Antitumor and antioxidant activity of *Polyalthia longifolia* stem bark ethanol extract

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**Abstract**

In the present study, the ethanol extract of stem bark of *Polyalthia longifolia* Benth. and Hook (Annonaceae) was screened for its *in vitro* and *in vivo* antitumor activity. *In vitro* cytotoxicity of *P. longifolia* extract was assessed in murine cancer cells and in human cancer cells by Trypan blue exclusion assay and MTT assay, respectively. *P. longifolia* extract showed concentration-dependent cytotoxicity in Ehrlich's ascites carcinoma (EAC) and Dalton's ascites lymphoma (DLA) cells with IC₅₀ values of 45.77 and 52.52 µg/mL, respectively. In the MTT assay, the IC₅₀ values of *P. longifolia* extract against HeLa and MCF-7 cells were 25.24 and 50.49 µg/mL, respectively. *In vivo* antitumor activity against Ehrlich's ascites tumor and Dalton's solid tumor models was assessed by administering 50 and 100 mg/kg of *P. longifolia* extract, i.p., for 7 consecutive days. *P. longifolia* extract, at a dose of 100 mg/kg, significantly enhanced mean survival time (MST) and marginally improved hematological parameters when compared to EAC control mice. And the same dose significantly reduced the tumor volume as compared to control DLA inoculated mice. Positive control, cisplatin (3.5 mg/kg, i.p., single dose), significantly enhanced MST and improved hematological parameters when compared to EAC and significantly reduced the tumor volume when compared to DLA control. *In vitro* antioxidant potential of *P. longifolia* extract was also determined owing to the role of reactive oxygen species in tumor initiation and progression. *P. longifolia* extract scavenged DPPH radicals, reduced ferric ions and inhibited lipid peroxidation with IC₅₀ values of 18.14, 155.41 and 73.33 µg/mL, respectively.

**Keywords:** *Polyalthia longifolia*; cytotoxicity; MTT assay; antitumor; antioxidant

**Introduction**

The search for a selective and less toxic molecule for cancer treatment is an ongoing process. Plants have played an important role as a source of effective anticancer agents and about 60% of the currently available anticancer drugs are derived from plant sources (Newman et al., 2003). The global trend is also towards natural bioactive substances due to their low toxicity and cost. The exploration of medicinal plants for their therapeutic efficacy still holds the hope for the treatment and prevention of cancer.

*Polyalthia longifolia* Benth. and Hook (Annonaceae) (PL) is a commonly planted tree useful for its effectiveness in alleviating noise pollution. In traditional medicine, the bark is used to treat skin diseases, fever, diabetes, hypertension, and helmenthiasis (Rastogi, 1997). Earlier work on *Polyalthia longifolia* reports the isolation of two clerodane diterpenoids with antifeedant properties from the acetone extract of leaves (Phadnis et al., 1988). The cytotoxic activity of methanol extract of PL stem parts has been reported against KB and P-388 cells (Wu & Duh, 1990). Diterpenoids isolated from the hexane extract of *P. longifolia* seeds demonstrated significant antibacterial and antifungal activities (Murthy et al., 2005). Malairajan et al. (2006) evaluated the analgesic activity of *P. longifolia* leaf ethanol extract. The anticancer potential of leaf extract of *P. longifolia* has also been tested in various cancer cell lines and the mechanism of apoptosis induction has also been...
reported (Verma et al., 2008). But so far the in vivo antitumor and antioxidant activity of the stem bark of PL has not been reported. Hence, the present study focuses the in vitro antioxidant potency and in vitro and in vivo antitumor activity of ethanol extract of *Polyalthia longifolia* stem bark.

**Materials and methods**

**Cell lines**

Dalton’s lymphoma ascites (DLA) and Ehrlich’s ascites carcinoma (EAC) originally obtained from Amala Cancer Research Center, Thrissur, India, were maintained and propagated as ascites tumor in Swiss albino mice by serial intra-peritoneal transplantation at the Central Animal Research Facility, Manipal University, Manipal, India. MCF-7 (human breast adenocarcinoma) and HeLa (human cervical tumor cells) cells procured from the National Center for Cell Science, Pune, India were sub-cultured every two to three days and maintained in 25 cm² tissue culture flasks (Tarsons Products, Kolkata, India) containing MEM medium supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL gentamicin sulfate at 37°C in a CO₂ incubator (NuAire, Plymouth, USA) in an atmosphere of humidified 5% CO₂ in 95% air.

**Animals**

Eight to ten week old Swiss albino mice weighing between 25 and 30 g were selected from an inbred colony maintained under the controlled conditions of temperature (23° ± 2°C), humidity (50% ± 5%) and light (14 h and 10 h of light and dark, respectively). The animals were provided with sterile food and water ad libitum. Four animals were housed in each polypropylene cage containing paddy husk as bedding. Animal care and handling was done according to the guidelines issued by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

**Plant material and extraction**

The fresh stem bark of *Polyalthia longifolia* was collected in and around Mysore, India, in the month of December and was authenticated by Dr. M. N. Naganandhini, Department of Pharmacognosy, J.S.S. College of Pharmacy, Mysore, Karnataka. Fresh bark of *Polyalthia longifolia* was shade dried at room temperature and powdered. The bark powder (100 g) was extracted with 95% ethanol in a Soxhlet extractor exhaustively. The extract was then cooled, filtered and concentrated in a rotary flash evaporator. The residue was first dried over a water bath and then in a desiccator over fused calcium chloride. The percentage yield of ethanol extract was found to be 16.8% w/w.

**Short-term cytotoxicity studies in DLA and EAC cells**

Short-term in vitro cytotoxicity of *P. longifolia* extract was assessed by the Trypan blue exclusion method (Sheeja et al., 1997). In brief, Dalton’s lymphoma ascites and Ehrlich ascites carcinoma (DLA/EAC) cells aspirated from the mice’s peritoneal cavities were washed with PBS two to three times and one million cells were incubated with different concentration of *P. longifolia* extract in 1 mL of PBS for 3 h at 37°C in sterile test tubes. After incubation, 100 μL of Trypan blue dye (0.4% in PBS) was added and the total number of dead (stained) and viable ( unstained) cells were counted using a hemocytometer, and percentage cytotoxicity was calculated using the formula:

\[
\text{% cytotoxicity} = 100 \times \frac{(T_{\text{dead}} - C_{\text{dead}})}{T_{\text{tot}}} 
\]

where \(T_{\text{dead}}\) is the number of dead cells in the treated group, \(C_{\text{dead}}\) is the number of dead cells in the control group and \(T_{\text{tot}}\) is the total number of dead and live cells in the treated group.

**MTT assay in cultured human cancer cells**

The effect of *P. longifolia* extract on growth of cancer cells (MCF-7 and HeLa) was assessed by MTT assay (Mossman, 1983). In brief, exponentially growing cells (1 × 10⁴ cells/well) were plated in 96-well plates and allowed to adhere for 24 h prior to extract addition. The extract was dissolved in 0.1% DMSO then diluted with the medium and filtered using 0.22 μ syringe filters. The cells were then exposed to different concentrations of extract (5-200 μg/mL) for 48 h. The cells in the control wells received medium containing the same volume of DMSO (0.1%). After the incubation, 100 μL of MTT reagent (1 mg/mL in PBS) was added and cells were incubated for an additional 4 h. The formazan produced by the viable cells was solubilized by addition of 100 μL DMSO. The suspension was placed on a micro-vibrator for 5 min and absorbance was recorded at 540 nm by the ELISA reader (BIOTEK-ELx800). The experiment was performed in triplicate. Doxorubicin was used as positive control. The percentage of growth inhibition was calculated with respect to vehicle control using the formula

\[
\text{% Inhibition} = \frac{(\text{Control absorbance} - \text{Blank absorbance}) - (\text{Test absorbance} - \text{Blank absorbance})}{\text{Control absorbance} - \text{Blank absorbance}} \times 100
\]
Acute toxicity

Median lethal dose for *P. longifolia* extract was determined by following the standard Organization of Economic Co-operation and Development (OECD) guidelines (2001). In brief, Swiss albino mice deprived of food for 18 h, were administered various doses of *P. longifolia* extract ranging from 500 to 2000 mg/kg. Animals were observed for any symptoms of toxicity continuously for 4 h then after 24 h and finally the number of survivors was recorded after 72 h.

Antitumor activity in EAC-induced ascites tumor in mice

Survival study

The tumor induction and propagation was carried out according to the method described by Jagetia and Baliga (2003). The known numbers of viable EAC cells (2.5 × 10⁶ cells/mouse) were injected intraperitoneally into each mouse in an aseptic condition and the day of tumor inoculation was considered as day zero. Twenty-four hours after tumor inoculation the tumor-bearing animals were randomly divided into desired groups of six each and treated with test compound or vehicle.

Group 1 (CMC): Animals were treated with 10 mL/kg CMC (0.5%), i.p.
Group 2 (extract): Animals were treated with 50 mg/kg *P. longifolia* extract, i.p.
Group 3 (extract): Animals were treated with 100 mg/kg *P. longifolia* extract, i.p.
Group 4 (Cisplatin): Animals were injected with cisplatin (CP) 3.5 mg/kg, i.p.

The *P. longifolia* extract was administered for seven days consecutively starting from day 1 of tumor inoculation. Cisplatin, a single dose of 3.5 mg/kg, i.p., was injected on day 1 which served as standard drug. Every third day animals were weighed to assess the tumor growth. The animals were monitored daily for 45 days and mortality was recorded to calculate the MST (mean survival time). The percentage increase in life span (ILS) was calculated by the formula:

\[\text{ILS} = \left( \frac{\text{MST in treated group} - \text{MST in vehicle group}}{\text{MST in vehicle group}} \right) \times 100\]

Whole blood count

Different sets of animal were used to assess hematological parameters. The experimental design was the same as described in the survival study. Blood was withdrawn on 8th day from retro-orbital plexus of mice. The total white blood cells (WBC), red blood cells (RBC) and haemoglobin content were determined using standard methods (Mukherjee, 1990).

Antitumor activity in DLA-induced solid tumor model in mice

Antitumor activity of *P. longifolia* extract was determined in DLA-induced solid tumor model as per the method of Rajesh Kumar et al., 2002. In brief, DLA cells (1 × 10⁶ cells per mouse) were inoculated subcutaneously into the hind limb of mice. After 24 h mice were randomized and divided into four groups of six each. Group I served as control and received 0.5% CMC, i.p. Group II received 3.5 mg/kg cisplatin, i.p., single dose on the first day. Groups III and IV received *P. longifolia* extract 50 and 100 mg/kg, i.p., respectively, for seven consecutive days. The diameter of the tumor was measured at five-day intervals for a period of 30 days and tumor volume was calculated using the standard formula

\[V = \frac{4}{3} \pi r_1^3 r_2\]

where \(r_1\) and \(r_2\) represent the radii of the tumor at two different planes.

Effect of *P. longifolia* extract on in vitro antioxidant activities

DPPH radical scavenging activity

DPPH scavenging activity of *P. longifolia* extract was determined by incubating equal volumes of different concentrations of *P. longifolia* extract with 100 μM DPPH in methanol at room temperature. Absorbance was recorded at 517 nm after 20 min using methanol as blank and percentage DPPH radical scavenging was calculated using the formula:

\[
\% \text{Antiradical activity} = \left( \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \right) \times 100
\]

The experiment was carried out in triplicate and ascorbic acid was used as standard (Sreejayan & Rao, 1996).

Ferric ion reduction activity

Electron donating capability was evaluated by ferric chloride reduction method. Reaction mixture contained 1 mL of phosphate buffer (pH 7.4), 100 μM Fe³⁺ and different concentrations of extract in 0.5 mL of PBS. After 3 min incubation, EDTA (100 M) and orthophenanthroline (300 M) were added, reaction was allowed for 10 min at room temperature and absorbance was recorded at 510 nm. Ascorbic acid was used as standard which is equivalent to 100% reduction of ferric ions, comparative reduction of Fe³⁺ by *P. longifolia* extract was calculated (Kunchandy & Rao, 1987).
Antitumor and antioxidant activity of Polyalthia longifolia

Anti-lipid peroxidation

Albino rats (180–200 g) of either sex were used for the study. Animals were anesthetized and perfused transcardially with ice-cold saline after which the brain was collected. The isolated tissue was weighed and 10% homogenate was prepared in 150 mM KCl. Inhibition of lipid peroxidation was determined in rat brain homogenate. The reaction mixture contained 0.1 mL FeCl₃ (1 mM), 0.1 mL ascorbic acid (1 mM), 0.1 mL of KCl (1.5 M), 0.1 mL of various concentrations of P. longifolia extract and 0.3 mL of brain homogenate (10%) in a final volume of 1 mL. After 20 min of incubation at room temperature, the reaction was stopped by addition of 1 mL of 15% TBA, 0.38% TCA and 0.05% BHT solution. Absorbance of supernatant was recorded at 532 nm after heating at 80°C for 15 min and centrifugation at 1000 rpm (Rajakumar & Rao, 1994). Anti-lipid peroxidation activity was calculated by the formula

\[
\% \text{ Anti-lipid peroxidation activity } = \left( \frac{C - S}{C} \right) \times 100
\]

where C is the absorbance of the control and S is the absorbance of the sample. Each experiment was carried out in triplicate and results were expressed as percentage anti-lipid peroxidation activity ± SEM.

Table 1. In vitro cytotoxic activity of P. longifolia extract on EAC and DLA cells by Trypan blue exclusion assay. EAC/DLA cells (1×10⁶) were incubated in 1 mL of PBS containing different concentrations of extract for 3 h at 37°C. After the exposure period, percentage cytotoxicity was calculated using Trypan blue dye. All values are expressed as mean ± SEM. The experiment was performed in triplicate.

| Concentration (µg/mL) | EAC | DLA |
|-----------------------|-----|-----|
| 10                    | 8.83±0.6 | 7±0.58 |
| 25                    | 25.11±1.63 | 21.67±0.88 |
| 50                    | 60.88±2.9 | 42.67±1.45 |
| 75                    | 88.21±0.25 | 80±1.15 |
| 100                   | 100 | 96±1.15 |

Table 2. Effect of P. longifolia extract on proliferation of human cancer cell lines assessed by MTT assay. A fixed number of cells (1×10⁶) grown in a 96-well culture plate were exposed to different concentrations of P. longifolia extract for 48 h followed by MTT addition and absorbance was recorded at 540 nm by ELISA reader. The IC₅₀ for standard doxorubicin in HeLa cells was 1.16 µg/mL. All values are expressed as mean ± SEM.

| Conc. (µg/mL) | % growth inhibition after 48 h incubation |
|---------------|-----------------------------------------|
|               | MCF-7 cells | HeLa cells |
| 5             | 5.38±0.51 | 9.35±0.9 |
| 10            | 11.99±0.23 | 24.85±1.88 |
| 25            | 26.17±0.34 | 45.33±2.46 |
| 50            | 46.16±0.85 | 70.06±2.19 |
| 100           | 63.52±0.7 | 92.43±0.31 |
| 150           | 92.31±0.4 | 97.91±0.38 |
| 200           | 95.87±0.52 | 98.03±0.8 |

Statistical analysis

Data obtained were expressed as mean ± SEM of indicated number of animals. Statistical analysis was carried out using one way ANOVA with Tukey’s post hoc test (GraphPad Prism version 4.03 for Windows, GraphPad Software, California, USA). A value of p < 0.05 was considered to be significant. Graphs were prepared by OriginLab Origin Pro 8.0 (OriginLab Software, Northampton, MA). The daily survival was determined by Kaplan Meir’s equation.

Results

In vitro cytotoxic activity

P. longifolia extract exhibited dose-dependent cell death in both EAC and DLA cells with an IC₅₀ value of 45.77 and 52.52 µg/mL in EAC and DLA, respectively (Table 1).

Effect on cultured human cancer cells

P. longifolia extract inhibited the proliferation in both the tested human cancer cell lines in a dose-dependent manner. However, the growth inhibition of HeLa cells was greater because of shorter doubling time as compared to MCF-7 cells. The IC₅₀ (concentration required to inhibit 50% of cell growth) value of P. longifolia extract in HeLa and MCF-7 cells was observed to be 25.24 and 50.49 µg/mL, respectively (Table 2). The percentage of DMSO (0.1%) used in the experiment did not affect the growth of the cells.

Toxicological study (median lethal dose)

In the toxicity study, no mortality occurred within 72 h under the tested doses. The P. longifolia extract was found safe up to 2000 mg/kg. On the basis of toxicological data, therapeutic doses were selected.

Effect on mean survival time

In the EAC vehicle control group (Group 1), the average life span of animals was found to be 17.21 ± 0.93 days. The average life-span of P. longifolia extract treated animals at both the doses (50 and 100 mg/kg) was 18.33 ± 0.65 and 22.28 ± 0.88 days. But only the higher dose was found significant (p < 0.05) when compared with control. The average life span of cisplatin treated mice was found to be 33.02 ± 1.03 days (Figure 1).
Effect on body weight changes
Substantial gain in body weight was observed in vehicle-treated mice with maximum gain of 16.35% on day 12. Cisplatin administration significantly reduced the weight gain as compared to control on all the tested days whereas the reduction in the weight gain was significant on days 9 and 12 as compared to control with *P. longifolia* extract treatment at both the tested doses (Figure 2).

**Effect on hematological parameters**
In the EAC vehicle control, the reduction in total RBC, hemoglobin (Hb) content and increase in total WBC count were significant when compared with normal mice. Cisplatin (3.5 mg/kg i.p., single dose) significantly normalized the EAC-induced hematological changes. *P. longifolia* extract at both the doses (50 and 100 mg/kg) significantly reversed the elevated WBC count. However, the increase in RBC and Hb content was not significant (Figure 3).

**Effect on DLA-induced solid tumor**
The tumor volume of vehicle-treated animals on day 30 after tumor inoculation was found to be 9.9 cc. The tumor volume was reduced to 8.67 and 6.93 cc by *P. longifolia* extract administration at the dose of 50 and 100 mg/kg, respectively. Cisplatin significantly reduced the tumor volume to 3.51 cc (Figure 4). The percentage reduction in the tumor volume of *P. longifolia* extract-treated animals was found to be 12.68% and 30.21%, whereas cisplatin reduced the tumor volume by 64.55%.

**Effect on free radical scavenging activity**
*P. longifolia* extract significantly scavenged DPPH radical and inhibited lipid peroxidation in rat brain homogenate with IC₅₀ values of 18.14 and 73.33 µg/mL, respectively. The IC₅₀ values of ascorbic acid and curcumin in DPPH radical scavenging and lipid peroxidation activity were 1.54 and 4.04 µg/mL, respectively. The IC₅₀ values of *P. longifolia* extract and ascorbic acid in ferric ion reduction were found to be 155.41 and 35.53 µg/mL, respectively (Table 3).

**Discussion**
Previously, *P. longifolia* extract has been reported to contain aporphine and azafluorene alkaloids (Wu & Duh, 1990), proanthocyanidine trimers, sitosterols, and clerodane diterpenes (Phadnis et al., 1988) which have antitumor activities (Zhang et al., 2005; Salatino et al., 2007; Shrivastava & Patel, 2007). Present in vivo studies revealed significant reduction in the body weights of EAC-bearing animals at a dose of 100 mg/kg. Another criterion for evaluating antitumor potential is prolongation of life span of tumor-bearing mice (Clarkson & Burchenal, 1965) with reversal of the elevated total levels of WBC (Oberling & Guerin, 1954). This parameter has also been fulfilled by *P. longifolia* extract as observed from the results.
Antitumor and antioxidant activity of Polyalthia longifolia

To further confirm antitumor activity, the effect of P. longifolia extract on solid tumor was assessed using DLA cells. Significant reduction in tumor volume was observed with P. longifolia extract treatment, which implies inhibition of DLA tumor growth. This indicates that the inhibitory effect of P. longifolia extract is not only due to its local cytotoxic effect but also due to its systemic action.

The short term in vitro cytotoxicity and antiproliferative data also supports the in vivo antitumor activity of P. longifolia extract against EAC and DLA, where P. longifolia extract caused significant cell death and inhibition of cancer cell growth.

Reactive oxygen species have multiple functions (Valko et al., 2006) and are implicated in tumor initiation and progression (Czapski & Goldstein, 1990; Sagun et al., 2006; Nishigori et al., 2004). Depleted endogenous antioxidant enzymes with enhanced free radical generation and MDA are well documented in carcinogenesis (Szatrowski & Nathan, 1991). Many tumor cells have pro-oxidant status and promote oxidative stress. This increases the surviving potential of the cancer cells by inducing mutations, activating redox signaling and stimulating pro-survival factors such as NFκB and AP-1 (Seeram et al., 2005).

Antioxidants alter the intracellular redox state, thereby enhancing the effects of cytotoxic therapy. We have observed that P. longifolia extract significantly scavenged DPPH, reduced ferric ion and inhibited lipid peroxidation which proves its antioxidant activity. It was reported that plant-derived extracts containing antioxidant principles showed cytotoxicity toward tumor cells (Jiau & Larry, 1977) and antitumor activity.
in experimental animals (Ruby et al., 1995). The cytotoxic and antitumor activity of plant-derived product is either through induction of apoptosis or inhibition of neovascularization (Ming et al., 1998). Plants with high phenol content are reported to possess effective antioxidant and antitumor properties (Lee et al., 2004) and *P. longifolia* extract has been found to have high phenol content from the antioxidant studies performed, hence could have antitumor activity.

A phytochemical study on the hexane extract of stem bark of PL has led to the characterization of various clerodane and *ent*-halimane diterpenes (Hara et al., 1995) which are active constituents that are reported to have antitumor activity (Seeram et al., 2005) and the MeOH extract of stem parts of PL have been reported to contain cytotoxic aporphine alkaloid liroidenine (Wu & Duh, 1990). The presence of any of these components may be attributed to the antitumor property of the extract. Since the present study focuses on the preliminary antitumor activity of *P. longifolia* extract, the tumor selective action and characterization of the active component of *P. longifolia* extract responsible for the activity are yet to be explored.

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**Declaration of interest**

The authors declare no conflict of interest. The authors themselves are responsible for the content and writing of the paper.

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