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Chemopreventive Effect of *Phyllanthus polyphyllus* Against N-Nitrosodiethylamine Induced Liver Tumors by Regulating Liver Enzymes, Lipid Peroxidation and Antioxidants

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Abstract: This study was aimed to evaluate the chemopreventive effect of methanol extract of *Phyllanthus polyphyllus* (MPP) against N-nitrosodiethylamine (DEN, 200mg/kg) induced experimental liver tumor in male Wistar rats. Administration of MPP (200 and 400mg/kg) effectively suppressed liver tumor induced by DEN as revealed by decrease in DEN induced elevated levels of alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), alkaline phosphatase (ALP), gamma glutamate transpeptidase (γGTP), lipid peroxidation (LPO) and alfa feto protein (AFP). The extract also produced an increase in total proteins and enzymatic antioxidants [superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)] and non enzymatic antioxidants [Reduced Glutathione (GSH), Vitamin C and vitamin E] levels when compared to liver tumor bearing animals. Our data suggest that MPP may extend its chemopreventive effect by modulating lipid peroxidation, liver enzymes and augmenting antioxidant defense system.

Key words: *Phyllanthus Polyphyllus* · N-Nitrosodiethylamine · Biochemical Parameters · Alpha Feto Protein · Lipid Peroxidation · Antioxidants

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major problem not only in developed countries but also in most undeveloped countries. It is induced by toxic industrial chemicals, air and water pollutants and also, food additives and fungal toxins [1]. Since the liver is the major site of metabolism of ingested materials, it is more susceptible to carcinogenic insult. Moreover, due to the high tolerance of liver, hepatocellularcarcinoma is seldom detected at the early stage and once detected treatment has a poor prognosis in most cases [2].

HCC is one of the ten most common human cancers, with a worldwide incidence of over one million cases every year [3]. It accounts for about 90% of all primary liver cancers. HCC, a fatal malignancy represents 4% of all malignant tumors. A large number of agents including natural and synthetic compounds have been identified as having some potential cancer chemopreventive value. Plants and plant products have been shown to play an important role in the management of various liver disorders.

Phyllanthus polyphyllus Linn (Euphorbiaceae) is a deciduous shrub or small tree found mostly in hill areas of South India and Ceylon. It is popularly known as Sirunelli in Tamil. Leaves are traditionally used for liver diseases by tribes of Kolli hills, Tamilnadu, India [4, 5]. The phytochemical studies of the plant have revealed the presence of benzenoid, 4-0-methyl galic acid, together with three arylnapthalide lignans, namely phyllamyricin, justicidin B and diphyllin. Its extract shows dose dependent inhibition of inflammatory mediators such as LPS/INF-γ stimulated by peritoneal exuded macrophages [6], monoacetylated triterpene arabinosides and terpenes found have cytotoxic activity against human cancer cell lines [7], antitumor activity against transplantable tumour,

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protective effect of human umbilical vein endothelial cells (HUVEC) against glycated protein-iron chelate induced toxicity [8-10]. The present study is aimed to evaluate the hepatoprotective and antioxidant activity of methanol extract of *Phyllanthus polyphyllus* against DEN induced Phenobarbital promoted liver tumours in male wistar rats.

MATERIALS AND METHODS

Collection of the Plant Material: Phyllanthus Polyphyllus (euphorbiaceae) collected in the month of November 2009 from kolli hills, Tamilnadu, India and identified by Botanical Survey of India, Coimbatore and Tamilnadu, India. A voucher specimen (PP 03) has been kept in our laboratory for future reference.

Preparation of Extract: The leaves of *Phyllanthus polyphyllus* were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No 40 and treated with petroleum ether for dewaxing as well as to remove chlorophyll and it was later packed into soxhlet apparatus with methanol and subjected to hot continuous percolation using Soxhlet apparatus. After the completion of extraction, it was filtered and the solvent was removed by distillation under reduced pressure. The extract was stored in desiccator.

Phytochemical Screening: The MPP extract was subjected to preliminary phytochemical investigations [11] and was found with the presence of various constituents like Alkaloids, Carbohydrates, Glycosides, Phenolic compounds, Tannins and Flavanoids.

Animals: Healthy Male Wistar albino rats (6-8 weeks old) were used throughout the study. The animals were purchased from King Institute of Preventive Medicine, Chennai-600 034 and maintained in a controlled environmental condition of temperature (23±2°C) and relative humidity (50-70%) on alternatively 12 hr light/dark cycles. All animals were fed standard pellet diet and water *ad libitum*. The research has followed the national ethical standards for the care and use of laboratory animals and it was approved by the Institutional Animal Ethics Committee (IAEC) constituted for the purpose.

Acute toxicity studies (LD_{50}): The oral acute toxicity study of the extract was carried out in Swiss albino mice using up and down procedure as per OECD, 2001 [12]. Mice received methanol extract at various doses (500-2,000 mg/Kg) orally by gavage. They were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noticed after 24 h. In the toxicity study, no mortality occurred within 24 h under the tested doses of MPC.

Sources of Chemicals: N-Nitroso Diethylamine [DEN], bovine serum albumin and 2, 4, 6-Trinitro benzene sulfonate, was obtained from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were of analytical grade obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India and Glaxo Laboratories, CDH division, Mumbai, India.

Experimental Protocol: The rats were divided into 4 groups, each group consisting of six animals. Group 1 served as control animals and were treated with distilled water orally for 20 weeks. Liver tumor was induced in group 2, 3 and 4 by single intraperitoneal injection of DEN at a dose of 200 mg/kg body weight in saline. Two weeks after the DEN administration, the carcinogenic effect was promoted by 0.05% Phenobarbital [13], which was supplemented to the experimental animals through drinking water for up to 20 successive weeks. Group 3 animals also treated with MPP (200 mg/kg b.wt, dissolved in 0.3% cmc) simultaneously for 20 weeks from the first dose of DEN and Group 4 animals treated with MPP (400 mg/kg b.wt, dissolved in 0.3% cmc) simultaneously for 20 weeks from the first dose of DEN. At the end of experiments, animals were fasted overnight and were killed by cervical decapitation. Blood was collected and serum separated out. The liver were immediately removed and suspended in ice cold saline. A 10% of liver homogenate was used for antioxidant studies.

Biochemical Analysis: Serum α-feto protein (AFP) was estimated by method described by Premalatha & Sachdanandam [14]. Alanine amino transferase (ALT) and aspartae amino transferase (AST) was assayed by the method of Reitman and Frankel [15]. Alkaline phosphatase (ALP) was assayed by the method of Kind and King [16]. The activity of γ-glutamyl transpeptidase was estimated according to the method of Orlowski and Meister [17]. Protein was estimated by the method of Lowry *et al*. [18]. A portion of 10% of liver homogenate was used for antioxidant studies such as lipid peroxidation by the method of Ohkawa *et al*., superoxide dismutase (SOD) by the method of Marklund and Marklund [19], catalase (CAT) by the method of Sinha [20], Glutathione peroxidase (GPx) by the method of Rotruck *et al*. [21], Glutathione Reductase (GR) by the method of Staal *et al*. [22], reduced glutathione (GSH) by the method of Moron *et al*. [23], ascorbic acid (Vit C) by the method of Omaye *et al*. [24] and vitamin E by the method of Desai [25].

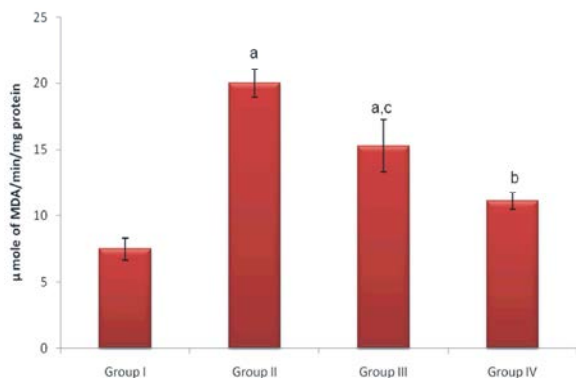

Fig. 1: Effect of MPP on level of α-feto protein against DEN induced liver cancer in rate

N=6; Each value is expressed as mean±S.E.M. Group I: control animals, Group II: Liver cancer bearing animals, Group III: MPP 200 mg/kg treated, Group IV: MPP 400 mg/kg treated

[a]P<0.001 Vs Control

[b]P<0.001 Vs Cancer bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test

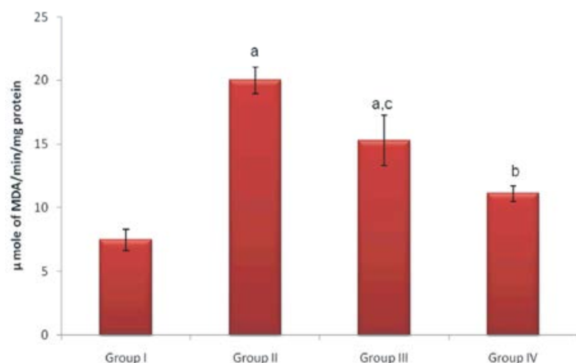

Fig. 2: Effect of MPP on level of Lipid peroxidation against DEN induced liver cancer in rate

N=6; Each value is expressed as mean±S.E.M. Group I: control animals, Group II: Liver cancer bearing animals, Group III: MPP 200 mg/kg treated, Group IV: MPP 400 mg/kg treated.

[a]P<0.001 Vs Control

[b]P<0.001; [c]P<0.01 Vs Cancer bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test.

Statistical Analysis: The values were expressed as mean±SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey-kramer multiple comparisons test. P values < 0.05 were considered as significant.

RESULTS

Serum Α-Feto Protein: Fig 1 showeded the level of α-feto protein (AFP) in the serum of the control and experimental animals. The group II showed the highest level of AFP (2.86±0.18) IU/ml. The other groups III and IV were significantly lowered AFP levels (p<0.001) on dose dependent manner when compared to the cancer bearing animals of group II. Among the cancer treated animals, the groups IV (400 mg/kg) more pronounce activity than group III (200 mg/kg). This result suggested that MPP could reduce back the level of AFP in the blood of cancerous rats near to the normal value.

Lipid Peroxidation: The level of LPO in liver tissues of control and experimental animals were depicted in Fig. 2. There found to be an increase in LPO in group II (*p*<0.001) cancer bearing rat when compared to control animals. These significant effects were reversed in MPP (200 and 400 mg/kg) treated group III and IV (*p*<0.001) on dose dependent manner.

Serum Transaminase: Table 1 shows the changes of AST and ALT determination after DEN administration. Both serum AST and ALT levels were markedly increased to their maximum value (U/L) at 165.74±2.38 and 297.62±3.63 respectively, at the end of the 20 weeks. In the normal group, serum AST levels were 65.72±1.10 and ALT levels were 96.15±2.20 U/L at the end of the 20 weeks, respectively. On the other hand, MPP treatment (200 and 400 mg/kg) produced dose-dependent reductions in AST and ALT levels. Both doses MPP in group III and group IV animals reversed these changes to near normal, but it was more effective in group IV than in group III animals.

Serum ALP, γGTP and Total protein: Table 1 showed the changes in hepatic ALP, γGTP and total protein activities in DEN treated rats. Activities of ALP, γGTP, had increased and decreased total protein at 20 weeks after DEN administration as compared with those of the control group I. On the other hand, the MPP reduced enzyme activities in a dose-dependent manner (200 and 400 mg/kg). However, the MP*P* extract (200 mg/kg) was slightly increased total protein activity. At higher dose (400 mg/kg), the MPP markedly attenuated the activity of total protein. This enzyme activity was completely restored to the normal level in group IV than III by treatment with the MPP at 200 mg/kg.

Table 1: Effect of MPP on serum AST, ALT, ALP, Total proteins and γGTP in DEN treated rats

Treatment	ALT U/L	AST U/L	ALP U/L	Total Proteins mg%	γGTP U/L
Group I (Control)	65.72±1.10	96.15±2.20	185.83±3.8	6.9±0.10	56.4±1.30
Group II (Cancer bearing animals)	165.74±2.38[a]	297.62±3.63 [a]	486.42±5.67 [a]	5.1±0.34 [a]	116±3.67 [a]
Group III (MPP 200 mg/kg)	120.76±1.74 [a,c]	186.47±2.17 [a,c]	316.87±4.63 [a,c]	5.8±0.15 [b]	89.6±1.31 [a,c]
Group IV (MPP 400 mg/kg)	98.45±1.25 [a,c]	129.23±1.34 [a,c]	242.19±3.28 [a,c]	6.3± 0.32[d]	70.9±1.26 [a,c]

N=6; each value is expressed as mean±S.E.M.

[a]P<0.001; [b]P<0.05 Vs Control

[c]P<0.001; [d]P<0.01 Vs Cancer bearing animals

Data were analyzed by one way ANOVA followed by Tukey Kramer multiple comparison test

Table 2: Effect of MPP on enzymic and non-enzymic antioxidants in liver of control and experimental animals

Treatment	SOD	CAT	GPx	GR	GSH	Vit -C	Vit -E
Group I (Control)	7.14±0.12	145±3.10	23.45±1.16	3.26±0.14	1.94± 0.06	0.75±0.005	0.45±0.002
Group II (Cancer bearing animals)	4.35±0.17 [a]	83±2.36 [a]	12.65±1.05 [a]	1.95±0.17 [a]	1.06±0.10 [a]	0.31±0.002 [a]	0.12±0.003 [a]
Group III (MPP 200 mg/kg)	5.47±0.23 [a,c]	96±2.97 [a,d]	16.52±1.25 [b]	2.63±0.09[b,d]	1.28±0.05 [a]	0.46±0.004 [a,c]	0.26±0.008 [a,c]
Group IV (MPP 400 mg/kg)	6.70±0.19 [c]	128±2.16 [a,c]	21.62±1.07 [c]	2.94±0.15 [c]	1.62±0.08 [c]	0.58±0.006 [a,c]	0.33±0.006 [a,c]

N=6; Each value is expressed as mean±S.E.M.

[a]P<0.001; [b]P<0.01 Vs Control

[c]P<0.001; [d]P<0.01 Vs Cancer bearing animals

Data were analysed by one way ANOVA followed by Tukey Kramer multiple comparison test

Enzymic and Non-Enzymatic Antioxidants: Table 2 represents the changes of enzymic and non-enzymic antioxidants of liver tissues of control and experimental animals. The enzymic and nonenzymic antioxidants such as Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione reductase, Reduced glutathione, vitamin C and vitamin E were significantly (p<0.001) reduced in group II animals when compared with group I animals. MPP (200 and 400 mg/kg) treated (Group III and Group IV) animals, these changes were brought back to near normal but in group IV it was more effective than group III [(p<0.001 and p<0.01].

Units: SOD-1U=amount of enzyme that inhibits the antioxidants of pyrogallol by 50%; CAT- µmoles of H_2O_2 consumed/min/mg protein; GPx-µmoles of GSH oxidized/min/mg protein; GSH-µg/mg protein; GR-nmoles of NADPH oxidized/min/mg protein; Vitamin C and Vitamin E- µg/mg protein.

DISCUSSION

In recent times, there is an increased risk of malignancy because of environmental pollution such as exposure to genotoxic and carcinogenic chemicals. This has created awareness to prevent the harmful effect of these chemical agents. This has lead to the development of several preventive agents. These agents were significantly reduced tumor incidence, delay tumor onset and also have minimal long-term toxicity. Any natural or synthetic agents, which exhibits any, or combination of these characteristics will qualify as a cancer-chemopreventive agent. The present study was undertaken to establish the cancer chemopreventive efficacy of MPP against DEN induced malignancy of liver.

α - fetoprotein (AFP), molecule carry the onco-fetal specificity Tumor marker. AFP is a serum protein that is detected in elevated concentration in conditions like hepatocellular carcinoma. AFP is a serum protein similar in size, structure and amino acid composition to serum albumin, but it is detectable only in minute amounts in the serum of normal adults. Elevated serum concentrations of this protein can be achieved in the adult by exposure to hepatotoxic agents (or) hepatocarcinogens and are frequently associated with HCC. It is a 72 KDa α-1 globulin with an uncertain biological function, is synthesized during embryonic life by fetal yolk sac, liver and intestinal tract. AFP has high specificity for hepatocarcinoma. Its serum concentration can be used to confirm hepatocarcinoma and for the diagnosis of tumor response to therapy. More than 90% of patients with hepatic cancer have increased serum AFP levels.

In the present study, a decrease in the levels of AFP following MPP (200 and 400 mg/kg) treatment indicates a positive prognosis. The decrease in the levels on MPP co-treatment prevents the neoplastic growth and reduces hepatic disorder, indicating that it possesses ant carcinogenic properties (Fig 1).

The role of transamination in biological systems is well known. It is apparent from transaminase substrate, i.e. oxaloacetic and pyruvic acids on one hand and glutaric and aspartic acids on the other hand, that transamination

is concerned with the interconversion of highly important metabolites. Elevated aminotransferase activities levels were observed in cancer bearing animals. Clinical diagnosis of neoplastic patients show an eight times increases over normal control patients [26]. Transaminase becomes gradually more pronounced towards the terminus, which indicates the severity of an advanced cancer condition. Increased transaminase activities in HCC have been reported by Rocchi et al., [27]. A good correlation exists between the activities of ALT and AST of tumor masses during therapy. The stable clinical and enzymatic pattern of these enzymes is noticed in patients with hepatic malignancy after chemotherapy, while patients failing to respond to drug showed progressive increase in the level of these enzymes. Similar results were observed by in dimethyl hydrazine-induced colon cancer in rats.

Diethyl nitrosamine (DEN) is a hepatotoxin and a carcinogen Similarly Phenobarbital is hepatotoxin and acts as promoter in hepatocarcinogenesis. An increase in AST, ALT activities in DEN induced animals correlate to the hepatotoxicity and carcinogenesis with the development of preneoplastic changes, increased severity and advanced stage of liver carcinoma. The lowering in the activities of AST and ALT on MPP treatment shows the hepatoprotective effect of MPP and inhibition of carcinogenesis (Table 1).

Alkaline phosphatase (ALP) is involved in transport of metabolites across cell membrane, protein synthesis, secretory activities and glycogen metabolism. Alkaline phosphatase is a membrane bound enzyme and its alteration is likely to affect the membrane permeability and produce derangement in the transport of metabolites. ALP was noticed in the serum and liver of hepatoma -bearing animals [28]. It was observed that the ALP activity was raised in the serum of cervical carcinoma patients. The rise in a activity of ALP in cancer bearing animals may be due to the disturbance in the secretory activity or in transport of metabolites or may be due to altered synthesis of certain enzymes in these conditions. ALP is used as a specific tumor marker during diagnosis in the early detection of cancer [29]. An increase in ALP activity on DEN administration may be due to altered synthesis of enzymes as in other hepatotoxic condition [30]. Activities of ALP are increased in precancerous lesion. In primary liver cell carcinoma and carcinoma of bile duct.The lowering of the activity of these enzymes significantly ($P<0.001$) indicates the inhibition of pre- cancerous transformation in the liver on MPP treatment in DEN+PB+MPP animals (Table 1).

Serum Gamma Glutamyl Transpeptidase (γGT) activity is higher in hepatocellular carcinoma. An increase in γGT activity paralleled with an increase in alkaline phosphatase activity is frequent in hepatocellular carcinoma. (Induction of γGT during hepatocarcinogenesis is frequent. An increased γGT activity causes resorption of GSH by preneoplastic foci rich in γGT that enhances cell proliferation and increases tumor promotion and favors transformation of preneoplastic foci to neoplasia [31]. Maintenance of γGT in serum on treatment with MPP (200 and 400 mg/kg) signifies its activities to modulate GSH transport and metabolism and thus prevent hepatocarcinogenesis. The increase in liver marker enzymes with a corresponding decrease shows the inhibitory effect of MPP in DEN induced hepatocarcinogenesis as evident from significant decreases in tumor marker (Fig 1).

Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals [32]. Administration of DEN has been reported to generate LPO products in general [33] and Phenobarbital enhanced the formation of the activated oxygen species in the preneoplastic nodules [34] in rat liver. Here the administration of DEN and Phenobarbital has shown to increase the level of liver tissue LPO during hepato carcinogenesis (Fig 2). This vigorous action may be lead by the uncompromised production of free radicals. It has been extensively reported that free radicals participated in DEN induced hepatocarcinogenesis [35, 36].

Nevertheless, by administration of MPP (200 and 400 mg/kg) in DEN induced and Phenobarbital promoted animals the level of LPO was decreased. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants [37]. This may represent the antioxidant potency of MPP and it might be an effective inhibitor in reducing TBARS formation. This scrutiny reveals that MPP is able to quench the LPO chain and is capable to shield the membrane from free radicals caused injuries.

The endogenous antioxidant system may counteract the ROS and reduce the oxidative stress with the enzymic antioxidants SOD, CAT and GPx. SOD accelerates the conversion of superoxide radical (O-2) to hydrogen peroxide while CAT or GPx converts H_2O_2 to H_2O. Depletion in the activity of these three antioxidant enzymes can be owed to an enhanced radical production during DEN and Phenobarbital metabolism. In this present observation an increase in MDA was presumably associated with increased free radicals, confirming the fact that these free radicals inhibited the activities of SOD,

CAT and GPx. Here the super oxide radical itself is also capable to inhibit the activity of SOD and CAT [38]. This is supported by earlier studies that showed during the DEN induced and Phenobarbital promoted hepato carcinogenesis [39]. The observed reduction in enzyme activities may be attributed to ROS; here the ROS themselves can reduce the activities of enzymes [40].

Activities of the enzymic antioxidants are reverted to near normal in MPP (200 and 400 mg/kg) treated animals. This indicates the antioxidant potency of the drug and so preventing the inactivity of these enzymes from ROS.

Vitamin E, Vitamin C and GSH are well known non enzymic antioxidant defense system of cells. Among these vitamin E is a well recognized, important biological free radical scavenger in the cell membrane [41]. It has been shown to provide protection against superoxides as well as H_2O_2 [42] and it contributes to membrane stability [43]. In hepatoma bearing animals the level of vitamin E was decreased considerably. Vitamin C is water soluble, antioxidant vitamin and can react with vitamin E radicals to regenerate vitamin E [44]. GSH, a non protein thiol is involved in many cellular processes including the detoxification of endogenous and exogenous compounds [45]. Accordingly GSH might be depleted partly by the Gpx mediated excess utilization of GSH. These three non-enzymic antioxidants are inter related by recycling processes.

Earlier report reveals that the levels of these non-enzymic antioxidants were also decreased in hepatoma bearing animals [35]. This observed reduction might be attributed to the utilization of these antioxidants to alleviate free radical induced oxidative stress. The increase in the level of these antioxidants after the administration of MPP (200 and 400 mg/kg) may be due to the direct reaction of MPP with ROS.

CONCLUSION

Our findings indicated that MPP inhibits the level of LPO and significantly increases the enzymic and non enzymic antioxidant defense mechanisms in DEN induced and Phenobarbital promoted experimental hepatocellular carcinogenesis. All these observations clearly indicate a chemopreventive function of the extract. Phytochemical studies have shown the presence of flavonoids in MPP. Flavonoids are known to possess antimutagenic and antimalignant effects [46]. Moreover, flavonoids have a chemopreventive role in cancer through the induction of enzymes affecting carcinogen metabolism and inhibit various activities of tumor promoters, which are involved in the process of carcinogenesis [47]. Chemopreventive effect of the MPP may be due to the presence of these compounds. Our results clearly indicate a significant chemopreventive effect of MPP. Further studies to characterise the active principles and to elucidate the mechanism action of MPP are in progress.

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