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Original Article

IN VITRO ANTIOXIDANT ACTIVITY OF METHANOLIC EXTRACT OF SHOREA ROBUSTA IN HEPATOCYTES

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ABSTRACT

Objective: The study was aimed to evaluate of *in vitro* antioxidant activity of *Shorea robusta* leaves (200 and 400µg/ml) through CCl_4 induced oxidative stress in hepatocytes.

Methods: The cytotoxicity of *Shorea robusta* on hepatocytes was evaluated by MTT assay [(3-(4,5 dimethylthiazole –2 yl)-2,5 diphenyl tetrazolium bromide) assay]. The percentage viability of the hepatocytes was carried out.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 °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 concentration of *Shorea robusta* (200 and 400µg/ml). After 60 min of CCl_4 intoxication, the oxidative stress markers were determined.

Results: The phytochemical screening revealed the presence of flavonoids, terpenoids, triterpenoids, polyphenol and tannins in Methanol extracts. The percentage viability of the hepatocytes was dose dependent. The entire variables tested i.e., SOD. CAT, GPx, reduced glutathione, vitamin C and vitamin E recorded a significant decline on CCl_4 treatment. However, treatment with *Shorea robusta* extract restored the levels to near normal value, suggesting the therapeutic effect of *Shorea robusta* to counter the oxidative stress. Among the two doses, the higer dose has potential antioxidant activity.

Conclusion: The results of the present study concluded that methanol extract of *Shorea robusta* exhibit a liver protective effect against CCl_4 induced oxidative stress and possessed anti-lipid peroxidative and antioxidant activities. The potential antioxidant activity of *Shorea robusta* leaves may be due to the presence of radical scavenging property of phenolic groups. Our results may aid in the development of new methods for enhancing the health of the hepatocytes.

Keywords: *Shorea robusta,* Hepatocytes, Carbon tetrachloride, Oxidative stress.

INTRODUCTION

Majority of the human diseases/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 an essential part of aerobic life and our metabolism [1]. 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 hundreds of diseases states which range from arthritis and connective tissue disorders to carcinogenesis, aging, physical injury, infection and acquired immunodeficiency syndrome [2].

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 [3, 4, 5]. 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 [6, 7]. 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, GSHPx) play an important role in the protection of cells and tissues against free radical mediated tissue damage [8, 9, 10]. 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 [11]. Natural antioxidants strengthen the endogenous antioxidant defences from ROS ravage and restore the optimal balance by neutralizing the reactive species. They are gaining immense importance by virtue of their critical role in disease prevention. The medicinal value of the chosen plant *Shorea robusta* has been extensively worked out. However its therapeutic efficacy in the state of oxidative stress has not been evaluated. Hence in the present study an attempt has been made to create an hepatocyte model with oxidative stress using CCl_4 and the therapeutic efficacy of the extract of *Shorea robusta* was evaluated and also determination of phytochemical screening of *Shorea robusta* leaves.

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride, thiobarbituric acid, 2,4-Dinitro phenylhydrazine, and glutathione were purchased from sigma chemical, Mumbai. All other reagents and chemicals used in this study were of analytical grade with high purity.

Collection of plant material

The collected plant was authenticated (RH021) by Dr.S.John Britto, The Director, the Rapiant Herbarium and centre for molecular systematics, St. Joseph's college Trichy-Tamilnadu.

Preparation of extract

The collected plant leaves of *Shorea robusta* were cut into small pieces and shade dried at room temperature and makes a fine powder using grinder mixture. The powder material of *Shorea robusta* was macerated with 70% methanol at room temperature for 3 days. After 3 days, the supernatant was transferred into china dish .The supernatant was completely removed by keeping the china dish over a boiling water bath at 45°C. A semi solid extract was obtained after complete elimination of alcohol. The obtained residue was kept in the refrigerator for further use.

In vitro antioxidant activity

The 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 1 g 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 °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 concentration of *Shorea robusta* (250 and 500µg/ml). After 60 min of CCl_4 intoxication, the oxidative stress markers were determined.

Biochemical estimations

MDA released from endogenous lipoperoxides, reflecting the lipid peroxidation process, were assayed in liver as described by Beuge and Aust, [12]. The activities of antioxidant enzymes SOD, Catalase and Glutathione peroxidase were determine by the method of Kakkar *et al*. [13], Beers and Sizer, [14] and Rotruck *et al*. [15] respectively. The levels of non-enzymatic antioxidants such as GSH, Vitamin C and Vitamin E were estimated by the method of Moron *et al*. [16], Omaye *et al*. [17] and Baker *et al*. [18] respectively. Determination of Mitochondrial Synthesis by Micro culture Tetrazolium (MTT) Assay (19).

Phytochemical Screening:

Phytochemical screening of the powdered leaf. The dry powdered leaves of *Shorea robusta* were extract with methanol for the presence of flavonoids, terpenoids, glycosides, saponins, tannins and proteins etc., using standard phytochemical procedures [18, 20].

RESULTS AND DISCUSSION

Preliminary phytochemical screening

The preliminary phytochemical test was performed on the methanolic extract of *Shorea robusta* leaves. Results of the test show the presence of the glycosides, polyphenol, flavonoids, tannin, terpenoids, triterpinoids, carbohydrate and protein present in the extract (Table-1). Among the various phytochemicals, tannin and flavonoids present in high concentrations as compared to other phytochemicals. Saponin, steroids, alkaloids and phlobatannins are absent. The detailed phytochemical investigation strengthens the resourcefulness of the extracts for the further pharmacological evaluations.

Table 1: Phytochemical Screening of *Shorea robusta* leaves

Phytochemicals	Methanol
Tannin	+++
Phlobatannins	-
Saponin	-
Flavonoids	+
Steroids	-
Terpenoid	+
Triterpenoids	+
Alkaloids	-
Carbohydrate	+++
Protein	++
Anthroquinone	+
Polyphenol	+
Glycoside	++

(+++ present in high concentration,++ present in less concentration, +present, -absent)

***In vitro* antioxidant activity of *Shorea robusta* on CCl_4 induced oxidative stress in hepatocyts**

The present study was carried out to evaluate the *In vitro* antioxidant activity of *Shorea robusta* on CCl_4 induced oxidative stress in hepatocytes. The concentration of MDA was significantly higher in hepatocyte of CCl_4 exposed rats, as compared to normal control animal (Table 3-5). These constituents were found to attain a near normal level in hepatocyte of CCl_4 + *Shorea robusta* treated rats. The decrease was dose depended. Conversely, GSH content in hepatocyte of Group II showed (Table 2) a significant decline when compared with control. But in Group III and IV GSH content was found to attain near normalcy. Activities of antioxidant enzymes are presented in Table 5. The activities of SOD, CAT and GPx recorded a significant increase in CCl_4 exposed rats, when compared with normal control. In CCl_4 + *Shorea robusta* treated hepatocytes, the activities of these enzymes attained a near-normalcy. Among the two doses, the higher dose has potential antioxidant activity. Table 2 represents the % of viability of control and experimental cell line. Group II CCl_4 induced oxidative stress showed a significant decreased in the % of cell viability when compared to Group I. Group III and IV CCl_4 induced oxidative stress group treated with (200 and 400µg/kg) *Shorea robusta* significantly increased in % of cell viability when compared to group II.

Table 2: shows the % of viability

Treatment group	Concentration	% of viability
Control (Untreated)	---	100
CCl_4	1%	43.21
CCl_4 + *Shorea robusta*	200µg/ml	76.45*

CCl_4 + *Shorea robusta*	400µg/ml	86.56*

Values were expressed as mean ± SD for triplicate in each group. * Significantly different from Group II (P < 0.05)

Table 3: Effect of Shorea *robusta* on MDA and GSH in experimental hepatocytes

Parameters	Group I	Group II	Group III	Group IV
MDA (nmol /L)	2.57±1.4	20.9±0.9*	3.32±1.38 **	3.18±0.90**
GSH (mg/dl)	6.27±0.34	4.3±0.45*	5.9±0.61**	6.4±0.45**

Values were expressed as mean ± SD, * Significantly different from Group I(P<0.05), ** Significantly different from Group II(P<0.05)

Table 4: Effect of *Shorea robusta* on vitamin C and vitamin E in hepatocytes

Parameters	Group I	Group II	Group III	Group IV
VitaminC (mg/dl)	5±0.60	3.23±0.04*	4.1±0.34**	5.3±0.42**
VitaminE (mg/dl)	2.55±0.36	1.59±0.12*	2.21±0.10 **	2.54±0.26**

Values were expressed as mean ± SD , * Significantly different from Group I(P<0.05), ** Significantly different from Group II(P<0.05)

Table 5: Effect of *Shorea robusta* on catalase, SOD and GPx activities in hepatocytes

Parameters	Group I	Group II	Group III	Group IV
Catalase(U/ml)	2.6±0.63	4.8±1.94*	2.5±0.63**	3.10±0.61**
SOD(U/ml)	0.56±0.14	1.31±0.22*	0.83±0.09**	0.48±0.09**
CPx (U/ml)	1.5±0.27	2.34±0.19*	1.8±0.24**	1.5±0.24**

Values were expressed as mean ± SD , * Significantly different from Group I(P<0.05),** Significantly different from Group II(P<0.05)

Ample experimental and epidemiological studies support the involvement of oxidative stress in the pathogenesis and progression of several chronic diseases [21]. It is now known that oxygen, indispensable for maintaining life, sometimes becomes toxic and results in the generation of most aggressive agents such as reactive oxygen species (ROS). The high reactivity of ROS may trigger a host of disorders in body resulting in tissue damage and necrosis in many instances [22]. It has been hypothesized that one of the principal causes CCl_4- induced liver injury is LPO by free radical derivatives of CCl_4. Thus the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4 –induced hepatopathy [23]. CCl_4 mediated oxidative stress was taken here as the experimental model for oxidative stress.

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 [24]. 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 [25]. In this context a marked increase in the concentration of MDA indicates oxidative stress in CCl_4 exposed rats when compared to control rats. Administration of *Shorea robusta* significantly decreased the level of MDA demonstrate the reduction of oxidative stress in *Shorea robusta* and 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. Decline in GSH in the liver of CCl_4 exposed rats, and its subsequent return towards near normalcy in CCl_4 and *Shorea robusta* treated hepatocytes 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 [26].

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 [27]. Ascorbate in turn is essential for the recycling of tocopherol radical to tocopherol [28]. In the present study, significantly decreased level of vitamin C and α-tocopherol in CCl_4 exposed rats, demonstrating the increased free radical accumulation in CCl_4 administered rats. The observed decline in glutathione level may contribute to the decrease in ascorbate as well tocopherol concentration in CCl_4 exposed rats. Supplementation of *Shorea robusta* to CCl_4 exposed rats improved vitamin C and α- tocopherol level as compared to control rats, which may be due to increase the GSH in *Shorea robusta* treated rats 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 [29]. 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 rats 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 of Lin *et al*. [30] 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 liver damage rats to prevent the accumulation of excessive free radicals and protects the liver from CCl_4 intoxication. This observation agrees with those of Gupta *et al*. [31] 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 withreduced 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 rats. 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 Venukumar and Latha [32] study.

The phytochemical screening revealed the presence of flavonoids, terpenoids, triterpenoids, polyphenol and tannins in methanol extracts. The entire variable tested i.e., SOD. CAT, GPx, reduced glutathione, vitamin C and vitamin E 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 two doses, the higer dose has potential antioxidant activity. In conclusion, it can be said that methanol extract of *Shorea robusta* exhibit a liver protective effect against CCl_4 induced oxidative stress and possessed anti-lipid 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*. Efforts are in progress here to isolate and purify the active principle involved in the antioxidant efficacy of this medicinal plant.

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