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Antitumor and Cytotoxic Effects of *Phyllanthus polyphyllus* on Ehrlich Ascites Carcinoma and Human Cancer Cell Lines

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To evaluate the antitumor and cytotoxic activity of methanol extract of *Phyllanthus polyphyllus* (MPP) in mice and human cancer cell lines, the antitumor activity of MPP was evaluated against an Ehrlich ascites carcinoma (EAC) tumor model. The activity was assessed using survival time, hematological studies, lipid peroxidation (LPO), antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione *S*-transferase (GST), solid tumor mass, and short-term *in vitro* cytotoxicity. The cytotoxic activity of MPP was evaluated using human breast cancer (MCF7), colon cancer (HT29), and liver cancer (HepG2) cell lines Oral administration of MPP (200 and 300 mg/kg) increased the survival time and significantly reduced the solid tumor volume in a dose-dependent manner. Hematological parameters, protein, and packed cellular volume (PCV), which were altered by tumor inoculation, were restored. MPP significantly decreased the levels of LPO, GPx, GST, and significantly increased the levels of SOD and CAT. In a cytotoxicity study against human cancer cell lines, MPP was found to have IC_{50} values of 27, 42 and 38 μg/ml on MCF-7, HT-29, and HepG2 cells respectively. MPP possessed significant antitumor and cytotoxic activity on EAC and human cancer cell lines.

Key words: *Phyllanthus polyphyllus*; Ehrlich ascites carcinoma; life span; antioxidants; cytotoxic

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

Plants have played an important role as a source of effective anticancer agents, and it is significant that 60% of currently used anticancer agents are derived from natural sources, including plants, marine organisms, and microorganisms.[3,4)] Plant-based medicine has definitely found a role in cancer treatment (chemotherapy), and the mechanism of interaction between many phytochemicals and cancer cells has been studied extensively.[5)] In particular, there is growing interest in the pharmacological evaluation of various plants used in, Indian traditional system of medicine.

Plant-derived natural products such as flavonoids, terpenoids, and steroids, *etc.* have received considerable attention in recent years due to their diverse pharmacological properties, including antioxidant and antitumor activity.[6,7)] Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases.

Phyllanthus polyphyllus Linn. (Euphorbiaceae) is a deciduous shrub or small tree found mostly in, hill area of South India and Ceylon. It is popularly known as Sirunelli in Tamil. Leaves are traditionally used for liver diseases by tribes in, Kolli Hills.[8,9)] Phytochemical studies have indicated the presence of benzenoid, 4-*O*-methyl gallic acid, together with three arylnapthalide lignans, phyllamyricin, justicidin B, and diphyllin, and

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the extract shows dose-dependent inhibition of inflammatory mediators, such as LPS/INF-γ stimulated peritoneal excluded macrophages[10] and monoacetylated triterpene arabinosides, and terpenes found have cytotoxic activity against human nasopharyngeal carcinoma (KB) and small-cell lung cancer (NCI-H187).[11] Moreover, various Phyllanthus species has been reported to work against tumors and to have cytotoxic activities.[12–15] Hence, the present study was carried out to evaluate the antitumor and cytotoxic activity together with the antioxidant status of methanol extract of *Phyllanthus polyphyllus* (MPP) against Ehrlich ascites carcinoma (EAC) in mice and human cancer cell lines.

Materials and Methods

Plant material and extraction. Leaves of *Phyllanthus polyphyllus* Linn. were collected in and around the Kolli Hills, Salem District, Tamil Nadu, India and were authenticated by Dr. G. Murthy, Botanical Survey of India, Coimbatore, Tamil Nadu, India. A voucher specimen (EPP-03) was kept in our laboratory for future reference. The leaves were dried in the shade and pulverized. The powder (250 g) was treated with petroleum ether (600 ml) for dewaxing as well as to remove chlorophyll. The powder was then packed into a soxhlet apparatus and subjected to continuous hot percolation using 450 ml of methanol (95% v/v) as solvent. The extract was concentrated under a vacuum and dried in a vacuum desiccator (yield, 4.1% w/w), and then suspended in 5% gum acacia for antitumor studies.

Animals. Swiss male albino mice (20–25 g) were procured from Tamil Nadu Veterinary College, Chennai, India, and used throughout the study. They were housed in standard microlon boxes and maintained on a standard laboratory diet and water *ad libitum*. The experiments were performed in accordance with the guidelines established by the European Community for the care and use of laboratory animals, and were approved by the Institutional Animal Ethics Committee (IAEC) of Vel's College of Pharmacy, Chennai, India.

Chemicals. 1-Chloro2,4-dinitro benzoic acid (CDNB), 5,5-dithio-bis-2-nitro benzoic acid (DTNB), reduced glutathione (GSH), and glutathione were purchased from Sisco Research Laboratories, Mumbai, India. Thiobarbituric acid was purchased from E-Merck, Mumbai, India. All other chemical used were of analytical grade.

Acute toxicity. The acute toxicity of the extract of *P. polyphyllus* was evaluated in mice by the up-and-down procedure.[16] Mice received alcohol 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 noted after 24 h. In the toxicity study, no mortality occurred within 24 h under the tested doses of MPP.

Cells. EAC cells were obtained through the courtesy of Amala Cancer Research Centre, Thrissur, India. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse.[17]

Effect of MPP on survival time.[18] Animals were inoculated at 1×10^6 cells/mouse on day 0, and treatment with MPP started 24 h after inoculation, at doses of 200 and 300 mg/kg/d p.o. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given out for 9 d. Median survival time (MST) of each group, containing 10 mice, was noted. The antitumor efficacy of MPP was compared with that of 5-fluorouracil (Dabur Pharmaceutical, Gaziabad, India; 5-FU, 20 mg/kg/d i.p. for 9 d). MSTs of treated groups were compared with those of control groups by the following calculation:

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

where T = number of d treated animals survived and C = number of d control animals survived.

Effect of MPP on hematological parameters.[18] In order to determine the influence of MPP on the hematological status of EAC bearing mice, comparisons were made among four groups (n = 5) of mice on the 14th d after inoculation. The groups comprised (1) tumor-bearing mice, (2 and 3) tumor-bearing mice treated with MPP (200 and 300 mg/kg/d p.o. for first 9 d), and (4) control mice. Blood was drawn from each mouse by retro orbital plexus method, and the white blood cell count (WBC), red blood cell count (RBC), hemoglobin, protein, and packed cell volume (PCV) were determined.[19–21] After blood collection, animals were sacrificed and livers were removed. A 10% liver homogenate was used for lipid peroxidation (LPO),[22] and antioxidants studies such as superoxide dismutase (SOD),[23] catalase,[24] glutathione peroxidation (GPx),[25] and glutathione *S*-transferase (GST)[26] were done.

Effect of MPP on solid tumors. The mice were divided into three groups (n = 6). Tumor cells (1×10^6 cells/mouse) were injected into the right hind limb of all the animals intramuscularly. Mice of group I were tumor control. Groups II and III received MPP (200 and 300 mg/kg) orally for 5 alternate d. Tumor mass was measured from the 11th d of tumor induction and was repeated every 5th d for a period of 30 d. The volume of tumor mass was calculated by 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 tumor mass.[27]

Effect of MPP on cytotoxicity in vitro. Short-term cytotoxicity was assessed by incubating 1×10^6 EAC

cells in 1 ml phosphate-buffered saline at varying concentrations (25–400 µg/ml) of the MPP at 37 °C for 3 h under a CO_2 atmosphere. The viability of the cells was determined by, trypan blue exclusion method.[28)]

Cytotoxic studies. Human breast cancer (MCF-7), colon cancer (HT-29), and liver cancer (HepG2) cells were obtained from, National Centre for Cell Science (Pune, India). Stock cells of these cell lines were cultured in RPMI-1640 or DMEM supplemented with 10% inactivated newborn calf serum, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin-B (5 µg/ml) under a humidified atmosphere of 5% CO_2 at 37 °C until confluent. The cells were dissociated in 0.2% trypsin, 0.02% EDTA in phosphate buffer saline solution. The stock culture was grown in 25 cm^2 tissue-culture flasks, and cytotoxicity experiments were carried out in 96-well microtiter plates (Tarsons India, Kolkata, India).

Cell lines in the exponential growth phase were washed, trypsinized and 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 (25–100 µg/ml) and 5-FU. Control wells received only maintenance medium. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 for a period of 72 h. At the end of, 72 h, cellular viability was determined by MTT assay.[29)]

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

Results

Effect of mean survival time (MST)

The effect of MPP on the survival of tumor-bearing mice showed MST for the control group to be 23 d, while it was 30 (30.43%) and 35 d (62.85%) respectively for the group treated with MPP (200 and 300 mg/kg/d p.o). These results are almost comparable to that for 5-FU, the standard drug, for which the MST was 42 d (Table 1).

Table 1. Effect of *P. polyphyllus* Treatment on the Survival of Tumor Bearing Mice

Treatment	MST (d)	Lifespan (%)
Tumor control (Saline 2 ml/kg, p.o.)	23 ± 1.10	—
5-FU (20 mg /kg i.p.)	42 ± 1.35[a]	82.60
P. polyphyllus (200 mg/kg p.o)	30 ± 1.24[b,c]	30.43
P. polyphyllus (300 mg/kg p.o)	35 ± 0.98[a,d]	62.85

n = 10; d of drug treatment = 9
[a]P < 0.001; [b]P < 0.01 *vs.* Tumor control; [c]P < 0.001; [d]P < 0.01 *vs.* 5-FU
Data were analyzed by one-way ANOVA followed by Tukey multiple comparison test.

Effect on hematological parameters

Hematological parameters of tumor-bearing mice on day 14 were found to be significantly altered from normal group (Table 2). There was a decrease in Hb, RBC, and lymphocytes in malignancy, accompanied by an increase in WBC, especially neutrophils, protein, and PCV. At the same time interval, MPP (200 and 300 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 significantly increased, by 24.28 ± 1.26, in EAC control group as compared to the normal group (P < 0.001). After administration of MPP at different doses (200 and 300 mg/kg) to EAC-bearing mice the levels of lipid peroxidation were reduced by 19.41 ± 0.87 and 13.14 ± 0.98 respectively as compared to EAC control group (P < 0.001). Inoculation with EAC drastically increased the GST and GPx content in EAC control group as compared with normal group (P < 0.001). Administra-

Table 2. Effect of *P. polyphyllus* on Hematological Parameters of EAC-Bearing Mice

Treatment	Hb (g %)	RBC (million /mm^3)	WBC (10^3 cells/mm^3)	Proteins (g %)	PCV (mm)	Differential count %		
						Lymphocytes	Neutrophils	Monocytes
Normal (saline 2 ml/kg)	13.4 ± 1.5	4.5 ± 0.67	8.6 ± 1.26	6.2 ± 0.54	16 ± 1.52	74 ± 3.52	15 ± 1.7	1 ± 0
Tumor-bearing mice (14 d)	8.7 ± 0.16	2.6 ± 0.76	14.7 ± 1.32[b]	12.2 ± 0.78[a]	28.3 ± 2.1[c]	58 ± 3.58	41 ± 3.2[a]	1 ± 0
P. polyhyllus 200 mg/kg	10.2 ± 1.64	3.6 ± 0.28	10.7 ± 1.2	10.2 ± 0.95[c]	21.0 ± 2.7	67 ± 3.64	32 ± 1.26[c,d]	1 ± 0
300 mg/kg	11.8 ± 0.72	4.15 ± 0.16	8.07 ± 1.1[e]	7.05 ± 0.6[d]	24.0 ± 1.57	73 ± 4.57	25 ± 1.45[d]	2 ± 0

N = 6 animals in each group; 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 analyzed by one-way ANOVA followed by Tukey multiple comparison test.

Table 3. Effect of *P. polyphyllus* on LPO, Antioxidants and GST Levels in EAC-Induced Tumor Bearing Mice

Treatment	Dose (mg/kg)	LPO	SOD	Catalase	GPx	GST
Normal (saline)	2 ml/kg	7.8 ± 0.46	38.47 ± 1.86	0.46 ± 0.008	16.75 ± 1.12	0.18 ± 0.04
Tumor control	—	24.28 ± 1.26^{a}	19.75 ± 0.92^{a}	0.18 ± 0.004^{a}	45.63 ± 1.87^{a}	0.33 ± 0.002^{a}
P. polyphyllus	200	$19.41 \pm 0.87^{a,d}$	$29.43 \pm 1.36^{a,c}$	$0.23 \pm 0.006^{a,c}$	$28.10 \pm 1.23^{a,c}$	$0.21 \pm 0.001^{a,c}$
	300	$13.14 \pm 0.98^{b,c}$	23.64 ± 1.14^{a}	$0.29 \pm 0.007^{a,c}$	$24.90 \pm 1.45^{b,c}$	$0.24 \pm 0.006^{a,c}$

N = 6; values are expressed as mean ± SEM.
$^{a}P < 0.001$; $^{b}P < 0.01$ *vs.* Normal; $^{c}P < 0.001$; $^{d}P < 0.01$ *vs.* Tumor Control
LPO, μ moles of MDA/min/mg protein
SOD, units/min/mg protein
CAT, μ mole of H_2O_2 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* on Solid Tumor Volume

Treatment	Dose (mg/kg)	Solid tumor volume (ml)			
		15th day	20th day	25th day	30th day
Tumor control (saline)	2 ml/kg	3.96 ± 0.27	4.14 ± 0.13	5.89 ± 0.42	6.56 ± 0.68
P. polyphyllus	200	2.47 ± 0.36^{a}	3.5 ± 0.27	3.9 ± 0.14^{a}	4.2 ± 0.18^{a}
	300	2.06 ± 0.10^{a}	3.12 ± 0.43	3.7 ± 0.44^{b}	4.17 ± 0.36^{b}

N = 6; values are expressed as mean ± SEM.
$^{a}P < 0.01$; $^{b}P < 0.05$ *vs.* control
Data were analyzed using one-way ANOVA followed by Dunnett test.

tion of MPP at doses of 200 and 300 mg/kg to the EAC-bearing mice decreased GST and GPx levels respectively as compared with EAC control group ($P < 0.001$) (Table 3).

The levels of superoxide dismutase (SOD) in the livers of EAC-bearing mice decreased ($P < 0.001$) in comparison with normal group. After administration of MPP at doses of 200 and 300 mg/kg, increased levels of SOD as compared to that of EAC control group were observed ($P < 0.001$). The catalase (CAT) level in EAC control group decreased ($P < 0.001$) as compared with normal group. Treatment with MPP increased CAT levels as compared to that of EAC ($P < 0.001$) (Table 3).

Effect on solid tumors and short-term cytotoxicity

The solid tumor volume increased by 6.56 ± 0.08 ml EAC-bearing mice, and treatment with MPP decreased ($P < 0.01$, $P < 0.05$) the tumor volume significantly to 4.2 ± 0.18 and 4.17 ± 0.36 ml respectively, in a dose-dependent manner at the end of 30 d (Table 4). The short term *in vitro* cytotoxicity study showed the GI_{50} of MPP to be 50 μg /ml.

Effect on human cancer cells

The cytotoxic activity of MPP on human breast cancer (MCF-7), colon cancer (HT-29) and liver cancer (HepG2) was evaluated by MTT assay. When the cells were treated for 72 h with various concentrations of ethanol extract (5–100 μg/ml), relative cell survival progressively decreased in a dose-dependant manner. The GI_{50} of MPP was found to be 27, 42, and 38 μg/ml on MCF-7, HT-29, and HepG2 cell lines respectively, comparable with or slightly weaker than those of 5-FU (0.61, 1.3, and 2.6 μg/ml). The total growth inhibition (TGI) of ethanol extract was found to be 68, 71, and 62 μg/ml on the MCF-7, HT-29, and HepG2 cell lines respectively. The LC_{50} of the ethanol extract was found to be > 100 μg/ml for all three cell lines. Based on cytotoxicity results, the extract produced a potent cytotoxic effect on these three human cancer cell lines (Table 5).

Discussion

The reliable criteria for evaluating an anticancer drug are prolongation of lifespan of the animal[30] and decrease in WBC count of blood.[31] Our results show an increase in lifespan accompanied by a reduction in WBC count in MPP treated mice. These results clearly demonstrate the antitumor effect of MPP against EAC.

The most common problems encountered in cancer chemotherapy are myelosuppression and anemia.[32,33] Anemia occurring in tumor-bearing mice is mainly due to reduction in RBC or hemoglobin production, and this may occur either due to iron deficiency or to hemolytic or other myelopathic conditions.[34] Treatment with MPP brought back the hemoglobin content, RBC and WBC counts to near normal. This indicates that MPPs have a protective effect on the hemopoietic system. Further, analysis of hematological parameters showed minimum toxic effects in mice treated with MPPs. In EAC-bearing

Table 5. Cytotoxic Effect of *P. polyphyllus* on Human Cancer Cell Lines

Cell lines	GI_{50} (μg/ml)		TGI (μg/ml)		LC_{50} (μg/ml)	
	MPP	5-FU	MPP	5-FU	MPP	5-FU
MCF-7	27	0.6	68	2.3	>100	<10
HT-29	42	1.3	71	4.8	>100	<10
HepG2	38	2.6	62	5.5	>100	<10

Average of 3 determinations, 3 replicates

GI_{50}, Drug concentration inhibiting 50% cellular growth following 72 h of drug exposure.

TGI, Total cellular growth inhibition.

LC_{50}, Concentration of drug resulting in a 50% reduction at the end of drug treatment as compared to that at the beginning.

mice, hematological parameters were reversed to normal by MPP administration (9 d).

Excessive production of free radicals resulted in oxidative stress, which leads to damage of macromolecules such as lipids, and can induce lipid peroxidation *in vivo*.[35] Increased lipid peroxidation causes degeneration of tissue. Lipid peroxide formed in the primary site is be transferred through the circulation and provokes damage by propagating the process of lipid peroxidation.[36] Malondialdehyde, the end product of lipid peroxidation has been reported to be higher in carcinomatous tissue than in non diseased organs.[35] Glutathione, a potent inhibitor of neoplastic process, plays an important role as an endogenous antioxidant system that is found particularly in high concentrations in liver and is known to have key functions in the protective process.

The free radical scavenging system, SOD, and catalase are present in all oxygen-metabolizing cells, and their function is to provide a defence against the potentially damaging reactivates of superoxide and hydrogen peroxide.[37] Decreased in SOD activity in EAC-bearing mice which might be due to loss of Mn-SOD activity in EAC cells and loss of mitochondria, leading to a decrease in total SOD activity in the liver. Inhibition of SOD and CAT activities as a result of tumor growth was also reported.[38] Similar findings were obtained in the present investigation with EAC-bearing mice. Treatment with MPP at different doses significantly increased the SOD and CAT levels in a dose dependent-manner.

Plant-derived extracts containing antioxidant principles showed cytotoxicity towards tumor cells[32] and antitumor activity in experimental animals,[39] antitumor activity of these antioxidants is either through induction of apoptosis[40] or by inhibition of neovascularization.[41] The involvement of free radicals in tumors is well documented.[42,43] The lowering of lipid peroxidation, GST, GPx and increase in levels of SOD and CAT in MPP-treated groups indicates its potential as an inhibitor of EAC-induced intracellular oxidative stress.

In EAC-bearing mice, there was a regular and rapid increase in ascitic fluid volume. Ascitic fluid is the direct nutritional source for tumor growth; it meets the nutritional requirements of tumor cells.[44] 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 EAC. MPPs were found to be cytotoxic against human cancer cell lines. The criteria of cytotoxicity activity for crude extracts, as established by the American National Cancer Institute (NCI), is an IC_{50} of $< 30\,\mu g/ml$ in the preliminary assay.[45] Confirmation of activity with an exposure time of 72 h establishes that the MPPs still showed the most activity for breast cancer ($GI_{50} = 27\,\mu g/ml$), but the GI_{50} of this plant extract was more than $30\,\mu g/ml$ for colon and liver cancer cell lines (42 and $38\,\mu g/ml$ respectively).

Preliminary phytochemical screening indicated the presence of alkaloids and flavonoids in MPPs. Flavonoids have been found to possess antimutagenic and antimalignant effects.[46,47] Moreover, they have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation[48] and angiogenesis.[49] Antitumor and cytotoxic properties of the extract may be due to these compounds. All these observations clearly indicate a significant antitumor and cytotoxic effect of the extract of the leaves of MPP. Further studies to characterize the active principles and to elucidate the mechanism of action are in progress.

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