

Antitumor and cytotoxic effects of *Phyllanthus polyphyllus* on dalton's ascitic lymphoma and human cancer cell lines

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ABSTRACT

The antitumor and cytotoxic activities of methanolic extract of Phyllanthus polyphyllus (MPP) were evaluated using mice and human cancer cell lines. The anticancer property of MPP was assessed against Dalton's ascitic lymphoma (DAL) tumor model by evaluating survival time, hematological parameters, lipid peroxidation (LPO), antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), solid tumor mass, and in vitro cytotoxicity (short-term). MPP (200 and 400 mg/kg) upon oral administration increased the survival time and reduced the solid tumor volume significantly in a dose-dependent manner. Packed cellular volume (PCV), protein, and hematological parameters were restored which were altered by tumor inoculation. MPP reduced the levels of LPO, GPx, GST, and increased the levels of SOD and CAT significantly. MTT assay was used to evaluate the cytotoxic activity using human cancer cell lines like cervical cancer cells (HeLa) and breast carcinoma cells (HBL-100). MPP showed IC₅₀ values of 170 and 130 µg/ml on HeLa and HBL-100 cells respectively. MPP was found to possess significant antitumor and cytotoxic activity on DAL and human cancer cell lines.

Keywords: *Phyllanthus polyphyllus*, Dalton's ascitic lymphoma, survival time, solid tumor volume, hematological parameters, HeLa and HBL cell lines, MTT assay

INTRODUCTION

Cancer is the leading cause of mortality worldwide and the failure of conventional chemotherapy in the major reduction of mortality indicates that new approaches are critically needed. The approach of chemotherapy serves as an effective alternative to control malignancy [1]. In experimental studies of cancer chemotherapy, attempts are made to identify the agents which can exhibit any or combination of the following characteristics: (i) prevent the tumor initiation, (ii) delay or arrest the tumor development, (iii) extend cancer latency periods, (iv) decrease cancer metastasis and mortality, and (v) prevent recurrence of secondary tumors. The focus of research in cancer chemotherapy in recent times includes the identification, characterization, and development of new cancer chemopreventive agents [2].

Plants have played a vital role as source of effective anticancer agents, and it is significant that 60% of currently used anticancer agents have derived from natural sources, including plants, marine organisms, and microorganisms [3,4]. Plant-based medicine has definitely found a role in cancer treatment, and the mechanism of interaction between cancer cells and many phytochemicals has been studied extensively [5].

Previous studies showed that, various *Phyllanthus* species have been reported to work against tumors and to have cytotoxic activities [6-8]. Based on ethnopharmacological information, the present study was carried out to assess

the antitumor and cytotoxic activity together with antioxidant status of methanol extract of *Phyllanthus polyphyllus* (MPP) against Dalton's Ascitic Lymphoma (DAL) in mice and human cancer cell lines like cervical carcinoma cells (HeLa) and human breast carcinoma cells (HBL-100).

MATERIALS AND METHODS

Plant material and extraction: Leaves of *Phyllanthus polyphyllus* were collected from the Kolli Hills, Tamil Nadu, India and were taxonomically identified and authenticated by Botanical Survey of India, Coimbatore, Tamil Nadu, and India. The leaves were dried and pulverized. The powder was then treated with petroleum ether for dewaxing and to remove chlorophyll. Then the powder was packed in a soxhlet apparatus and was extracted using methanol (solvent). The extract was then concentrated under vacuum and dried in a desiccator (yield, 5.2% w/w), and suspended in 5% gum acacia for antitumor studies.

Animals: Swiss male albino mice weighing 20–25 g were procured from Tamil Nadu Veterinary College, Chennai, India. They were housed in standard microcolon cages and maintained on a standard laboratory diet and water *ad libitum*. The experiments were approved by the Institutional Animal Ethics committee (IAEC).

Acute toxicity: The acute toxicity of the extract of *P. polyphyllus* was evaluated in mice as per OECD guidelines. Mice received alcohol extract at various doses (50-2,000 mg/kg) orally. They were observed for the toxic symptoms continuously for first 4 h after dosing, and finally the number of survivors was noted down after 24 h. In the toxicity study, no mortality occurred within 24 h under the tested doses of MPP.

Cells: DAL cells were obtained from Amala Cancer Research Centre, Thrissur, India. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse [9].

Effect of MPP on survival time [10]: Animals were inoculated on day 0 with 1×10^6 cells/mouse, and treated orally with MPP 24h after inoculation at doses of 200 and 400 mg/kg/day. The control group was administered with the same volume of distilled water. All the treatments were done for 9 days and observed for 45 days. Median survival time (MST) of each group (n=6) was noted. The antitumour efficacy of MPP was compared with that of 5-Fluorouracil (Dabur Pharmaceutical, Gaziabad, India; 5-FU, 20mg/kg/d i.p. for 9 days) as a reference standard. MSTs of the treated groups (T) were compared with MSTs of the control groups (C). The increase in life span was calculated using the following formula.

$$\text{Increase of life span} = \frac{T - C}{C} \times 100$$

Where T = number of days survived by treated animals and C = number of days survived by control animals.

Effect of MPP on hematological and antioxidant parameters [10]: In order to determine the influence of MPP on the hematological status of DAL bearing mice, the four groups (n= 6) of mice were compared on the 14th day after inoculation. The four groups comprised (1) tumour bearing mice, (2 &3) tumour bearing mice treated with MPP (200 and 400mg/kg/day p.o. for first 9 days), and (4) control mice. Using retro orbital plexus method, blood was drawn from DAL mouse and the white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin, protein and packed cell volume (PCV) were determined [11-13]. After blood collection, animals were sacrificed and livers were removed and preserved in tris HCl buffer pH (7.4). A 10% liver homogenate was utilized for estimating lipid peroxidation (LPO) [14], and antioxidant studies such as superoxide dismutase (SOD) [15], catalase [16], glutathione peroxidation (GPX) [17], glutathione S-Transferase (GST) [18].

Effect of MPP on solid tumors: Mice were divided into three groups (n=6). Tumour cells (1×10^6 cells/mice) were intramuscularly injected into the right hind limb of all the animals. Mice of group I were tumour control. Group II and III received MPP (200 and 400mg/kg) orally for 5 alternate days. Tumour mass was measured from 11th day of tumour induction and was repeated every 5th day for a time period of 30 days. The volume of tumour mass was calculated using the formula $V = 4/3\pi r^2$ where r is the mean of r_1 and r_2 , which are two independent radii of the tumour mass [19].

Effect of MPP on cytotoxicity *in vitro*: Short-term cytotoxicity was assessed by incubating 1×10^6 DAL cells in phosphate buffer saline (1 ml) with varying concentrations (50-800 $\mu\text{g/ml}$) of the MPP at 37^o C for 3 h in CO₂ atmosphere. The viability of the cells was determined by trypan blue exclusion method [20].

Cytotoxic studies: Human cervical carcinoma cells (HeLa) and human breast carcinoma cells (HBL-100) were obtained from National Centre for Cell Science (Pune, India). Stock cells of these cell lines were cultured in RPMI-1640, penicillin (100 IU/ml), streptomycin (100µg/ml), and amphotericin-B (5µg/ml) under a humidified atmosphere of 5% carbondioxide at 37°C until confluent. The cells were then dissociated in 0.2% trypsin, 0.02% EDTA in phosphate buffer saline solution. The stock culture was developed in 25 cm² tissue-culture flasks, and cytotoxicity experiments were carried out in 96-well micro titer plates (Tarsons India, Kolkata, India).

Cell lines in the exponential growth phase were washed, trypsinized, and then resuspended in complete culture media. Cells were plated at 10,000 cells/well in 96-well microtiter plates and incubated for 24 h, during which a partial monolayer formed. They were then exposed to various concentrations of the extract (31.25-500µg/ml) and 5-FU. Control wells received only maintenance medium. The plates were incubated for a period of 72 h at 37°C in a humidified incubator with 5% carbondioxide. At the end of 72 h, determination of cellular viability was done by MTT assay [21].

Statistical analysis: All values were expressed as mean±SEM. The data was statistically analyzed by one-way ANOVA, followed by Tukey multiple comparison test and data for solid tumors were analyzed by Dunnett test. P values<0.05 were considered significant.

RESULTS

Effect on mean survival time (MST): The effect of MPP on the survival of tumour-bearing mice exhibited MST for the control group to be 21d, while it was 32 d (52.38%) and 35 d (66.66%) respectively for the group treated with MPP (200 and 400 mg/kg/d p.o.). The results are almost comparable to that of the standard drug, 5-FU, for which the MST was 38 d (Table 1).

Effect on hematological parameters: Hematological parameters of tumour-bearing mice were found to be altered significantly from normal group on the day 14 (Table 2). There was a decrease in Hb, RBC, and lymphocytes in malignancy, along with an increase in WBC, especially neutrophils, protein, and PCV. At the same time interval, MPP (200 and 400 mg/kg/d p.o) treatment changed those altered parameters significantly (P < 0.001), to near normal in a dose-dependent manner.

Effect on antioxidant parameters: The levels of lipid peroxidation in liver tissue were increased significantly, by 21.28 ± 1.14 , in DAL control group as compared to the normal group (P<0.001). After administration of MPP at different doses (200 and 400 mg/kg) to DAL-bearing mice, the lipid peroxidation levels were reduced by 18.31 ± 0.97 and 12.9 ± 0.83 respectively as compared to DAL control group (P < 0.001) (Table 3).

The levels of superoxide dismutase (SOD) in the livers of DAL bearing mice decreased (P<0.001) in comparison with normal group. After administration of MPP at the dose of 200 and 400 mg/kg, increased levels of SOD as compared to that of DAL control group were observed (P<0.001). The catalase (CAT) level in DAL control group decreased (P<0.001) as compared with normal group. Treatment with MPP at the dose of 200 and 400 mg/kg significantly increased CAT levels respectively when compared to that of DAL (P<0.001) (Table 3).

Inoculation with DAL drastically increased the GST and GPx content in DAL control group as compared with normal group (P<0.001). Administration of MPP at doses of 200 and 400 mg/kg to the DAL-bearing mice decreased GST and GPx levels respectively as compared with DAL control group (P<0.001) (Table 3).

Effect on solid tumors and short-term cytotoxicity: The solid tumour volume was increased by 6.05 ± 0.35 in DAL bearing mice, and treatment with MPP decreased (P<0.01, P<0.05) the tumour volume significantly to 4.45 ± 0.13 ml and 4.25 ± 0.21 ml respectively, in a dose dependent manner at the end of 30 days (Table 4). The short term *in vitro* cytotoxicity study showed the IC₅₀ of MPP to be 120 µg/ml.

Effect on human cancer cells: The cytotoxic activity of MPP on human cervical carcinoma (HeLa) and human breast cancer (HBL-100) cell lines were evaluated by MTT assay. When the cells were treated for 72 h with various concentrations of methanol extract (31.25-500 µg/ml), the relative cell survival progressively decreased in a dose-dependent manner. The GI₅₀ of MPP was found to be 48 and 44µg/ml on HeLa and HBL-100 cell lines respectively, comparable with or slightly weaker than those of 5-FU (2.5 and 1.3µg/ml). Among the tested cell lines, extract of PP was more selective cytotoxic against HBL-100 cell line than HeLa (Table 5).

Table 1. Effect of *P. polyphyllus* treatment on the survival of Tumour Bearing Mice

Treatment	MST (d)	Life Span (%)
Tumour control (Saline 2 ml/kg, p.o.)	21 ± 1.12	-
5-FU (20 mg/kg, i.p.)	38 ± 1.40 ^a	80.95
MPP (200 mg/kg p.o)	32 ± 1.67 ^a	52.38
MPP (400 mg/kg p.o)	35±1.26 ^a	66.66

n = 6 animals; Days of drug treatment = 9

^a*P* < 0.01 vs Tumour control

Data were analyzed by using one way ANOVA followed by Dunnett test.

Table 2. Effect of *P. polyphyllus* on Haematological Parameters

Treatment	Hb (g %)	RBC (million/mm ³)	WBC (10 ³ cells/mm ³)	Proteins (g %)	PCV (mm)	Differential Count %		
						Lymphocytes	Neutrophils	Monocytes
Normal mice	15.6 ± 0.28	4.6 ± 0.12	7.5 ± 0.25	7.2 ± 0.33	16 ± 0.57	72 ± 1.31	27 ± 1.6	1 ± 0
Tumour bearing mice (14 days)	8.7 ± 0.10 ^a	2.6 ± 0.07 ^a	20.2 ± 2.5 ^a	13.2 ± 1.0 ^a	26 ± 1.36 ^b	22 ± 1.45 ^a	76 ± 2.10 ^a	2 ± 0
MPP (200 mg/kg p.o)	11.3 ± 0.35 ^{a,c}	3.9 ± 0.10 ^{b,c}	14.1 ± 0.62 ^{b,c}	10.8 ± 0.2 ^{a,c}	20 ± 2.30	67 ± 1.3 ^c	32 ± 1.0 ^c	1 ± 0
MPP (400 mg/kg p.o)	13.5 ± 0.42 ^{a,c}	4.2 ± 0.18 ^c	11.08 ± 1.04 ^d	8.6 ± 0.12 ^c	18 ± 2.0 ^c	69 ± 1.7 ^c	30 ± 1.4 ^c	1 ± 0

N = 6 animals in each group; Days of drug treatment = 9; Values are expressed as mean ± SEM.

^a*P* < 0.001; ^b*P* < 0.01 vs Normal mice; ^c*P* < 0.001; ^d*P* < 0.01; ^e*P* < 0.05 vs Tumour mice.

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

Table 3. Effect of *P. polyphyllus* LPO, Antioxidants and GST Levels in DAL-Induced Tumour Bearing Mice

Treatment	Dose (mg/kg)	LPO	SOD	Catalase	GPx	GST
Normal (saline)	2 ml/kg	8.5 ± 0.16	35.68 ± 1.26	1.86 ± 0.008	18.75 ± 1.12	0.28 ± 0.004
Tumor control	-	21.28 ± 1.14 ^a	20.75 ± 0.92 ^a	0.78 ± 0.002 ^a	49.67 ± 1.37 ^a	0.53 ± 0.002 ^a
<i>P. polyphyllus</i>	200	18.31 ± 0.97 ^a	28.37 ± 1.36 ^{a,c}	0.93 ± 0.006 ^{a,d}	31.10 ± 1.13 ^{a,d}	0.43 ± 0.003 ^{a,d}
	400	12.9 ± 0.83 ^{b,d}	31.45 ± 1.42 ^{c,d}	1.29 ± 0.005 ^{a,d}	24.50 ± 1.45 ^{b,d}	0.32 ± 0.006 ^{a,d}

N = 6; values are expressed as mean ± SEM.

^a*P* < 0.001; ^b*P* < 0.05; ^c*P* < 0.01 vs. Normal; ^d*P* < 0.001; ^e*P* < 0.01 vs. Tumor Control

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

LPO: μ moles of MDA/ min/mg protein; SOD: units/min/mg protein; CAT: μ mole of H₂O₂ consumed/ min/mg protein

GPx: μ moles of GSH oxidised/min /mg protein; GST: μ moles of CDNB conjugation formed/min /mg protein

Table 4. Effect of *P. polyphyllus* solid tumor volume

Treatment	Dose (mg/kg)	Solid tumor volume (ml)			
		15 th day	20 th day	25 th day	30 th day
Tumor control (saline)	-	3.88 ± 0.12	4.12 ± 0.10	5.72 ± 0.16	6.05 ± 0.35
MPP	200	2.39 ± 0.10 ^a	3.45 ± 0.23 ^b	3.83 ± 0.12 ^a	4.45 ± 0.13 ^a
	400	2.16 ± 0.13 ^a	3.24 ± 0.11 ^a	3.77 ± 0.14 ^b	4.25 ± 0.21 ^a

N = 6 animals; Values are expressed as mean ± SEM.

^a*P* < 0.01; ^b*P* < 0.05 vs. Tumour Control.

Data were analyzed by using one way ANOVA followed by Dunnett test.

Table 5. Cytotoxic effect of *P. polyphyllus* on Human cancer cell lines

Compound	HeLa	HBL-100
	IC ₅₀ (μg/ml)	IC ₅₀ (μg/ml)
MPP	48	44
5-FU	2.5	1.3

Average of 3 determinations, 3 replicates

IC₅₀, Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure.

DISCUSSION

The reliable criteria for evaluation of an anticancer drug are prolongation of lifespan of the animal [22] and decrease in WBC count of blood [23]. Our study results show an increase in life span accompanied by a reduction in WBC count in MPP treated mice. These results clearly demonstrate the antitumour effect of MPP against DAL.

Myelosuppression and anaemia are the most common problems encountered in cancer chemotherapy [24, 25]. Occurrence of anaemia in tumour bearing mice is mostly due to decrease in production of RBC or haemoglobin, and this may happen either due to iron deficiency or haemolytic or other myelopathic conditions [26]. Treatment with MPP restored the haemoglobin content, RBC count and WBC count to near normal. This demonstrates that MPP has a protective action on the haemopoietic system. Further, upon analysis of hematological parameters, minimum toxic

effect was seen in mice treated with MPP in DAL bearing mice, hematological parameters were restored to normal by MPP administration (9 days).

Production of excessive free radicals result in oxidative stress, which causes damage to macromolecules like lipids, and can lead to lipid peroxidation *in vivo* [27]. Increased lipid peroxidation causes degeneration of tissue. Lipid peroxide formed in the primary site is transferred through the circulation and provokes damage by propagating the process of lipid peroxidation [28]. Malondialdehyde, the end product of lipid peroxidation has been reported to be higher in carcinomatous tissue than in non diseased organs [27]. Glutathione, a potent inhibitor of neoplastic process, that is seen particularly in high concentrations in liver, is known to have key functions in the protective process as it plays a vital role as an endogenous antioxidant system.

All oxygen metabolizing cells contain SOD and catalase, which act as free radical scavenging system by providing defence against the potentially damaging reactivates of superoxide and hydrogen peroxide [29]. Loss of mitochondria and loss of Mn SOD activity in DAL cells might lead to a decrease in SOD activity in DAL bearing mice. The inhibition of CAT and SOD activities as a result of tumor growth was also reported [30]. Similar observations were found in the present study with DAL bearing mice. Treatment with MPP at different doses increased the levels of SOD and CAT significantly in a dose dependent-manner.

Plant derived extracts comprising antioxidant principles showed antitumor activity in experimental animals [31] and cytotoxicity towards tumor cells [32]. These antioxidants exhibited antitumor activity either through apoptosis-induction [33] or by neovascularisation inhibition [34]. The role of free radicals in cancer is well documented [35, 36]. The lowering of lipid peroxidation, GST, GPx and increasing of SOD and catalase levels in MPP treated groups indicates its potential as an inhibitor of DAL induced intracellular oxidative stress.

In DAL -bearing mice, there was a rapid and regular increase in ascitic fluid volume. The direct nutritional source for tumor growth is ascitic fluid; it meets the nutritional requirements of tumor cells [37]. MPP treatment decreased the volume of solid tumor and the viable cancer cell count, and increased the lifespan. It may be concluded that MPP decreases the nutritional fluid volume and thereby arrests tumor growth and increases the lifespan. There was a reduction in solid tumor volume in mice treated with MPP ($P < 0.001$). The present study reveals that the extract was cytotoxic towards DAL. MPPs were found to be cytotoxic against human cancer cell lines. Among the tested cell lines, extract of PP showed greater cytotoxicity against HBL-100 cell line than HeLa.

Preliminary phytochemical screening indicated the presence of alkaloids and flavonoids in MPPs. Flavonoids have been found to possess antimutagenic and antimalignant effects [38, 39]. Moreover, they have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [40] and angiogenesis [41]. Antitumor and cytotoxic properties of the extract may be due to these compounds.

CONCLUSION

The results of the present study clearly indicate significant antitumor and cytotoxic effects of the methanolic extract of the leaves of *Phyllanthus polyphyllus*. Further studies to characterize the active principles and to elucidate the mechanism of action are in progress.

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