



Preventive Effect of *Shorea Robusta* Bark Extract against Diethylnitrosamine-Induced Hepatocellular Carcinoma in Rats

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Abstract

Antioxidants are one of the key players in tumorigenesis, several natural and synthetic antioxidants were shown to have anticancer effects. The aim of the present study is to divulge the preventive nature of *Shorea robusta* bark extract (SRBE) during diethylnitrosamine (DEN)-induced liver cancer in male Wistar albino rats. Administration of DEN to rats resulted in increased serum marker enzymes aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), and gamma glutamyl transpeptidase (GGT). The levels of lipid peroxides elevated with subsequent decrease in the tissue antioxidants like superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione peroxidase (GPx), and glutathione reductase (GR). SRBE supplementation (500mg/kg body weight) significantly attenuated these alterations, thereby showing potent anticancer effect in liver cancer. These findings suggest that SRBE prevents lipid peroxidation, hepatic cell damage, and protects the antioxidant system in DEN-induced hepatocellular carcinogenesis.

Keywords: *Shorea robusta* bark extract, hepatocellular carcinoma, diethylnitrosamine, antioxidants, lipid peroxidation.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent cancers among humans, with 0.50–1 million newly diagnosed cases each year¹. The highest frequencies are found in sub-Saharan Africa and far eastern Asia, where hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are endemic and in regions where food contaminated with Aflatoxin B1 is consumed². HCC incidence appears to be rising, even in countries with relatively low incidence, especially in Southern Western Europe and Asia³.

Carcinogenesis may arise as a result of chemical or biological damage to normal cells in a multistep process that involves changes at the initiation level followed by promotion and progression which lead to malignancy. The promotional stage of cancer is reversible stage and appears to be most appropriate target stage for chemopreventive intervention⁴ chemoprevention is one of the strategies by which we can revert or delay the response of carcinogen. Cancer chemopreventive agents are able to reduce the incidence of tumorigenesis by intervening in one or more stages of carcinogenesis initiation, promotion or prolongation⁵.

Animal experimental models are particularly useful for the study of neoplasm in humans. As experimental model of human HCC, we used rats treated with DEN which induces poor, moderate and well differentiated forms of HCCs with histological features similar to those of the human tumors⁶. Hence, the model of DEN-induced liver cancer is considered as

one of the most accepted and widely used experimental models to study about the hepatocarcinogenesis⁷.

In developing countries about 35% of prescribed drugs are derived from natural products. Many investigations are being carried out worldwide to discover naturally occurring compounds which can suppress or prevent the progress of carcinogenesis⁸ It is well known that many anticancer compounds derived from plants include Taxol from Pacific Yew tree, Vinblastine and Vincristine from *Catharanthus roseus*, Rohitukine from *Dysoxylum binectariferum*, Broccoli and Red Cabbage⁹ diterpene from *Tinospora cordifolia*¹⁰, derivatives of Podophyllin from *Podophyllum peltatum* and Campothecin from *Camptotheca acuminata*¹¹. It is important to continue efforts aimed at discovering anticancer agents based on natural products¹².

In recent years, there has been considerable emphasis on the identification of plant products with antioxidant property, as free radicals are considered to play a major role in most of the diseases including cancer. The medicinal value of the chosen plant *Shorea robusta* bark has been extensively worked out. However, its therapeutic efficacy in anticancer activity has not been evaluated. *Shorea robusta* is a tropical hardwood found and developed in Southeast Asia. It prospers most commonly in Indonesia but can also be seen in Malaysia, the Philippines and certain parts of Southern India. Bark is a dark brown and thick, with longitudinal fissures deep in poles, becoming shallow in mature trees; provides effective protection against fire. Traditionally the plant is used for dysentery, Ulcers, Jaundice,

wounds, gonorrhoea and leprosy. The bark used as astringent, acrid, cooling, antihelminthic, alexeteric, anodyne, constipating, urinary astringent, union promoter depurative and tonic¹³. The major chemical constituents of *Shorea robusta* are reported to contain flavonoids, steroids, terpenoids, phenols and cardioglycosides^{14,15}.

Material and Methods

Animals: Male albino rats of Wistar strain approximately 3-4 months young rats (weighing approximately 140-160g) and 24-26 months old rats (weighing approximately 380-410g) were used in this study. They were healthy animals procured from Sri Venkateswara enterprises, Bangalore, India. The animals were housed in spacious polypropylene cages bedded with rice husk. The animal room was well ventilated and maintained under standard experimental conditions (temperature 27±2°C and 12 hours light / dark cycle) throughout the experimental period. All the animals were fed with standard pellet diet (Gold Mohur, Mumbai, India) and water *ad libitum*. They were acclimatization to the environment for 1 week prior to experimental use. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Chemicals: Nitro blue tetrazolium (NBT), ethylene diamine tetraacetic acid (EDTA), trichloro acetic acid (TCA), thiobarbituric acid (TBA), 1-chloro-2,4-dinitro benzene (CDNB), 5,5'-dithio-bis (2-nitrobenzoic acid), glutathione (reduced), glutathione (oxidized), Diethylnitrosamine (DEN) 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.

Plant materials and preparation of plant extract: The plant bark of *Shorea robusta* was collected from Sengipatti, Thanjavur District in TN, India. The collected plant materials were washed, sliced and completely dried in a hot-air oven at 37°C. The dried materials were ground into a fine powder and used for extraction. Three hundred grams (300g) of the powdered plants were extracted with ethanol (70%) using "Soxhlet Apparatus" for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in refrigerator until used. The extract contains both polar and non-polar phytochemicals. For experiments 500mg/kg body weight of *Shorea robusta* bark extract (SRBE) was used. This effective dose was selected based on dose dependent studies of SRBE carried out in our laboratory.

Dosage Fixation: Different doses of *Shorea robusta* bark extract (SRBE) (50mg, 100mg, 250mg, 500mg and 750mg/kg body weight) were treated for 4 weeks in rats. (The effective dose of HEE was assessed based on the contents of liver and kidney

lipid peroxidation (oxidative damage marker). Supplementations of *Shorea robusta* bark extract (SRBE) at doses of 250, 500mg and 750mg/kg body weight for 4 weeks were found to be effective in aged rats. Among these doses, the minimal effective dose 500mg was fixed as therapeutic dosage for the subsequent studies.

Experimental Design: Body weights of the animals were recorded and they were divided into 4 groups of 6 animals each as follows.

Group 1: Normal control rats will be fed with standard diet and served as a control, which received saline.

Group 2: Rats treated with *Shorea robusta* bark alone by oral gavage daily at a dose of 500 mg/kg body weight (based on effective dosage fixation studies) for 16 weeks.

Group 3: Rats induced with hepatocellular carcinoma by providing 0.01% DEN through drinking water for 16 weeks.

Group 4: Rats pretreated with *Shorea robusta* bark intragastrically at the dose of (500mg/kg body weight) for one week before the administration of DEN and continued till the end of the experiment (i.e., 16 weeks).

Collection of Samples: On completion of the experimental period, animals were anaesthetized with thiopentone sodium (50mg/kg). The blood was collected with or without EDTA as anticoagulant. Blood, plasma and serum were separated for the estimation of various biochemical parameters. The Liver was dissected out, washed in ice-cold saline, and weighed. A known weight of them was used for homogenate preparation and used for various biochemical analyses.

Evaluation of biochemical parameters: The total protein in the liver was estimated by the method of Lowry et al. The activities of marker enzymes aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), and γ -glutamyl transpeptidase (GGT) were assayed in the serum by the method¹⁶⁻¹⁸. MDA released from endogenous lipoperoxides, reflecting the lipid peroxidation process, were assayed in liver and serum as described by¹⁹. The activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH)—were estimated in the liver tissue homogenate by the method of Kakkar *et al.* (1984)²⁰⁻²³.

Statistical analysis: Values were expressed as mean \pm SD for six rats in the 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 comparisons²⁶. Statistical analysis carried out by Ms-Windows based graph pad InStat software (Graph Pad Software, San Diego, CA, USA) 3 version was used. A value of $p < 0.001$ was considered statistically significant.

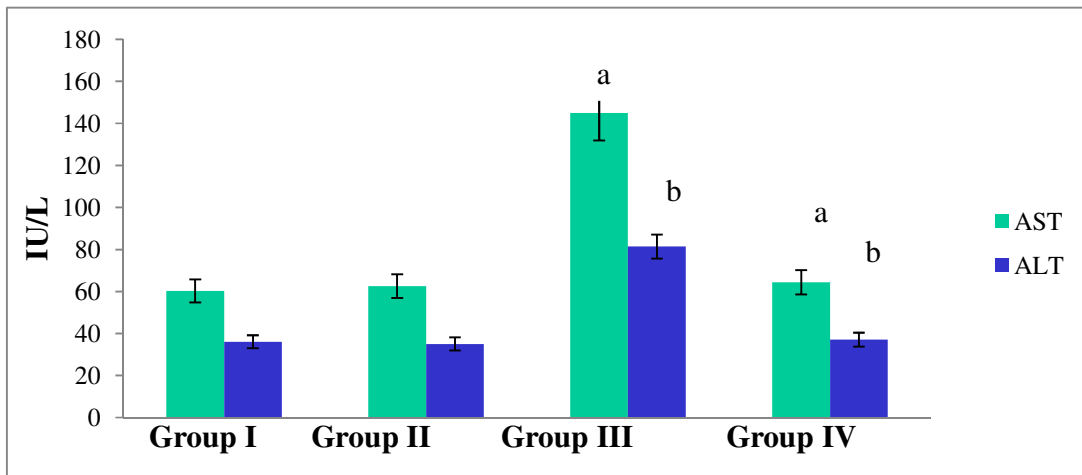
Results and Discussion

Effect of SRBE on the activities of marker enzymes: Figure 1 to 3 portrays the effect of SRBE on the activities of marker enzymes AST, ALT, LDH, and GGT in the serum of control and experimental group of rats. These marker enzymes are significantly increased in DEN-induced group 3 animals when compared with group 1 normal control animals. SRBE -treated group 4 showed a significant decrease in the activities of these enzymes when compared with group 3 DEN-induced animals. This reveals that SRBE has restoration potential of liver tissue.

Assessment of lipid peroxidation: Figure 4 shows the level of LPO in the liver of control and experimental groups of animals which was analyzed for oxidative stress. In DEN-induced group 3 animals, there is a significant increase in the levels of lipid peroxides when compared with group 1 normal control animals. This could be a tumor burden. Whereas in SRBE - treated group 4 animals, there is a significant decrease in the levels of lipid

peroxides when compared with group 3 tumor-bearing animals. However, animals treated with SRBE alone (group 2) did not show any significant changes when compared with group 1 control animals.

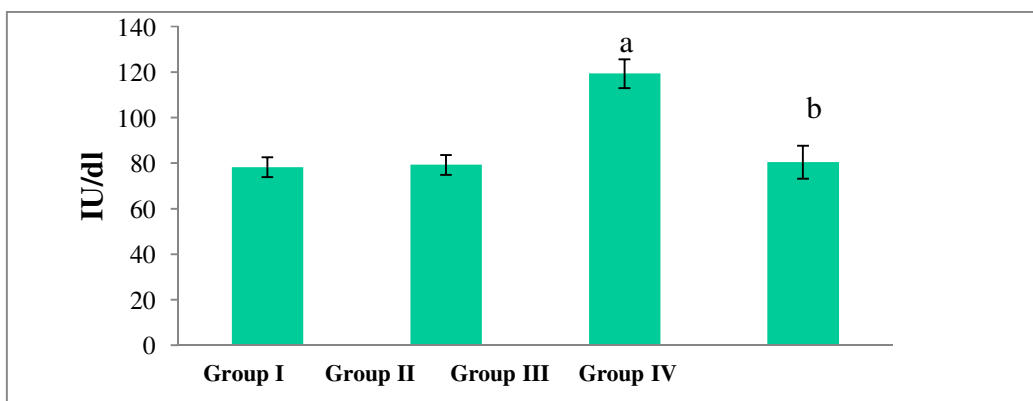
Evaluation of antioxidant status in liver: Figure 5 to 7 depicts the antioxidant status in the liver of control and experimental group of animals. DEN-induced group 3 animals exhibited a significant decrease in the activities of SOD and CAT when compared with group 1 normal control animals, SRBE -treated group 4 showed a significant increase in the activities of SOD and CAT when compared with group 3 DEN-induced animals. The activities of GPx, GR, and GSH also significantly decreased in DEN induced group 3 tumor-bearing animals when compared with group 1 control animals. In SRBE -treated group 4 animals, there is a significant increase in the activities of GPx, GR, and GSH when compared with group 3 DEN-induced animals. No adverse effect was observed in group 2 animals.



^a*p* <0.001 significantly different compared with group I and II control animals, ^b*p* <0.001 significantly different compared with group III animals.

Figure-1

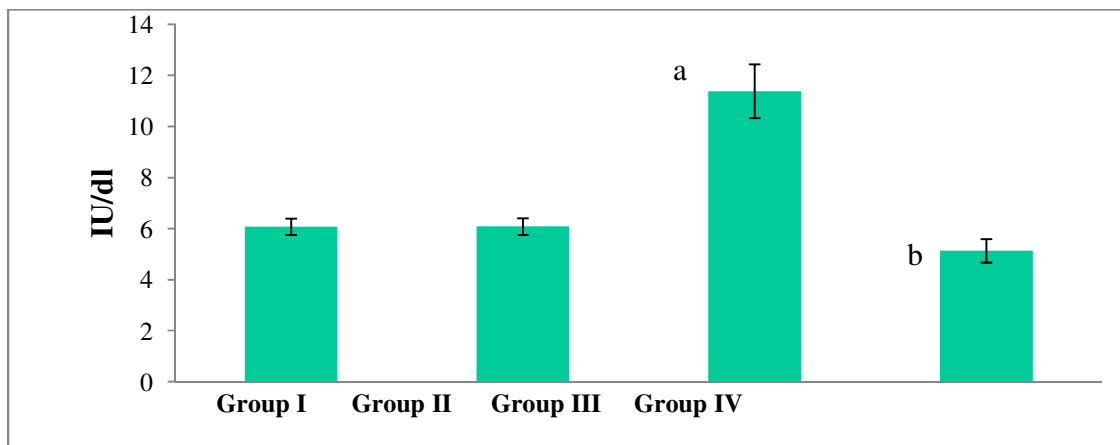
Effect of SRBE on AST and ALT activity in control and experimental rats



^a*p* <0.001 significantly different compared with group I and II control animals, ^b*p* <0.001 significantly different compared with group III animals.

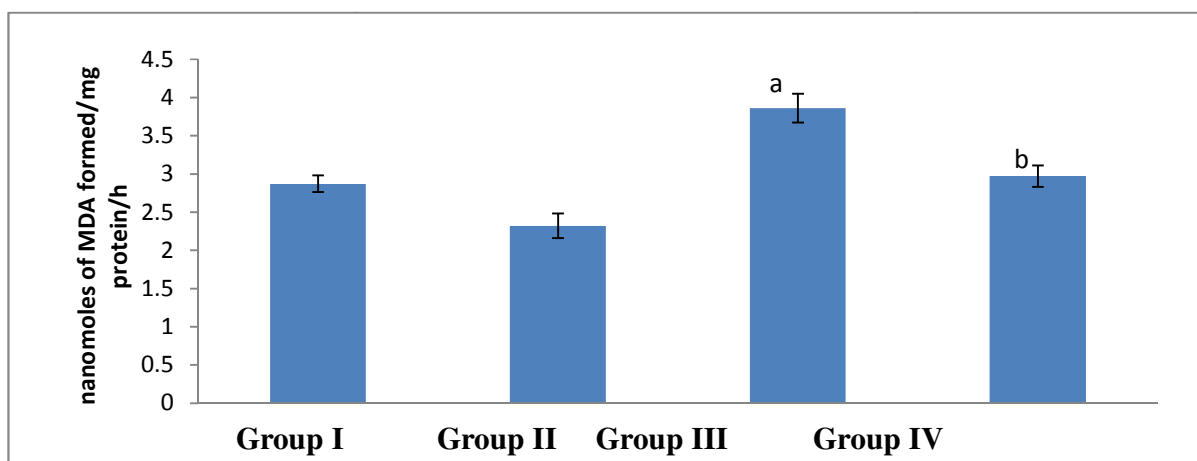
Figure-2

Effect of SRBE on LDH activity in control and experimental rats



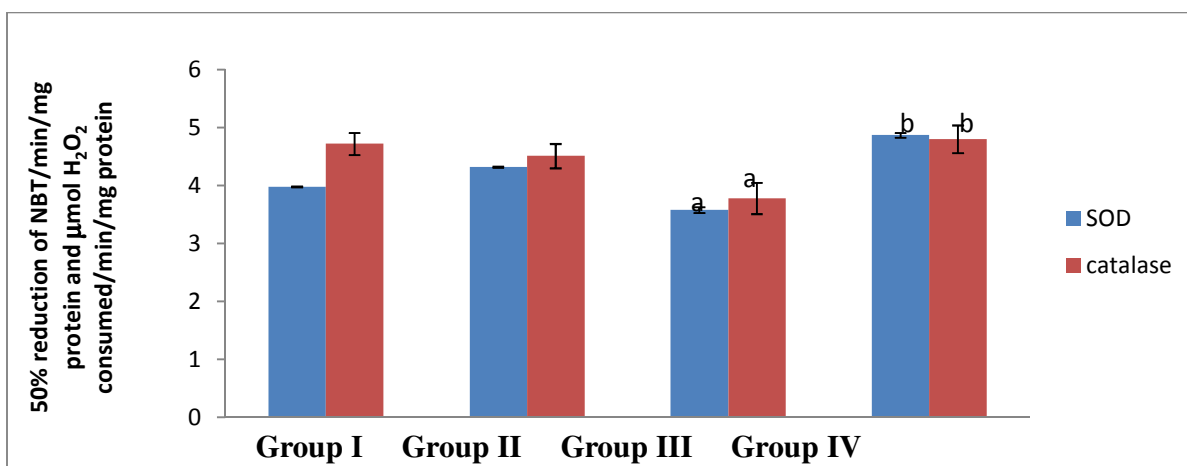
^a $p < 0.001$ significantly different compared with group I and II control animals, ^b $p < 0.001$ significantly different compared with group III animals

Figure-3
 Effect of SRBE on GGT activity in control and experimental rats



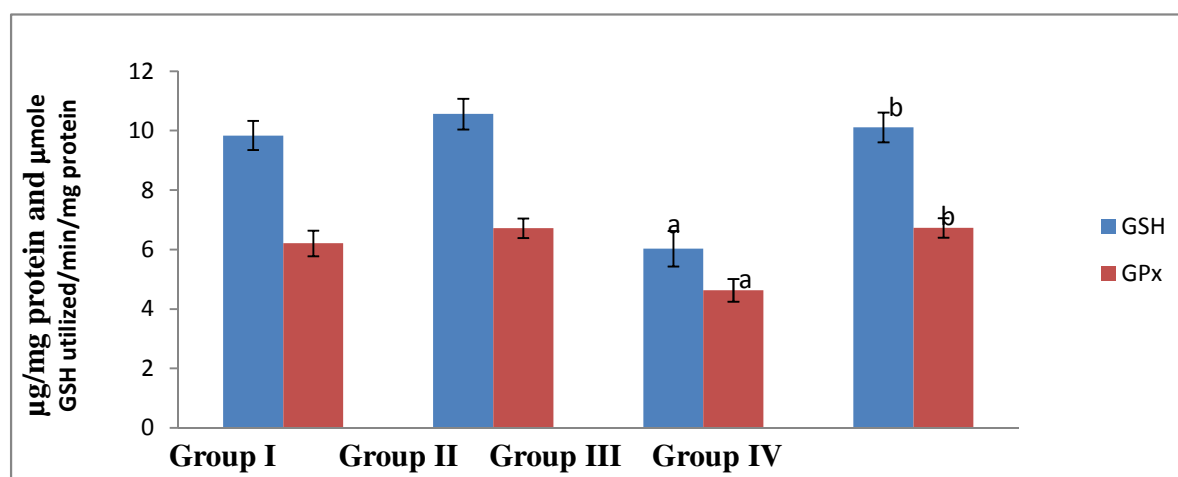
^a $p < 0.001$ significantly different compared with group I and II control animals, ^b $p < 0.001$ significantly different compared with group III animals

Figure-4
 Effect of *Shorea robusta* on liver MDA in control and experimental rats



^a $p < 0.001$ significantly different compared with group I and II control animals, ^b $p < 0.001$ significantly different compared with group III animals

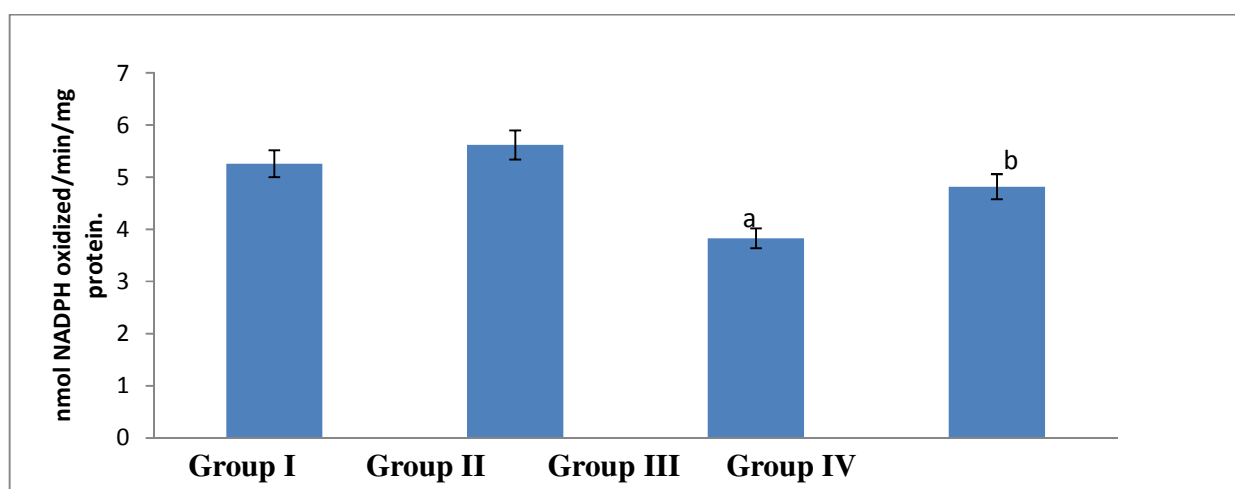
Figure-5
 Effect of *Shorea robusta* on liver SOD and Catalase in control and experimental rats



^a $p < 0.001$ significantly different compared with group I and II control animals, ^b $p < 0.001$ significantly different compared with group III animals

Figure-6

Effect of *Shorea robusta* on liver GPx and GSH in control and experimental rats



^a $p < 0.001$ significantly different compared with group I and II control animals, ^b $p < 0.001$ significantly different compared with group III animals

Figure-7

Effect of *Shorea robusta* on liver GR in control and experimental rats

Hepatic injury caused by DEN generally reflects instability of liver metabolism which leads to distinctive changes in the serum enzyme activities²⁴. Intracellular enzymes, such as transaminases, LDH, and GGT are useful indicators for liver function; their increased levels are indicators of liver damage. Aminotransferases (AST and ALT) are a reliable marker enzymes of liver and they are the first enzymes to be used in diagnostic enzymology when liver damage has occurred²⁵. Because of their intracellular location in the cytosol, toxicity affecting the liver with subsequent breakdown in membrane architecture of the cells leads to their spillage into serum, and their concentration rises in the latter. The discharge of LDH reflects a nonspecific alteration in the plasma membrane integrity and/or permeability. LDH is a familiar sensitive marker of solid neoplasm²⁶ and many studies revealed increased LDH activity in various types of tumor²⁷. GGT is an enzyme

embedded in the hepatocyte plasma membrane, mainly in the canalicular domain; again the liberation of this enzyme into serum indicates damage to the cell and thus injury to the liver. It is point outing that serum GGT activity is considered to be one of the best indicators of liver damage²⁸. In the present study, SRBE treatment significantly attenuated the increased activities of these enzymes.

Oxidative stress is associated with damage to a wide range of macromolecular species including lipids, proteins, and nucleic acids thereby producing major interrelated derangements of cellular metabolism including peroxidation of lipids. Free radicals and nonradicals oxidizing species were produced in animals treated with carcinogens, and also in human tissues²⁹. Reactive oxygen species (ROS) is formed from endogenous or exogenous sources are highly reactive, toxic, and mutagenic³⁰.

DEN has been shown to generate free radicals³¹ an uncompromising free radical generation in the liver overwhelms the antioxidant status and ultimately proceeds to oxidative stress paving way to carcinogenesis³². Lipid peroxidation plays an important role in carcinogenesis³³ is the most studied biologically relevant free radical chain reaction and measured as malonaldehyde (MDA). Induction of DEN has been reported to generate lipid peroxidation products like malondialdehyde and 4-hydroxy nonenal that may interact with various molecules leading to cause oxidative stress and carcinogenicity³⁴. Increased level of MDA was recently reported during DEN-induced hepato carcinogenesis. This dynamic action may further lead to uncompromised production of free radicals overwhelming the cellular antioxidant defense³⁵. It has been extensively reported that free radicals participated in DEN-induced hepatocarcinogenesis. MDA generation at the initiation stage can be prevented by free radicals scavengers and antioxidant action of SRBE. Animals treated with SRBE exhibited significantly lowered the levels of MDA, both in liver and serum, when compared with animals induced with DEN. This shows the antilipid peroxidative role of carvacrol that is probably mediated by its ability to scavenge free radical generation.

Antioxidants possess a variety of biological activities, including the induction of drug-metabolizing enzymes, inhibition of prostaglandin synthesis, inhibition of carcinogen- induced mutagenesis, and scavenging of free radicals³⁶. Antioxidants may protect membrane from ROS toxicity by prevention of ROS formation by the interruption of ROS attack, by facilitating the repair caused by ROS and by providing cofactors for the effective functioning of other antioxidants³⁷. Development of life threatening diseases like cancer is linked to the availability of these antioxidants⁴¹. Natural antioxidants are capable of inhibiting the ROS production and thereby reducing the associated intracellular oxidative stress³⁸.

SOD is the first line of defense in the antioxidant system against the oxidative damage mediated by superoxide radicals³⁹. Superoxide dismutases catalyze the dismutation of superoxide radical to hydrogen peroxide and water⁴⁰. Furthermore, CAT or GPx catalyze the transformation of H₂O₂ to harmless byproducts. Glutathione, a cysteine-containing tripeptide, is required to maintain the normal reduced state of cells and to counteract all the deleterious effects of oxidative stress. GSH is said to be involved in many cellular processes including the detoxification of endogenous and exogenous compounds. DEN, an electrophilic carcinogen may interact with the large nucleophilic pool of GSH thereby reducing the macromolecule and carcinogen interaction⁴¹. In SRBE treated animals, there was a significantly higher level of GSH in liver when compared to DEN-induced animals consistent with the idea of attenuation of DNA-carcinogen interaction and thereby averting a favorable environment for carcinogenesis.

Decreases in the activities of SOD, CAT, GPx, GR, and GSH are seen in tumor cells. The compounds that can scavenge

excessive free radicals in the body are suggested to hinder the process of carcinogenesis⁴². Such studies support our findings as we had seen a significant decrease in the activities of antioxidant enzyme in liver of animals treated with carcinogen in comparison with normal animals. On the other hand, there is a significant increase in the activities of antioxidant enzymes in liver of the animals administered both SRBE and carcinogen when compared with animals administered carcinogen alone.

Conclusion

In conclusion, the present study demonstrates that the SRBE possesses potent free radical scavenging and antioxidant activities. From the results, it is evident that SRBE is capable of modulating the levels of MDA and significantly increases the endogenous antioxidant defense mechanisms in DEN-induced hepatocellular carcinogenesis. Our results also show that the significant increase in the levels of serum markers was prevented by SRBE treatment. Then, we suggest that SRBE may be developed as an effective chemotherapeutic agent.

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