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Chemopreventive effect of *Phyllanthus polyphyllus* against N-nitrosodiethylamine induced liver tumors in wistar rats

Naina Mohamed Pakkir Maideen[1,*], Ravichandiran Velayutham[1] and Gobinath Manavalan[2]

***Corresponding author:**

Naina Mohamed Pakkir Maideen
[1]Department of Pharmacology, College of Pharmacy, Vels University, Velan Nagar, Pallavaram, Chennai-600 117, India.
pnmcologist@yahoo.com
Phone: +91-44 -2266 2500
Fax : +91-44 -2266 2513
[2]Ratnam Institute of Pharmacy, Pidathapolur (V & Post), Muthukur (M), Nellore-524 346, Andhrapradesh, India.

Abstract

The aim of the study is to evaluate the chemopreventive effect of methanol extract of *Phyllanthus polyphyllus* (MPP) against N-nitrosodiethylamine (DEN, 200mg/kg) induced experimental liver tumors in male wistar rats. Administration of *Phyllanthus polyphyllus* (200 and 400mg/kg) effectively suppressed liver tumor induced by DEN as revealed by decrease in DEN induced elevated levels of mitochondrial lipid peroxidation (LPO), deoxy ribonucleic acid (DNA), ribo nucleic acid (RNA) and liver weight. The extract produced an increase in mitochondrial enzymatic antioxidants (Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and non enzymatic antioxidants (Reduced Glutathione (GSH)) levels when compared to liver tumor bearing animals. Our data suggest that MPP may extend its chemopreventive effect by modulating the levels of mitochondrial lipid peroxidation, DNA and RNA and liver weight and augmenting mitochondrial antioxidant defense system.

Keywords: *Phyllanthus polyphyllus*, Liver Tumor, N-nitrosodiethylamine, Lipid Peroxidation, Antioxidants.

Introduction

Hepatocarcinoma is induced by toxic industrial chemicals, air and water pollutants and also, food additives and fungal toxins [1]. A large number of agents including natural and synthetic compounds have been identified as having some potential cancer chemo preventive 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 [2]. 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 [3], monoacetylated triterpene arabinosides and terpenes found to have cytotoxic activity against human cancer cell lines [4]. Its extract also showed antitumour activity against transplantable tumour, protective effect of human umbilical vein endothelial cells (HUVEC) against glycated protein-iron chelate induced toxicity and hepatoprotective effects [5-7]. The present study is aimed to evaluate the Chemopreventive effect of *Phyllanthus polyphyllus* against N-nitrosodiethylamine induced hepatic carcinogenesis in wistar rats.

Materials and Methods

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 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.

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.

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 four 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 with 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, which was supplemented to the experimental animals through drinking water for up to 20 successive weeks [8]. Whereas Group 2 animals receive DEN alone, Group 3 animals were treated with MPP (200 mg/kg b.wt, dissolved in 0.3% cmc) simultaneously for 20 weeks from the first dose of DEN (as in Group II) and Group 4 animals treated with MPP (400 mg/kg b.wt, dissolved in 0.3% cmc) (as in Group III) simultaneously for 20 weeks from the first dose of DEN (as in Group II). At the end of experiments, animals were fasted overnight and were killed by cervical decapitation. The liver was immediately removed and weighed. A portion of liver suspended in ice cold saline and 10% of liver homogenate was used for the analysis of lipid peroxidation (LPO) [9], superoxide dismutase (SOD) [10], Catalase (CAT) [11], Glutathione peroxidase (GPx) [12], Glutathione Reductase (GR) [13] and Reduced glutathione (GSH) [14]. The nucleic acids were extracted by the method of Schneider [15]. Deoxy ribonucleic acid (DNA) was estimated by the method of Burton [16] and Ribonucleic acid (RNA) was estimated by the method of Rawal et al. [17].

Statistical analysis

The values were expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. P values < 0.05 were considered as significant.

Results And Discussion

Lipid peroxidation (LPO)

The levels of LPO in mitochondria of control and experimental animals were depicted in Fig.2. There found to be an increase in LPO in group II ($p<0.001$) tumor bearing rats when compared to control animals. These significant effects were reversed in MPP (200 and 400 mg/kg) treated groups III and IV ($p<0.001$) on dose dependent manner. MPP treated group IV shows more restoration than treated Group III in revising these changes.

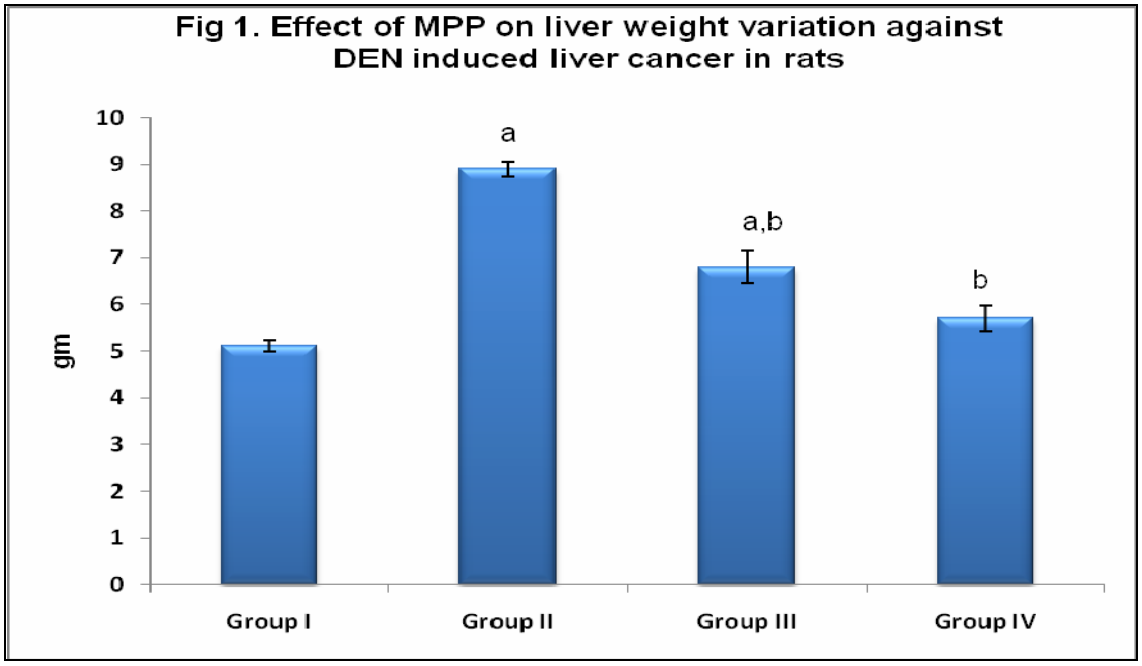
Table 1. Effect of MPP on the activities of liver mitochondrial antioxidant enzymes in control and experimental animals.

Treatment	SOD	CAT	GPx	GR	GSH
Group I (Control)	5.68 ± 0.15	76 ± 1.12	3.76 ± 0.14	2.71 ± 0.06	1.28 ± 0.09
Group II (Cancer bearing animals)	3.45 ± 0.16[a]	33 ± 1.40 [a]	1.84 ± 0.09 [a]	1.58 ± 0.04 [a]	0.73 ± 0.06 [a]
Group III (MPP 200 mg/kg)	4.10 ± 0.18 [c]	48 ± 1.34 [a,d]	2.37 ± 0.15 [a,e]	2.09 ± 0.04 [a,d]	0.98 ± 0.05 [c]
Group IV (MPP 400 mg/kg)	4.92 ± 0.25 [e]	59 ± 1.16 [a,d]	3.16 ± 0.07 [b,d]	2.36 ± 0.08 [a,d]	1.13 ± 0.04 [d]

N=6; each value is expressed as mean ± S.E.M.
[a]P<0.001; [b]P<0.01; [c]P<0.05 Vs Control
[d]P<0.001; [e]P<0.05 Vs Cancer bearing animals
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;

Mitochondrial Enzymic and Non-Enzymic Antioxidants

Table 1 shows the activities of mitochondrial antioxidant enzymes of liver tissues of control and experimental animals. Antioxidant enzymes were significantly ($p<0.001$) reduced in group II animals when compared with group I animals. These enzyme activities were increased in MPP treated Group III, (200 mg/kg) and Group IV (400 mg/kg) animals when compared to group II animals. Both doses of MPP in group III and group IV animals reverted these changes to near normal but it was more effective in group IV than in group III animals.



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.

Liver weight

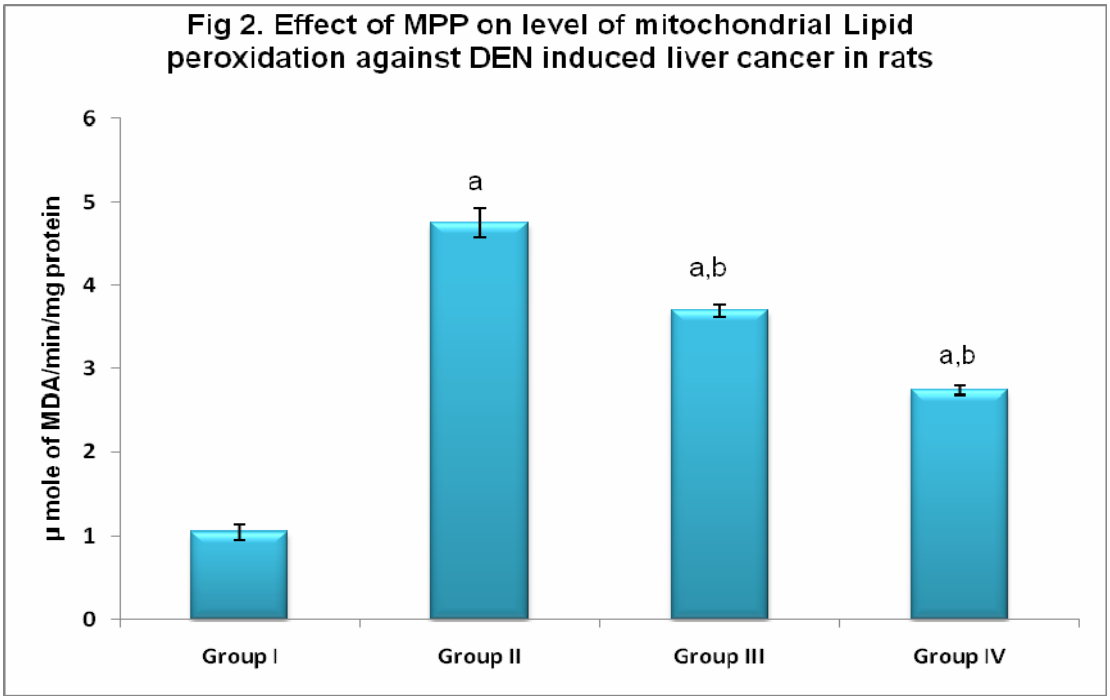
Different groups of rat showing their liver weight is depicted in Fig1. The liver weight of group II animals was higher than that of control (Group I) animals. On treatment with MPP (200 and 400 mg/kg), there found to be a significant ($p<0.001$) reduction in the liver weight in group III and IV animals when compared with group II animals.

Nucleic acid contents

The levels of nucleic acid content i.e., DNA and RNA are represented in fig.3. Cancer bearing animals showed a significantly increased nucleic acid contents in liver tissues ($p<0.001$). MPP (200 and 400 mg.kg) treatment resulted in a significant decrease in the levels of nucleic acid contents in group III ($p<0.05$) and group IV ($p<0.01$ and $p<0.05$) animals. MPP treated group IV shows more restoration than treated Group III.

Chemoprevention is defined as the use of naturally occurring or synthetic agents to prevent, inhibit or reverse the process of carcinogenesis. Chemoprevention trials are undertaken on the basis of the hypothesis that interruption of the biological mechanisms involved in carcinogenesis will inhibit the process and therefore reduce cancer incidence. This hypothesis provides a framework for the design and evaluation of chemoprevention trials including the rationale for the selection of agents that are likely to inhibit biological processes and the development of intermediate markers associated with carcinogenesis.

Development of intermediate markers for chemoprevention trials is crucial. Changes in cancer incidence that are associated with preventive treatment do not become apparent for many years and monitoring intermediate markers that are modulated by chemoprevention treatment, and correlate with a reduction in cancer incidence, would enable a more expeditious evaluation of potentially active chemopreventive agents [18].

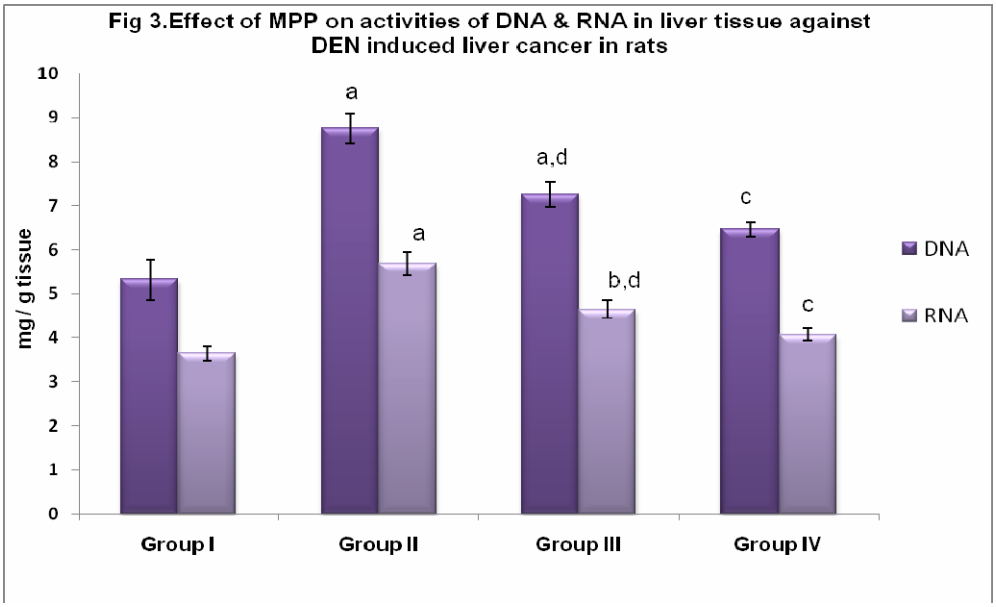


N=6; Each value is expressed as mean ± S.E.M. Group I: control animals, Group II: Liver cancer bearing animals, Group III: MPP 200 mg/kg treated, Group IV: MPP 400 mg/kg treated.
[a]P<0.001 Vs Control; [b]P<0.001 Vs Cancer bearing animals.

Lipid peroxidation is regarded as one of the basic mechanisms of tissue damage caused by free radicals [19]. Administration of DEN has been reported to generate LPO products in general [20] and phenobarbital enhanced the formation of the activated oxygen species in the preneoplastic nodules [21] in rat liver. Here the administration of DEN and phenobarbital has shown to increase the level of mitochondrial LPO during hepato carcinogenesis (Fig 2). This vigorous action may be caused by the uncompromised production of free radicals. It has been extensively reported that free radicals are participated in DEN induced hepatocarcinogenesis [22, 23].

The administration of MPP (200 and 400 mg/kg) in DEN induced and phenobarbital promoted animals, the level of LPO was found to be decreased. LPO can be prevented at the initiation stage by free radical scavengers and antioxidants [24]. 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. And 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 [25]. This is supported by earlier studies of DEN induced and Phenobarbital promoted hepato carcinogenesis [26]. The observed reduction in enzyme activities may be attributed to ROS; here the ROS themselves can reduce the activities of enzymes [27]. 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.



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; [b]P<0.05 Vs Control
[c]P<0.001; dP<0.05 Vs Cancer bearing animals.

Protectivity of enzymic antioxidant system by MPP can be explained by the following mechanisms. The extract itself may scavenge free radicals and/or prevent the antioxidants from ROS and additionally the plant extract can act by upregulating endogenous antioxidant defences. MPP absorbed during parsley intervention resulted in significant increases in antioxidant enzymes, Glutathione reductase and superoxide dismutase in erythrocytes of human subjects [28]. GSH is well known non enzymic antioxidant defence system of cells. It has been shown to provide protection against super oxides as well as H_2O_2 [29] and it contributes to membrane stability. GSH, a non protein thiol is involved in many cellular processes including the detoxification of endogenous and exogenous compounds [30]. Accordingly GSH might be depleted partly by the GPx mediated excess utilization of GSH. Earlier report reveals that the levels of these non-enzymic antioxidant was also decreased in hepatoma bearing animals. 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. Our findings indicate that MPP inhibits the level of LPO and significantly increases the enzymic and non enzymic antioxidant defence mechanisms in DEN induced and Phenobarbital promoted experimental hepatocellular carcinogenesis.

Nucleic acid content of tumor is found to be an important indicator of prognosis, because it is well correlated with the size of the tumor in the cancerous condition [31]. In diseased state, the degree of malignancy increases with the defective abnormalities in DNA. Reports reveal that abnormal amount of DNA was observed in various cancers including breast carcinoma, endometrial carcinoma and lung carcinoma [32]. In the present study, an increased activity was observed in DEN induced liver cancer animals and this may be due to the over expression of many enzymes which are necessary for DNA synthesis in tumor cells.

RNA levels were found to be increased in the cancerous condition as DNA and RNA are directly related to each other, an abnormally increased content of DNA may lead to an increased transcription, which in turn increased RNA content in tumor cells. The mechanisms by which tea polyphenols may act includes the inhibition of promutagen activation, the inactivation of mutagens and carcinogens, blocking and scavenging of reactive molecules, modulation of DNA replication or repair, inhibition of promotion, and inhibition of invasion and metastasis of tumor cells. These mechanisms are currently being progressively clarified. Most of the reports on mechanisms, however, still remain as suggestive or speculative [33]. In MPP (200 and 400 mg/kg) treated animals, the nucleic acid levels were decreased due to its inhibition of mutagenesis process.

Conclusion

All these observations clearly indicate a significant chemopreventive effect of Methanol extract of *Phyllanthus polyphyllus*. Further studies to characterize the active principles and to elucidate mechanism of action are in progress.

Authors' Contributions

NMPM carried out the hematological, biochemical and histopathological studies, participated in the sequence alignment and drafted the manuscript. RV participated in the design of the study and performed the statistical analysis. GM participated in design of study and helped to draft the manuscript.

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