliable liver-protective drugs in the allopathic medical practices, herbs play a vital role in the management of liver disorders. Many indigenous plants are used for the treatment of liver disorders.\(^7\) Centella asiatica, commonly used as a medicinal herb native to India, Sri Lanka, Australia, Iran, Malaysia, Papua New Guinea, and other parts of Asia. The presence of different
glycosides, asiaticosides and bacoside A and B are chemicals useful for treating leprosy, skin diseases and to improve memory. It is used for blood conditions, liver and nervous system conditions, cardiovascular conditions, gastrointestinal conditions, glandular conditions, immune system conditions, cough problems, liver conditions, respiratory tract conditions, and tissue development. Centella asiatica extract exhibited hepatoprotective action against carbon tetrachloride-induced liver injury. Therefore, the present study was designed with an aim to investigate the possible mechanism of Centella asiatica involve in the anti-TB drugs-induced hepatotoxicity in rats.

Materials and Methods

Animals

Wistar albino rats, weighing 150-250 g, of either sex were used in the present study. The animals were housed under standard laboratory conditions. The study was approved by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) having Registration number and date of registration: 1099/c/07/CPCSEA, dated 27.07. CPCSEA guidelines were followed for animal handling and treatment.

Drugs

Isoniazid (50 mg/kg, p.o.) + Rifampicin (50 mg/kg, p.o.), Silymarin (50 mg/kg, p.o.) and Centella asiatica (20, 40 mg/kg/p.o.) were used in the present study. Centella asiatica was obtained as a gift sample from Sanath Products Limited, Vikas Marg, Delhi. Isoniazid, Rifampicin and Silymarin were purchased from Sigma Chemicals, USA.

Induction of hepatotoxicity

The hepatotoxicity was induced in Wistar albino rats by oral administration of isoniazid & rifampicin for a period of 4 weeks.

Experimental protocol

Animals were randomly divided into five groups comprising of six animals in each group and treated in the following way. Group I: Normal control (normal saline 2 ml/kg, p.o.) daily, Group II: hepatotoxic control (INH + RIF, p.o. for 4 weeks), Group III: Silymarin (50mg/kg), Group IV & V – Centella asiatica (20, 40mg/kg, p.o.).

Estimation of SGOT, SGPT, Total Bilirubin & Alkaline phosphatase (ALP) in Serum

Blood was collected from retro-orbital plexus under light ether anesthesia and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation at 3000 rpm at 30 °C for 15 min and used for the further estimation. The concentrations of serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), total bilirubin and alkaline phosphatase in serum were estimated with commercial biochemical kits (Span diagnostics Ltd., Surat, Gujarat, India). The assay was carried out as per the procedure given by the manufacturer. The absorbance of the color development due to the enzymatic reaction was read using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).

Biochemical estimation in liver tissue

The liver were removed, rinsed in isotonic saline and weighed. A 10% (w/v) tissue homogenate was prepared with 0.1M phosphate buffer (pH 7.4). The post nuclear fraction was obtained by centrifugation of the homogenate at 12000xg for 20min at 4 °C. An aliquot was used for the estimation of biochemical estimations.

Measurement of lipid peroxidation

The quantitative measurement of lipid peroxidation in the whole liver was measured according to the method of Wills.10 The amount of malondialdehyde formed was measured by the reaction with thiobarbituric acid at 532nm using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). The results were expressed as nano mole of malondialdehyde per milligram protein using the molar extinction coefficient of chromophore (1.56×10M⁻¹ cm⁻¹).

Catalase estimation

The catalase activity was assayed by the method of Luck11, where in the breakdown of hydrogen peroxides (H₂O₂) is measured at 240 nm. Briefly, assay mixture consisted of 3 ml of H₂O₂ phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and change in absorbance was recorded at 240 nm UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). The results were expressed as micromole H₂O₂ decomposed per milligram of protein/min.

Glutathione estimation

Reduced glutathione was estimated according to the method of Ellman.12 Homogenates were precipitated with 1.0 ml of 4% sulfosalicylic acid by keeping the mixture at 4°C for 1 hour and the samples were immediately centrifuged at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer of pH-8 and 0.2 ml of 0.01 M dithiobisnitrobenzoic acid (DTNB). The yellow color developed was read immediately at 412 nm using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). The results were expressed as nano moles of reduced glutathione per milligram of protein.

Superoxide dismutase activity (SOD)

Superoxide dismutase activity was assayed according to the method of Kono13 wherein the reduction of nitrozobluetetrazolium (NBT) was inhibited by the superoxide dismutase, is measured at 560 nm using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan). Briefly, the reaction was initiated by the addition of the hydroxylamine hydrochloride to the mixture containing nitrozobluetetrazolium (NBT) and sample. The results were expressed as unit/mg protein.

Isolation of mitochondria

Liver was removed and stored at −70°C until analysis could be completed. Liver tissues were excised washed thoroughly in ice cold saline to remove the blood. They were gently blotted between the folds of a filter paper and weighed in an analytical balance. The 10% of the liver homogenate was prepared in 50 mmol/L phosphate buffer (pH 7.0) containing 0.25 mol/L (w/v) sucrose and the mitochondrial pellets were prepared from the
liver homogenate according to the method as described in our previous publication. The isolated mitochondria were suspended in 50 mmol/L phosphate buffer (pH 7.0). The mitochondrial fraction was frozen and thawed 3–5 times to release the enzymes (except complex IV, which was extracted with 0.5% tween 80 in phosphate buffer, v/v).  

**Complex-I (NADH Dehydrogenase activity)**

The method involves the catalytic oxidation of NADH to NAD+ with subsequent reduction of cytochrome-C. The reaction mixture contained 0.2 M glycy1 buffer pH 8.5, 6 mM NADH in 2 mM glycy1 buffer and 10.5 mM cytochrome-C. The reaction was initiated by the addition of a requisite amount of solubilised mitochondrial sample. The absorbance change was measured at 550 nm for 2 min by using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).

**Complex-II (Succinate Dehydrogenase)**

The method involves the oxidation of succinate by an artificial electron acceptor, potassium ferricyanide. The reaction mixture contained 0.2 M phosphate buffer pH 7.8, 1% BSA, 0.6 M succinic acid and 0.03 M potassium ferricyanide. The reaction was initiated by addition of the mitochondrial sample and the absorbance change was measured at 420 nm for 2 min by using UV-spectrophotometer (Shimadzu 1800, Tokyo, Japan).

**MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay**

The MTT assay is based on the reduction of (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-H-tetrazolium bromide (MTT) by hydrogenase activity in functionally intact mitochondria. The MTT reduction rate was used to assess activity of the mitochondrial respiratory chain in isolated mitochondria. Briefly, 100 µl mitochondrial samples were incubated with 10 µl MTT for 3 h at 37°C. The blue formazan crystals were solubilized with dimethylsulfoxide and measured by an ELISA reader with a 580 nm filter using Bio-rad microplate absorbance reader for 96-well plate.

**Histopathology of liver tissue**

The whole liver was dissected out and fixed overnight at 4°C in the buffer containing 4% (w/v) paraformaldehyde. The liver was then washed with 0.1 M PBS (pH 7.4) for 1 h, dehydrated in alcohol, and then embedded in paraffin wax. Serial coronal sections (5 µm thickness) of whole liver were then obtained & stained with haematoxylin & eosin dye.

**Statistical analysis**

The results were expressed as the mean ± standard error of means (SEM). The results were analyzed using one-way ANOVA followed by post-hoc analysis using Tukey’s Multiple Comparison Test. The p value < 0.05 was considered to be statistically significant.

**Results**

**Effect of treatment on mitochondrial enzyme complex levels in liver**

In the present study, the administration of INH and RIF significantly decreases the mitochondrial enzyme complex (I, II and IV) and MTT (mitochondrial redox) activity in liver as compared to normal control group. Silymarin and centella asiatica (40) pre-treatment significantly (P < 0.05) restored mitochondrial complex enzyme activity (I, II and IV) and MTT activities as compared to the hepatotoxic control group. However, Centella asiatica (20) treatment showed no significant change in the effect on mitochondrial complex activities as compared to hepatotoxic control group (Figure 1).

**Effect of treatment on serum glutamic oxaloacetate transaminase (SGOT), glutamic pyruvate transaminase (SGPT), Total bilirubin and alkaline phosphatase in serum**

In the present study, INH and RIF significantly (P < 0.05) elevated the levels of SGOT, SGPT, total bilirubin and alkaline phosphatase in serum as compared to normal control group. Silymarin and centella asiatica (40) pre-treatment significantly (P < 0.05) decreased the serum SGOT, SGPT, total bilirubin and alkaline phosphatase as compared to the hepatotoxic control group. However, Centella asiatica (20) treatment showed no significant change in the effect on serum SGOT, SGPT, total bilirubin and alkaline phosphatase as compared to hepatotoxic control group (P < 0.05) (Figure 2).
**Effect treatment on histopathological studies**

Photomicrographs of rat's liver tissue of normal control group showed liver with normal hepatocytes any apoptotic and necrotic cells. INH and RIF -induced hepatotoxicity group showed necrosis of cells and inflammatory cell. *Centella asiatica* (40 mg/kg) treatment showed less denaturation with small amount of inflammatory cell and less hyalinization as compared to INH and RIF -induced hepatotoxic cells (Figure. 3).

**Effect of treatment on oxidative stress in liver**

In the present study, INH and RIF treatment produced oxidative stress as evidenced by a significant increase in lipid peroxidation, depletion of reduced glutathione level and catalase activity as compared to normal control group. Silymarin and *Centella asiatica* (40) treatment showed significantly attenuation of elevated lipid peroxidation, and restored reduced glutathione, superoxide dismutase and catalase activity as compared to hepatotoxic control group. *Centella asiatica* (20) treatment showed no significant change in the oxidative stress as compared to hepatotoxic control group (Figure 4a, b, c, d).
INH and RIF are potential entities to cause hepatotoxicity, when in combination; their toxic effect is enhanced in a synergistic manner. The exact mechanism of hepatotoxicity by these drugs is not clear, but several researchers have suggested that hepatotoxicity is mediated through release of reactive/toxic metabolites binding covalently with liver cell macromolecules causing liver injury. Secondly, cytochrome P450 2E1 is involved in induction of hepatic damage. Third reason is oxidative stress for destruction and damage to cell membranes. Finally, an alteration of various cellular defense mechanisms involves enzymatic and non-enzymatic components such as reduced GSH have been reported in INH and RIF induced hepatotoxicity. In the present study, hepatotoxicity was successfully induced in Wistar rats by administration of INH and RIF. Hepatotoxicity was indicated by marked rise in the normal upper limits of serum SGOT, SGPT, total bilirubin and alkaline phosphatase. The reduced GSH mediated by free radical injury leads to progress in lipid peroxidation. Pretreatment with *Centella asiatica*, the levels of these marker enzymes in serum were near normal, this may be a consequence of the stabilization of plasma membrane as well as repair of hepatic tissue damage caused by anti-TB drugs. Hepatotoxicity is characterized by cirrhotic liver condition which in turn increased the bilirubin release, & was observed in anti-TB drug treatment. Pretreatment with *Centella asiatica* restored the level of bilirubin to the normal level, suggesting that *Centella asiatica* stabilizes biliary dysfunction of rat liver. During the metabolism of INH, hydrazine is produced directly (from INH) or indirectly (from acetyl hydrazine). From earlier study it is evident that hydrazine plays a role in INH-induced liver damage in rats. INH and RIF are reported to result in higher rate of inhibition of biliary secretion and an increase in liver cell lipid peroxidation, and cytochrome P450 and is thought to involve the synergistic effect of RIF on INH. INH is metabolized in the liver primarily by acetylation and hydrolysis, and its acetylated metabolites that are thought to be hepatotoxins. Previous reports in rats suggest that the hydrazine metabolite of INH which has subsequent effect on CYP2E1 induction & involved in the development of INH-induced hepatotoxicity, and also oxidative stress as one of the mechanism for INH + RIF induced hepatic injury. Oxidative stress causes the cellular damage and hepatotoxicity by inducing the reactive oxygen species that oxidizes vital cellular components such as lipids, proteins and DNA. Numerous studies suggested that oxidative damage related to pathogenesis of hepatotoxic diseases. In the present study, there was elevation in lipid peroxidation, & decreased levels of reduced glutathione and catalase in liver. *Centella asiatica* prevents lipid peroxidation due to its metal chelating action. *Centella asiatica* is a phenolic compound, known for its free radicals scavenging property responsible for antioxidant properties. Mitochondria are the main intracellular sites of ROS generation and are also targets for oxidative damage in liver. Mitochondrial dysfunction (i.e. decrease in the mitochondrial complexes-I, II, IV and MTT levels) and overproduction of ROS plays a key role in progression of chronic hepatitis and ethanol-induced liver injury. In the present study, there was consistent decrease in the mitochondrial complexes in liver by administration of INH + RIF. Further, the present study reported that *Centella asiatica* pretreatment restores mitochondrial complexes. In INH + RIF induced hepatic injury the levels of diagnostic marker enzymes viz. SGOT, SGPT, total bilirubin and alkaline phosphatase were elevated in serum. These enzymes enter into the blood stream thus increasing their concentration in the serum. The elevated levels of SGOT, SGPT, total bilirubin and alkaline phosphatase in serum are important measures of both early and late phases of hepatic injury. Further, the present study purposed that *Centella asiatica* pretreatment attenuated SGOT, SGPT, total bilirubin and alkaline phosphatase in serum. Hepatocytes of the normal control group showed a normal lobular architecture of the liver. In the hepatotoxic control the liver showed cell swelling, congestion and feathery degeneration of the liver cells. Silymarin pretreated group showed normal hepatocytes without degeneration and their lobular architecture was normal. *Centella asiatica* (40) showed minimal changes and their lobular architecture was normal. These above findings indicated the hepatoprotective effect of *Centella asiatica*. In summary, the present study showed that *Centella asiatica* has a hepatoprotective effect against INH + RIF induced hepatic injury in rats. It was further demonstrated that the beneficial effects of *Centella asiatica* are probably due to its ability to suppress various antioxidant and apoptotic pathways. These findings indicate that *Centella asiatica* could be useful intervention in the management of hepatic diseases.

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Conflict of interest

Authors declare no conflict of interest.

References


