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In vitro antioxidant and hepatoprotective activity of *Shorea robusta* on Carbon tetrachloride induced hepatocytes

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ABSTRACT

Liver regulates various important metabolic functions. Hepatic damage is associated with distortion of these metabolic functions. Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used in the treatment of liver diseases are inadequate and sometimes can have serious side effects. The allopathic medicine has little to offer for the alleviation of hepatic ailments whereas the most important representatives are of phytoconstituents. The study was aimed to evaluation of in vitro hepatoprotective activity of Shorea robusta leaves through CCl4 induced toxicity in hepatocytes. The phytochemical screening revealed the presence of flavonoids, terpenoids, triterpenoids, polyphenol and tannins. All the variables tested as MDA, SOD, Catalase, GPx, Vitamin C and E, Protein, ALP, GOT and GPT recorded a significant alteration observed in CCl4 exposed rats. However treatment with Shorea robusta extract restored the level to near normal was observed. The potential hepatoprotective activity of Shorea robusta leaves is due to the presence of phytochemical constitution present in plant. Some of these phytochemical such as flavonoids and polyphenolic compounds have possessed hepatoprotective activity.

Keywords: *Shorea robusta*, Phytochemical, Hepatocytes, Carbon tetrachloride,

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INTRODUCTION

Medicinal plants are assuming greater importance in the primary health care of individuals and communities in many developing countries. There has been an increase of demand in international trade because of very effective, cheaply available, supposedly have no side effects and used as alternative to allopathic medicines. Medicinal plants are believed to be much safer and proved elixir in the treatment of various ailments (Kalaiarasan and Nethaji, 2015).

Liver is considered to be one of the most vital organs that functions as a centre of metabolism of nutrients such as carbohydrates, proteins and lipids and excre-tion of waste metabolites. Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide etc., chronic alcohol consumption and microbes is well-studied. Enhanced lipid peroxidation during metabolism of ethanol may result in development of hepatitis leading to cirrhosis (Wolf,

1980). A number of natural products are used in the traditional medicinal system for various ailments. Alternative medicine for treatment of various diseases is getting more popular and no sideeffects. Therefore, agents of natural origin with no side effects are required as substitute chemical therapeutics. Search for herbal remedies with potentmodulatory activity received momentum recently. In the present study to unvestigate in *vitro* antioxidant and hepatoprotective activity of *Shorea robusta* leaves on carbon tetrachloride induced hepatocytes.

MATERIALS AND METHODS

Plant materials

The fully mature *Shorea robusta* leaves were collected from Tamil University, Thanjavur District, Tamil Nadu, India. The leaves were identified and authenticated (M001) by Dr.S.John Britto, The Director, Rabiant Herbarium and Centre for Molecular Systematics, St. Joseph's College Tiruchirappalli, Tamil Nadu, India. A voucher specimen has been deposited at the Rabiant Herbarium, St. Josephs College, Tiruchirappalli, Tamil Nadu, India.

Chemicals

Nitro blue tetrazolium (NBT), ethylene diamine tetraacetic acid (EDTA), trichloro acetic acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dintiro benzene (CDNB), 5,5'-dithio-bis (2-nitrobenzoic acid), glutathione (reduced), glutathione (oxidized), phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH), carbon tetrachloride, 1, 1-diphenyl-1, 2-picryl hydrazine (DPPH) and L-ascorbic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade and were obtained from Glaxo Laboratories, Mumbai, India and Sisco Research Laboratories, Mumbai, India.

Preparations of alcoholic extract (Suganya *et al.*, 2014)

The collected *Shorea robusta* leaves were washed several times with distilled water to remove the traces of impurities from the leaves. The leaves were dried at room temperature and coarsely powdered. The powder was extracted with 70 % ethanol for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The *Shorea robusta* leaves extract (SRLE) was stored in refrigerator until used.

In vitro hepatoprotective activity

The *in vitro* hepatoprotective activity was carried out by the method of Nandhini *et al.* (2014). The rat liver was excised and weighed in a tared beaker of cold calcium-free Locke's solution. Sufficient solution was removed to give a ratio of 1g of liver to 10 ml of final suspension. The liver and solution were then transferred to a homogenizer tube and the liver broken up by pressing down with a loose fitting Lucite pestle. This was followed by twenty even up and down strokes by hand. Shreds of connective tissue containing many cells remained after this treatment, but they were readily removed by straining through bolting silk. Experience has shown that further homogenization to release more whole cells. The isolated hepatocytes were cultured in Ham's F12 medium, supplemented with 10 % newborn calf serum, antibiotics, dexamethasone and bovine insulin. The cell suspension was incubated at 37 ^{o}C for 30 min in a humidified incubator under 5 % CO_2. After incubation of 24 hrs, the hepatocytes were exposed to the fresh medium containing CCl_4 (1 %) along with different concentrations of *Shorea robusta*. Group I served as normal, Group II served as control, Group III to V served as different concentrations (100, 200 and 300 µg/ml)of plant extract. After 60 min of CCl_4 intoxication, the hepatic markers, oxidative markers and antioxidants were determined.

Biochemical Estimations

Malondialdehyde was estimated by the thiobarbituric acid assay method of Beuge and Aust (1978). Superoxide dismutase activity was determined by the procedure of Kakkar *et al.* (1984). The activity of catalase was assayed by the method of Beers and Sizer (1952).The activity of glutathione peroxidase was assayed by the method of Rotruck *et al.* (1973). The GOT was estimated by the method of Reitman and Frankel (1957). The GPT was estimated by the method of Reitman and Frankel *(*1957). The level of ascorbic acid was estimated by the method of Omaye *et al.* (1979). α-tocopherol was estimated by the method of Baker *et al.* (1980). Protein was estimated by the method of Lowry *et al.* (1951). The alkaline phosphatase activity was estimated by the method of King and King's (1954).

Statistical analysis:

Values were expressed as Mean ± SD for each group and statistical significant differences between mean values were determined by one way analysis of variance (ANOVA) followed by the

Tukey's test for multiple comparisons (Harvey and Paige, 1998). The results were statistically analyzed by Graphpad Instat Software (Graphpad Software, San Diego, CA, USA) version 3 was used and $p< 0.05$ was considered to be significant.

RESULTS

Antioxidant and hepatoprotective activity of *Shorea robusta* on CCl_4 induced hepatocytes

The study was aimed to evaluation of *in vitro* hepatoprotective activity of *Shorea robusta* leaves (100, 200 and 300 µg/ml) through CCl_4 induced toxicity in hepatocytes. All the variables tested as MDA, GOT, GPT, ALP, Protein, SOD, Catalase, GPx, Vitamin C and Vitamin E' recorded a significant alteration observed in CCl_4 exposed hepatocytes. However treatment with *Shorea robusta* extract restored the level to near normal was observed (Fig 1 - 3).

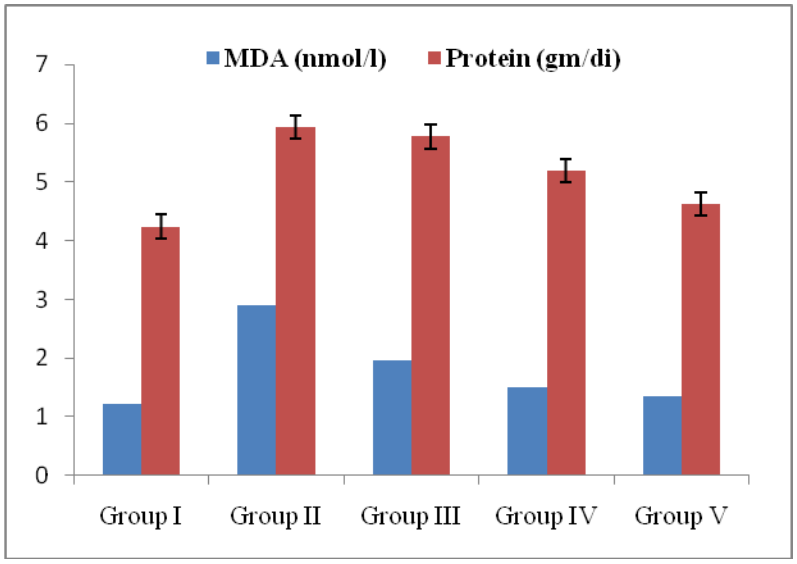

Fig 1 Effect of *Shorea robusta* on MDA and Protein in CCl_4 induced hepatocytes

Group I served as normal,
Group II served as control,
Group III to V served as different concentrations (100, 200 and 300 µg/ml) of plant extract.

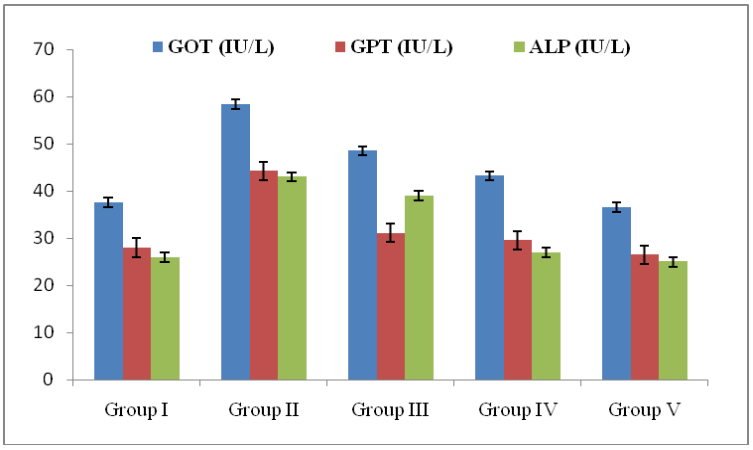

Fig 2 Effect of *Shorea robusta* on liver markers in CCl_4 induced hepatocytes

Group I served as normal,
Group II served as control,
Group III to V served as different concentrations (100, 200 and 300 µg/ml)of plant extract.

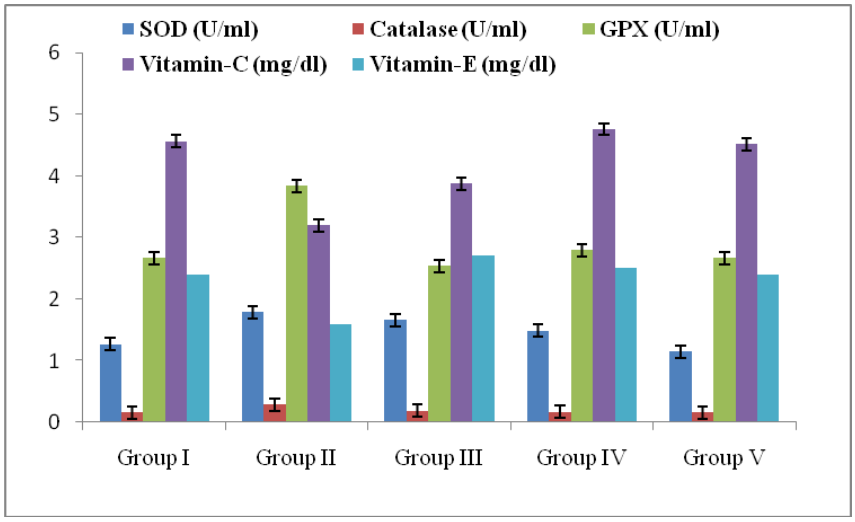

Fig. 3 *In vitro* antioxidant activity of *Shorea robusta* on CCl_4 induced hepatocytes

Group I served as normal,
Group II served as control,
Group III to V served as different concentrations (100, 200 and 300 µg/ml)of plant extract.

DISCUSSION

Shorea robusta leaves appear to have a broad spectrum of activity for several diseases. The leaves of *Shorea robusta* have been used in physicochemical analysis, phytochemical screening, *in-vitro* antioxidant activity, hepatoprotective, anti-inflammatory and cytoprotective activity. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. They may protect cells from damage caused by unstable molecules known as free radicals. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and metabolism. Majority of the diseases are mainly linked to oxidative stress due to free radicals (Velavan *et al.*, 2007).

Majority of the human diseases or disorders are mainly linked to oxidative stress due to imbalance between pro-oxidant (free radicals) and antioxidant homeostatic phenomenon in the body. Free radicals are fundamental to any biochemical process and represent essential part of aerobic life and our metabolism (Tiwari, 2001). The most common Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet-}$) anion, hydrogen peroxide (H_2O_2), peroxyl ($ROO^{\bullet}$) radicals and the very reactive hydroxyl ($OH^{\bullet}$) radicals. The nitrogen derived free radicals are nitric oxide ($NO^{\bullet}$) and peroxy nitrite anion ($ONOO^{\bullet}$). ROS have been implicated in over a hundred of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome (Joyce, 1987).

It is well established that CCl_4 induces hepatotoxicity by metabolic activation; therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl_4 is bio transformed by the cytochrome P_{450} system in the endoplasmic reticulum to produce trichloromethyl free radical ($CCl_3^{\bullet}$). Trichloromethyl free radical then combined with cellular lipids and proteins in the presence of oxygen to form a trichloromethyl peroxyl radical, which may attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethylperoxyl free radical leads to elicit lipid peroxidation, the destruction of Ca^{2+} homeostasis and finally, results in cell death (Clawson, 1989; Recknagel *et al.*, 1989). These result in changes of structures of the endoplasmic reticulum and other membrane, loss of enzyme metabolic enzyme activation, reduction of protein synthesis and loss of glucose-6-phosphatase activation, leading to liver damage (Wolf *et al.*, 1980; Azri *et al.*, 1992).

The harmful effect of reactive oxygen species is neutralized by a broad class of protective agents called antioxidants, which prevents oxidative

damage by reacting with free radicals before any other molecules can become a target. The non-enzymatic antioxidants (Vitamin E, C, reduced glutathione etc.) and antioxidant enzymes (SOD, CAT, GPx) play an important role in the protection of cells and tissues against free radical mediated tissue damage (Rayg and Husain, 2002). Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this type of damage. As plants produce a lot of antioxidants to control the oxidative stress, they can represent a source of new compounds with antioxidant activity. A number of plants and plant isolates have been reported to protect free radical induced damage in various experimental models (Scartezzini and Speroni, 2000).

The study of lipid peroxidation is attracting much attention in recent years due to its role in disease processes. Membrane lipids are particularly susceptible to LPO due to the presence of polyunsaturated fatty acids. It has been implicated in the pathogenesis of a number of diseases and clinical conditions (Kale and Sitasawad, 1990). Malondialdehyde (MDA) is a commonly used biomarker of lipid peroxidation, which arises from the breakdown of lipid peroxyl radicals, is one of the indicators of oxidative stress. Measured levels of MDA can be considered a direct index of oxidative injuries associated with lipid peroxidation (Halliwell, 1991). In this context a marked increase in the concentration of MDA indicates oxidative stress in CCl_4 exposed group when compared to control group. Administration of *Shorea robusta* significantly decreased the level of MDA in dose dependent manner demonstrate the reduction of oxidative stress in *Shorea robusta* injected in CCl_4 exposed hepatocytes.

GSH is a major non-protein thiol in living organism, which plays a central role of co-ordinating the body's antioxidant defense process. It is implicated in the cellular defense against xenobiotics and naturally occurring deleterious compounds such as free radicals. Glutathione status is a highly sensitive indicator of cell functionality and viability. Perturbation of GSH status of a biological system has been reported to lead to serious consequences (Pastore, 2003). Decline in GSH in the hepatocyte of CCl_4 exposed hepatocytes and its subsequent return towards near normaly in leaves extract of Shorea *robusta* treated group reveal antioxidant effect of *Shorea robusta*. Explanations of the possible mechanism underlying the hepatoprotective properties of drugs include the prevention of GSH depletion and destruction of free radicals (Fraga *et al.*, 1987).

Ascorbate (vitamin C) plays an important role with the lipophilic antioxidant α tocopherol in protecting the membrane from oxidative stress. Recycling of ascorbic acid requires GSH, which reduces dehydroascorbate to ascorbate (Winkler, 1992). Ascorbate in turn is essential for the recycling of tocopherol radical to tocopherol (Packer *et al.*, 1997). In the present study, significantly decreased level of vitamin C and α-tocopherol in CCl_4 exposed group, demonstrating the increased free radical accumulation in CCl_4 administered group. The observed decline in glutathione level may contribute to the decrease in ascorbate as well tocopherol concentration in CCl_4 exposed group. Supplementation of leaves extract of *Shorea robusta* to CCl_4 exposed rats improved vitamin C and α-tocopherol level as compared to control group, which may be due to increase the GSH in *Shorea robusta* treated group improve the recycling of vitamin C and α- tocopherol.

Biological systems protect themselves against the damaging effects of activated species by several means. These include free radical scavengers and chain reaction terminators; enzymes such as SOD, CAT and GPx system (Proctor and McGinness, 1986). The SOD dismutases superoxide radicals O_2^- into H_2O_2 plus O_2, thus participating with other antioxidant enzymes, in the enzymatic defense against oxygen toxicity. In this study, SOD plays an important role in the elimination of ROS derived from the peroxidative process of xenobiotics in liver tissues. In our study, restored the activity of this enzyme in CCl_4 administered group revealed that MDA and oxidative stress elicited by CCl_4 intoxication have been nullified due to the effect of *Shorea robusta*. This observation perfectly agrees with those Suganya *et al.* (2014) study.

CAT is a key component of the antioxidant defense system. Inhibition of these protective mechanisms results in enhanced sensitivity to free radical induced cellular damage. Excessive production of free radicals may result in alterations in the biological activity of cellular macromolecules. Therefore, the reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxide. Administration of *Shorea robusta* restored the activities of catalase in CCl_4 induced hepatocyte damage to prevent the accumulation of excessive free radicals and protects the hepatocyte from CCl_4 intoxication. This observation agrees with those of Nandhini *et al.* (2014) study.

GPx is a seleno-enzyme two third of which (in liver) is present in the cytosol and one-third in the mitochondria. It catalyses the reaction of

hydroperoxides with reduced glutathione to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide. In our study, decline in the activity of this enzyme in CCl_4 administered group. The observed decrease of GPx activity suggests that the *Shorea robusta* have efficient protective mechanism in response to ROS. And also, these findings indicate that *Shorea robusta* may be associated with decreased oxidative stress and free radical-mediated tissue damage. This observation consisted with those of Nandhini *et al*. (2014) and Suganya *et al*. (2014) study.

In the assessment of liver damage by carbon tetrachloride, the determination of enzyme activities such as aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) is largely used. Activities of AST, ALT and alkaline phosphatase (ALP) are the most frequently utilized indicators of hepatocellular injury. Necrosis or membrane damage releases the enzymes into circulation and therefore, they can be measured in hepatocyte. ALT is more specific to the liver and is thus a better parameter for detecting liver injury. Elevated levels of hepatocyte enzymes are indicative of cellular leakage and loss of functional integrity of the cell membrane in the liver (Wolf, 1999). The mechanism by which alkaline phosphatase reaches the circulation is uncertain; leakage from the bile canaliculi into hepatic sinusoids may result from leaky tight junctions and the other hypothesis is that the damaged liver fails to excrete alkaline phosphatase made in the bone, intestine and the liver (Thapa and Walia, 2007). Total protein levels, on other hand, are related to the function of hepatic cells i.e they reveal the functional status of the hepatic cells. Decreased levels of total protein is indicative of the failure of the biosynthetic function of the hepatocyte (Crawford *et al.*, 2004).

In the present study, the CCl_4 treated hepatocyte showed a significant elevation in the activities of GOT, GPT, alkaline phosphatase while significantly decreasing the levels of total protein as compared to the normal control hepatocyte, thereby indicating oxidative damage. Co-treatment with *Shorea robusta* at doses of 100, 200 and 300 µg/kg, significantly prevented the rise in the levels of the marker enzymes, as well as it significantly prevented the decrease in the total protein. The diminished rise of hepatocyte enzymes, together with the diminished fall in the levels of total protein in the *Shorea robusta* treated groups, is a clear manifestation of the hepatoprotective effect of the *Shorea robusta*. Present finding is in agreement with Nandhini *et al*. (2014) and Suganya *et al*. (2014) studies.

The phytochemical screening revealed the presence of flavonoids, terpenoids, triterpenoids, polyphenol and tannins in *Shorea robusta* extract. The entire variable tested i.e., SOD. CAT, GPx, reduced glutathione, vitamin C, GOT, GPT, alkaline phosphatase and protein recorded a significant alteration on CCl_4 treatment. However, treatment with herbal extract restored the levels to near normal value, suggesting the therapeutic effect of *Shorea robusta* to counter the oxidative stress. Among the three doses, the higher dose has potential antioxidant and hepatoprotective activity. In conclusion, it can be said that ethanol extract of *Shorea robusta* exhibit a hepatocyte protective effect against CCl_4 induced oxidative stress and possessed anti-lipids peroxidative and antioxidant activities. This indicates that the lipid peroxidation and oxidative stress elicited by CCl_4 intoxication had been nullified due to the effect of *Shorea robusta*.

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