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Protective Role of Ethanolic Extract of TridaxProcumbens in Experimental Hepatocellular Carcinoma in Rats

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Abstract: *The present investigation was carried out to evaluate the hepatoprotective and antioxidant efficacy of Tridaxprocumbens (TP) against DEN induced hepatocellular carcinogenesis in experimental animals. The DEN induced cancer bearing animals were treated with 200 mg/ kg body weight of TP extract for 21 days. The levels of lipid peroxidation, protein carbonyls and liver marker enzymes such as AST (Aspartate transaminase), ALT (Alanine transaminase) and ALP (Alkaline phosphatase) were markedly increased in carcinogen administered animals. In contrast, the activity of the enzymic-antioxidants (Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx)) and non-enzymic antioxidants (Reduced glutathione (GSH), Vitamin C and Vitamin E) status both in liver were decreased in cancer bearing animals. Oral administration of TP to cancer bearing animals show significantly decreased levels of LPO, protein carbonyls and liver marker enzymes with simultaneous increase in the activities of enzymic and non-enzymic antioxidant levels, when compared with cancer bearing animals. From the obtained results it is concluded that TP is capable of restoring the liver architecture and also increase the antioxidant status during experimentally induced hepatocarcinogenesis.*
Keywords:*Antioxidant, Tridaxprocumbens, DEN, Liver cancer*

I. Introduction

Medicinal plants are playing a key role in the human health care. About 80% of the world population rely on traditional medicine, predominantly plants(1). These practices incorporated ancient beliefs and were passed on from one generation to another by oral tradition and/or guarded literature. Even though effective in treating various ailments very often these drugs are unscientifically exploited and/or improperly used. Therefore, these plant drugs deserve detailed studies in the light of modern science. It is estimated that in Indian villages about 7,500 plants are used in local health traditions. Out of these, the real medicinal value of over 4,000 plants is either little known or hitherto unknown to the mainstream population(2).

The liver cancer is the sixth most common cancer in worldwide, but because of very poor prognosis, it is the third most common cause of death from cancer in developingcountries (3). Liver is the most common site for metastasis from a variety of organs such as lung, breast, colon and rectum (4,5,6). Hepatocellular carcinoma **(**HCC) is a cancer arising from hepatocytes, the major cell type of the liver.Hepatocellular cancer formations are multifunctional process and possible mechanisms leading to those diseases have not been clarified yet.

Most of the hepatotoxic chemicals damage liver cells by inducing lipid peroxidation and other oxidative damages. The free radical damage is the most important contributor to the disease. Enhanced lipid peroxidation produced during the liver microsomal metabolism of ethanol results in hepatitis and cirrhosis (7). It has been estimated that about 90% of the acute hepatitis and cirrhosis are high risk for HCC due to viruses such as hepatitis B virus (HBV) and Hepatitis C virus (HCV) (8).N–Nitrosodiethylamine (DEN) is one of the most important environmental carcinogen in N-Nitrosamine class, which primarily induce liver cancer by oxidative damage in the DNA of hepatocyte cell (9). HCC's limited treatment remedy and the poor prognosis emphasize the importance in developing an effective chemoprevention for this disease. In addition, oxidative stress has recently been suggested to participate in both the metabolism (activation and detoxification) and the carcinogenic actions of nitrosoamines, including DEN (10).

Many medicinal plants have an advantage in drug discovery based on their use by humans for more than thousands of years. Tridaxprocumbens Linn (family Asteraceae, fig.1), one of the Indian plant used in ayurvedic preparations, which have hepatoprotective property. Several studies have been demonstrated the antioxidant and anti-tumor activity, anti-arthritic, anti-pyretic and anti-inflammatory activity of this plant (11,12,13). The leave extract has shown to have immunomodulatoryactivity, anti-stress effect and antioxidant activity (11,14,15,16). In previously many reports revealed that TP is used in treating various forms of cancer (17,12). Antioxidants are good markers of free radical induced tissue damage of the cells. Therefore, an assay of lipid peroxidation, protein carbonyls, liver marker enzymes and antioxidants were performed to ascertain the role of TP in decreasing the macromolecular damages and improving the antioxidant status in experimentally induced hepatocellular carcinoma.

Fig:1Tridaxprocumbens Linn

II. Materials And Methods

Animals

Male albino rats of wistarstrainwere used throughout the study. The animals were purchased from Central Animal House facility, University of Madras, Chennai-113and maintained in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed with standard pellet diet (Gold Mohor rat feed, Ms. Hindustan Lever Ltd., Mumbai) and water *ad libitum*. The animals were acclimatized to the laboratory conditions for one week before starting the experiment.

Drugs and chemicals

N-Diethylnitrosoamine (DEN), Bovine Serum Albumin (BSA) and antioxidant enzymes were obtained from Sigma Chemical Co. (St. Louis, USA). Staining solution was purchased from Ranbaxy Chemicals Ltd., (Mumbai, India). All the other chemicals used in the study were of analytical grade.

Plant material and Extraction

The plant, T. procumbens was identified at the Herbarium of the Siddha Central Research Institute, Chennai. The plant was air dried and pulverized into coarse powder. The air dried and pulverized plant material (1000 g) was exhaustively extracted with ethanol (1000 ml, BDH) using a soxhlet extractor for 24 h. The extract was concentrated in vacuum using a rota vapor and used for treat the liver cancer in experimental rats.

Experimental design

The rats were randomly divided into five groups (6 rats each): group 1- normal; group 2- Liver cancer; group 3- Cancer rats pre-treated with TP (200 mg/kg b.w) for 21 days; group 4- Cancer rats post-treated with TP (as group-3); and group 5- drug control rats. After the experimental period of 15 weeks, the rats were anesthetized with diethyl ether and sacrificed by cervical decapitation. The liver tissue excised was washed with ice cold saline. A portion of the liver was then homogenized in 0.1M Tris buffer and the homogenate was used for biochemical estimation.

Biochemical assays

The levels of protein in the liver homogenate [18] were estimated. Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) (19), Alkaline phosphatase (ALP) (20) in liver; Lipid peroxidation (LP) in terms of thiobarbituric acid reacting substances (TBARS) (21). The protein carbonyl was quantified by the method of Levine et al (50). The activities of antioxidant enzymes superoxide dismutase (SOD) [22], catalase (CAT) [23], glutathione peroxidase (GPx) [24], gluathionereductase (GR) [25], and glucose-6phosphate dehydrogenase (G6PD) [26] were assayed in the liver tissue. Further, the levels of non-enzymatic antioxidants such as total reduced glutathione (GSH) (27), Vitamin C (Vit C) (28) and Vitamin E (Vit E) (29) were also estimated.

Liver histopathology

Histological evaluation of a portion of the liver and portion of specimen fixed in 10% formalin and embedded in paraffin wax. Sections were cut at 4µm in thickness, stained with hematoxylin and eosin (H&E 45X) and viewed under light microscope for histological changes.

Statistical Analysis

The data for various biochemical parameters were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). Values were considered statistically significant when $p < 0.05$ (30).

III. Results

Table 1 shows the body weight and liver weight of control and experimental group of animals. In group 2 animals there is a significant decrease in the final body weight when compared with groups 1 and 4. The group 4 TP pretreated animals showed a significant increase in the final body weight when compared with group 2

animals. In group 2 animals the relative liver weight is significantly increased when compared with group 1 animals and there is a significant decrease in the liver weight in TP treated groups 4 and 5 animals when compared with group 2 animals.

Graph 1. Shows the effect of TP on liver marker enzymes of control and experimental animals. The activity of AST and ALT was significantly decreased in the liver tissue of animals from group 2 as compared with group 1 (graph1) TP-treated animals (groups 4 and 5) showed a significant increase in the level of transaminases as compared with group 2. The activity of ACP, ALP, LDH, GGT and 5'NT was significantly increased in the liver tissue of animals from group2 as compared with group 1. There was a significant decrease in the activity of these enzymes in TP-treated groups as compared with group 2.

Table 2.shows the activities of SOD, CAT, GPx, GR and the levels of GSH in the liver of control and experimental group of animals. In group 2 DEN induced animals there is a decrease in the activities of SOD, CAT, GPx, GR and in the levels of GSH when compared with group 1 control animals. In TP treated groups 4 and 5animals there is an increase in the activities of SOD, CAT, GPx, GR and in the levels of GSH when compared with tumor bearing group 2 animals.

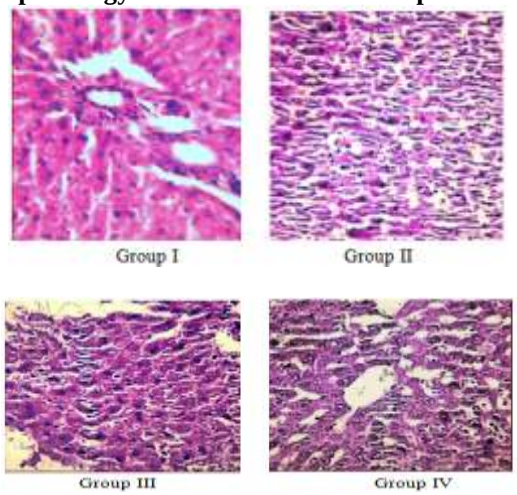
Graph 2.show the levels of lipid peroxidation and protein oxidation in liver of control and experimental rats in each group. In induced rats, the lipid peroxidation and protein oxidation levels have significantly increased when compared to control rats. Whereas pre-treated and post-treated rats show these levels to be statistically ($P < 0.001$, $P < 0.05$ and $P < 0.01$) and significantly decreased when compare to induced rats group II, but in TP alone treated rats, these levels near to control rats.

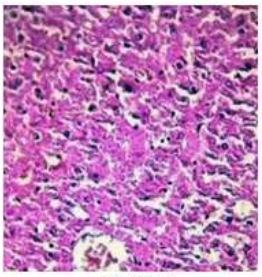
Table3. shows the levels of lipid Peroxidation (LPO) and protein carbonyl in liver of control and experimental rats in each group. In tumour induced rats (Group-II), the lipid Peroxidation and protein carbonyl levels in liver were significantly ($P < 0.001$) increased when compared to control rats (Group-I), whereas pretreated and post-treated rats (Group-III &IV) showed a statistically ($P < 0.001$) significant decrease when compared to tumour induced rats (Group II). These levels were brought back to near to normal levels in TP alone treated rats (Group-V).

Histopathology

Plate 1. Histopathological examination of liver sections from control group1 animals revealed normal architecture and Cells with granulated cytoplasm and small uniform nuclei. Group 2 animals revealed loss of architecture, showed a tendency to spread by intrahepatic veins, both hepatic and portal with significant tumor thrombi within portal vessels, the histologic appearance of hepatocellular carcinoma is also extremely variegated. Many different histologic patterns may be seen. The tumor cells are growing in nests and thick cords that are separated from one another by thin walled sinusoids. Cytology of the tumor cells shows some resemblance to normal hepatocytes but they are slightly larger, have more irregular nuclei, and prominent nuclei. There are also numerous mitotic figures. Group5 animals exhibited normal architecture indicating the non-toxic nature of TP. Whereas group3 animals pretreated with TP showed few neoplastically transformed cells and hepatocytes maintaining near normal architecture, group4 animals posttreated with TP showed loss of architecture, comparative less tendency to spread by intrahepatic veins, both hepatic and portal vessels.

Plate1.Histopathology of liver of control and experimental animals





Group V

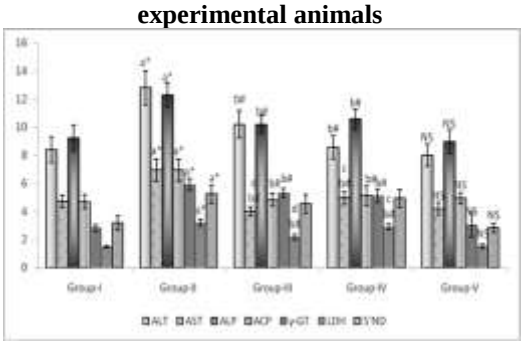
Plate1. Interpretation: Group-I-Photomicrographof liver of control group shows hepatocytes in a normal architecture (H&E 45X). Group-II- Photomicrograph of liver of DEN induced hepatocellular carcinoma group shows degeneration of hepatocytes, increase in nuclear size, hyperchromatism, hyperplasia and nodular collection of epithelial cells. Group-III- Photomicrograph of liver of hepatocellular carcinoma animals pre-treated with TP group shows reduction in the degeneration of hepatocytes and hyperchromatism. Group-IV- Photomicrograph of liver of hepatocellular carcinoma animals post-treated with TP group shows greater reduction in chromatin condensation and almost normal architecture Group-V- Photomicrograph of liver of hepatocellular carcinoma animals treated with TPalonegroup shows good reduction in the degeneration and hyperchromatism.

Table 1: Effect of plant extract on body weight and liver weight in control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
Body wt (gm)	181.04±10.03	137.02±9.01 a	154.04± 11.02^{ab}	158.12± 9.1^{abc}	179.12±10.10^{NS}
liver wt (gm)	4.64±0.44	6.80± 0.54 ^{a}	5.13± 0.47 ^{ab}	4.68± 0.46 $^{a\,bc}$	4.53± 0.42^{NS}

Each value is expressed as mean ± S.D. for six male wistar rats in each group.
a: compared with Group I; b: compared with Group II; c: compared with Group III;
d: compared with Group IV; NS- Not significant;
Statistical significance: *p<0.001, $^{@}p$<0.01, #p<0.05.

Graph1: Effect of plant extract on the activity of some marker enzymes in liver of control and experimental animals

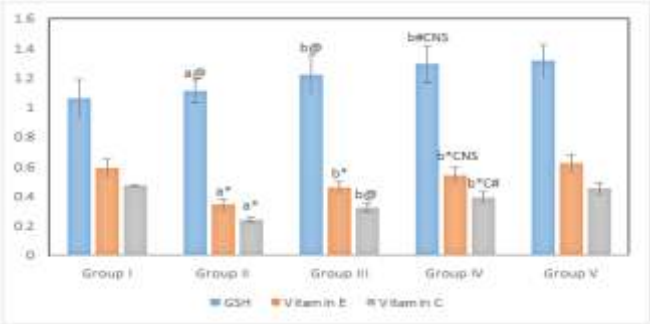


Each value is expressed as mean ± S.D. for six male wistar rats in each group.
a: compared with Group I; b: compared with Group II; c: compared with Group III;
d: compared with Group IV;
Units - ALT, AST and LDH:µmoles of pyruvate liberated/min/mg protein
ALP and ACP:µmoles of phenol liberated/min//mg protein
5'ND - nmoles of Pi liberated /min/mg protein
Statistical significance: *p<0.001, $^{@}p$<0.01, #p<0.05, NS-Not significant

Table: 2Effect of plant extract on enzymic antioxidants in the liver of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
SOD	3.47 ± 0.40	1.63 ± 0.18^{a*}	3.37 ± 0.36^{b*}	3.44 ± 0.40$^{b*\ NS}$ c	3.52 ± 0.34
CAT	236.00±19.00	108.00±13.00^{a*}	148.00±14.00$^{b@}$	226.00±21.00$^{b*\ \#}$ c	241.00± 25.00
GPx	33.60 ± 3.05	11.70 ± 2.19^{a*}	24.60 ± 4.63^{b*}	29.57±3.18$^{b*\ *}$ +C	34.20 ± 3.76
GR	2.15 ± 0.30	1.07 ± 0.01^{a*}	1.54 ± 0.19^{b*}	1.78 ± 0.19$^{b*\ NS}$ c	2.17 ± 0.30

Graph 2: Effect of plant extract on non enzymic antioxidants in the liver of control and experimental animals



Each value is expressed as mean ± SD for six rats in each group.
Units - SOD : units/min/mg protein ; CAT : µmoles of H_2O_2 liberated/min/mg protein ;
GPx :µmoles of GSH oxidised/min/mg protein ; GSH, Vitamin C, Vitamin E : µg/mg protein
a - as compared with Group I; b - as compared with Group II
Statistical significance - $^{\#}p<0.001$, $^{@}p<0.01$, *p<0.05.

Table:3. Effect of plant extract on the levels of Lipid Peroxidation and protein carbonylin liver of control and experimental animals

Particulars	Group I	Group II	Group III	Group IV	Group V
LPO	1.30 ±0.14	3.40 ± 0.26 a#	2.34 ± 0.22b#	3.02 ± 0.25b#	2.03 ± 0.17b#
Protein carbonyl	1.13 ± 0.1	2.11 ± 0.21a*	1.34 ± 016 b*	1.41 ± 015b# cns	1.09 ± 0.07

Each value is expressed as mean ± SD for six rats in each group.
Units:Plasma:nmoles of MDA liberated/mg protein;Liver:nmoles of MDA liberated/mg proteina - as compared with Group I; b - as compared with Group II
Statistical significance - $^{\#}p<0.001$, $^{@}p<0.01$, *p<0.05.

IV. Discussion

The ancient Ayurvedic physician understood the delicate cellular mechanisms of the body and deterioration of the functional efficiency of the body tissues. They developed certain dietary and therapeutic measures to arrest/delay aging and rejuvenating whole functional dynamic of the body organs.In Nigeria, Tridaxprocumbens is traditionally used in the treatment of fever, typhoid fever, cough, asthma, epilepsy and diarrhea (31,14). Now there are plenty of research articles on this plant, its parts being used as drug which haveimmunomodulatory and anti-inflammation and protective effects, but still understanding the basic molecular mechanism is unknown.

Nowadays a majority of the diseases are reported to be due to the shift in the balance of the pro-oxidant and the oxidant homeostatic phenomenon in the body by increased generation of the free radicals caused by excessive oxidative stress (32,33). Free radicals are natural by-products of our own metabolism. These are electrically charged molecules that attack our cells, tearing through cellular membranes to react and create havoc with the nucleic acids, proteins and enzymes present in the body is known as oxidative stress by causing cells to lose their structure, functions and eventually destroy them. The antioxidant defense systems can only protect the body only when the amount of the free radicals is within the normal physiological level. But when the balance is shifted to more free radicals, it leads to oxidative stress, which may result in tissue injury and subsequent disease (34).Over about 100 disorders have been reported as reactive oxygen species (ROS) mediated.

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 lipid peroxidation. Free radicals and non-radical oxidizing species are regularly produced in animals treated with carcinogens, and also in human tissues (35). DEN has been shown to generate an uncompromised free radical in the liver overwhelms the antioxidant status and ultimately proceeds to oxidative stress paving way to carcinogenesis (36,37). In recent years much research has been dedicated in identifying the plant components which contribute in combating the oxidative stress and free radical induced damage, which is mainly the first

step of chemical carcinogenesis.Lipid peroxidation has been shown to perturb the bilayer structure and modify membrane fluidity (38) and may lead to the formation of several toxic byproducts such as malondialdehyde and 4-hydroxynonenal. They attack cellular targets including DNA, inducing mutagenicity and carcinogenicity (39). Increased level of lipid peroxidation was recently reported during DEN induced hepatocarcinogenesis (40).InHCC there is a disequilibrium between oxidant and antioxidant balance which is tilted towards oxidant side (41). This may be the reason for the elevated lipid peroxidation level in the liver of DEN treated animals. In the current study, the focus was to assess the levels of oxidative stress markers, lipid peroxides and protein carbonyls in the liver of DEN induced male Wistar rats. In line with this finding there is a significant increase in the levels of lipid peroxidation in the liver of the animals induced with DEN. However, in animals treated withTridaxProcumbens (both pre and post) exhibited significantly low levels of lipid peroxidation in the liver compared to animals induced with DEN, showing the anti-lipidperoxidative role of TP and is probably mediated by it stability to inhibit free radical generation.

Proteins are important targets of oxidative modifications. Protein carbonyl is a product of irreversible non enzymatic oxidation or carbonylation of protein and indicates free radical generation in cells (42). Oxygen radicals generated as byproducts of cellular metabolism or from carcinogenic assault result in functional changes in structural & enzymatic proteins (43). The presence of carbonyl group has been used as a marker of reactive oxygen mediated protein oxidation (44). There was a significant increase in the levels of protein carbonyls in DEN induced animals which was restored to lower levels on treatment with TP. TP has shown to decrease oxidative stress or MDA products by increasing the antioxidant enzymes like SOD, catalase and thiol of the cell might alsobe a reason for decreased protein carbonyl levels, since carbonyl groups in proteins are mainly introduced by MDA produced during lipid peroxidation (45).

Antioxidants may protect membrane from ROS toxicity by prevention of ROS formation by scavenging the reactive metabolites and converting them to less reactive molecules (46). The superoxide anion, hydrogen peroxide and the hydroxyl radical are the major ROS which function in concert to induce LPO of cell membrane lipids. The toxic peroxidative products cause widespread cellular injury (47). Natural antioxidants are capable of inhibiting the ROS production and thereby reducing the associated intracellular oxidative stress (48,49). From this we observed that the levels of Vitamin C, E and GSH levels were decreased in cancer induced animals. On treatment with TP the enzymic and non-enzymic antioxidants levels were brought to near normal. This may be due to the antioxidant property of TP. But still there is lacuna in the existing knowledge because the proper mechanism of the action of this plant is not clear. Even though continuously used in traditional practices, the clinically efficacy of this preparations have not been scientifically validated. The personalized medicine is still followed by Ayurvedic, making it difficult for the global acceptance, as the exact mechanism for their uses are not clear. Thus, a furtherstudiesgoing on to isolate active principles from this plant parts and their pharmacological validation in terms of modern medicine will be of great medicinal importance in future.

V. Conclusion

From these observations it can be concluded thatTridaxProcumbens may suppress the formation of DEN induced hepatocarcinogenesis in rats by alleviating lipid peroxidation through scavenging of free radicals, or by enhancing the activity of antioxidants, which may involve the free radical scavenging mechanism. The precise molecular mechanism of Tridaxprocumbens against DEN induced liver cancer is under way.

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