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Anti-proliferative effect of leaf extracts of *Eucalyptus citriodora* against human cancer cells *in vitro* and *in vivo*

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Six different extracts from *Eucalyptus citriodora* leaves were investigated for their anticancer effect. Extracts were prepared using a range of polar and non-polar solvents to leach out maximum active components. Phytochemical analysis of the extracts revealed the presence of anthraquinones, cardiac glycosides, flavonoids, saponins and tannins. Cytotoxic activity of different extracts was tested *in vitro* against seven human cancer cell lines from seven different tissues, such as SW-620 (colon), HOP-62 (lung), PC-3 (prostate), OVCAR-5 (ovary), HeLa (cervix), IMR-32 (neuroblastoma) and HEP-2 (liver). The ethyl acetate, chloroform and 50% methanolic extract displayed highest anti-proliferative effect in a dose-dependent manner. *In vivo* anti-tumor activity was evaluated against murine tumor (solid) model of Ehrlich ascites carcinoma and Sarcoma 180. The results showed that ethyl acetate and aqueous extracts suppressed the growth of Ehrlich ascites carcinoma (29.79% and 18.48%, respectively), but showed little growth inhibition in case of Sarcoma 180 (13. 86% and 8.57%, respectively). The activity might be due to the flavonoids, tannins and saponins that are present in all the extracts of the plant. Further investigation is required for the isolation of active principle(s) from the ethyl acetate extract, which has shown significant *in vitro* and *in vivo* anticancer potential.

Keywords: *Eucalyptus citriodora,* Cytotoxicity, Human cancer cell lines, Murine tumor model, Ethyl acetate extract

Cancer has remained a major cause of death and the number of individuals living with cancer is continually increasing. Chemotherapy is a major treatment modality for cancer and various anticancer drugs like taxol, vincristine, adriamycin, 5-flurouracil, etoposide are used for the treatment[1,2]. However, most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic, teratogenic effects in non-tumor cells[3,4]. These side effects limit the use of chemotherapeutic agents despite their high efficacy in treating target malignant cells. Thus, there is a need for alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action for treatment of cancer.

The plant kingdom has long history for the treatment of cancer and some medicinal plants like *Catharanthus roseus, Podophyllum emodii, P. peltatum, Taxus brevifolia, Camptotheca acuminata* and *Ocrosia elliptica* have provided active principles that are used to control the advanced stages of malignancies in clinical settings[5]. Due to enormous propensity and their capability to synthesize a variety of structurally diverse bioactive compounds, plants remain the potential source for isolating chemical constituents with antitumor and cytotoxic activities[6,7]. Natural products now have been contemplated of exceptional value in the development of effective anticancer drugs with minimum host cell toxicity. In fact, increased efforts are being made to isolate bioactive products from medicinal plants for their possible utility in cancer treatment[8].

Eucalyptus citriodora (family Myrtaceae) commonly known as lemon scented gum is a tall evergreen tree, native to Queensland, Australia and has been introduced in different countries, including India for commercial cultivation[9]. The tree is highly valued for its citronellal rich essential oil extracted from its leaves. Dried leaves of *E. citriodora* have the highest mean percentage of essential oil with the yield of 0.4% in comparison with other species[10], which has

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shown potential as insect repellant[11] and is used as an alternative in the management of poultry red mite[12]. The essential oil form leaves has also shown anti-inflammatory[13], antibacterial[14] and acaricidal[15] properties, whereas essential oil extracted from the fruits, flowers and bark possesses significant antifungal property[16,17]. The leaf extracts have also been used to treat influenza, chest problems and skin rashes[18,19]. In addition to the essential oil, eucalyptin, β-sitosterol and triterpenoids have also been isolated from the leaves of the plant[20]. Eucalyptol (1, 8-cineole) is an active ingredient of the eucalyptus oil that inhibits cytokine production and arachidonic acid metabolism in human blood monocytes *in vitro*[21].

In the present study, the *in vitro* cytotoxic effect of various extracts from *E. citriodora* leaves has been investigated against human cancer cell lines from colon, lung, prostate, ovary, cervix, neuroblastoma and liver origin and *in vivo* effect against solid tumor murine models, namely Ehrlich ascites carcinoma and Sarcoma-180.

Materials and Methods

Chemicals

RPMI-1640 medium, Dulbecco's minimum essential medium (DMEM), dimethyl sulfoxide (DMSO), EDTA, fetal calf serum (FCS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin, 5-flurouracil, camptothecin, propidium iodide, DNase-free RNase were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally.

Plant material and preparation of extracts

Eucalyptus citriodora leaves were collected from the Chatha farm of Jammu, Jammu & Kashmir, India in the month of January and authenticated at site by the taxonomist of the Indian Institute of Integrative Medicine, Jammu, India. A voucher specimen was deposited at the herbarium of the Institute (vide collection no. 17671, Acc. no. 19153). Freshly collected leaves were chopped, shade-dried and crushed into powder (green color). Powdered dried (at 50°C) leaf material was then extracted with different solvents at room temperature to obtain six different extracts, *viz.*, *n*-hexane, chloroform, ethyl acetate, methanolic, 50% methanolic, aqueous (using sequential extraction procedure) for bioevaluation.

For *n*-hexane extract, dried ground leaves (100 g) was percolated with *n*-hexane, vigorously shaken and after standing for 30 min, supernatant was decanted. The procedure was repeated thrice using fresh *n*-hexane every time and the combined *n*-hexane soluble portion was evaporated to dryness under reduced pressure at 50°C in rotary vacuum evaporator. The dried isolate (green color paste) was scrapped-off. Nitrogen was blown in the container before capping. The extract was stored at -20°C in desiccator.

For chloroform extract (dark green paste), the residue left after removing the *n*-hexane soluble part was further macerated with chloroform four-times (4 × 100 ml). Combined chloroform soluble portion was then concentrated to dryness under reduced pressure by repeating the above mentioned procedure. The residue left after removing the *n*-hexane and chloroform soluble part was suspended in water (200 ml). Suspension was taken in a separating funnel and extracted four-times (each time with 100 ml) with ethyl acetate. The solvent was removed by reduced pressure evaporation at 50°C to obtain ethyl acetate extract (green color paste). The rest of the procedure was same as mentioned above. Similarly, methanolic (dark green-brown paste) and 50% methanolic extract (green paste) were prepared. Finally, aqueous extract (brown) was obtained by collecting filtered water fraction, centrifuged at 1000 rpm for 20 min, dried by freeze dryer and stored at -20°C.

The aqueous extract was dissolved in sterile water, 50% methanolic extract in 50% DMSO and remaining extracts (hexane, chloroform, ethyl acetate, methanolic) were dissolved in DMSO to form stock solutions of 20 mg/ml. Stock solutions were prepared at least one day in advance and 1% gentamycin was added to control microbial contamination in complete growth medium *i.e.* used for dilution of stock solutions to prepare working test solutions of 200 μg/ml.

The leaf extracts were screened for the presence of secondary metabolites such as alkaloids, anthraquinones, cardiac glycosides, flavonoids, saponins, steroids and tannins using standard methods[22,23].

Cell lines and cultures

The human cancer cell lines were obtained from National Centre for Cell Science, Pune, India and National Cancer Institute, Frederick, USA. The human colon (SW-620), lung (HOP-62), prostate (PC-3) and ovary (OVCAR–5) cells were grown and

maintained in RPMI-1640 medium (pH 7.4), whereas DMEM was used for cervix (HeLa), neuroblastoma (IMR-32) and liver (HEP-2) cells. The media was supplemented with FCS (10%), penicillin (100 units/ml), streptomycin (100 µg/ml) and glutamine (2 mM).

Preparation of positive controls

Positive controls like adriamycin, 5-fluorouracil, mitomycin-C were prepared in distilled water, while taxol was prepared in DMSO. These were further diluted in gentamycin medium to obtain desired concentrations of 2×10^{-5} M and 2×10^{-6} M.

Cytotoxic activity

***In vitro* assay**

Test material was subjected to *in vitro* anticancer activity against various human cancer cell lines[24]. In brief, the cells were grown in tissue culture flasks in growth medium at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator (Hera Cell; Heraeus; Asheville, NCI, USA). The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in PBS containing 0.02% EDTA) and suspended in growth medium. Cells with more than 97% viability (trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100 µl of cells (10^5 cells/ml) was transferred to a well of 96-well tissue culture plate. The cells were allowed to grow for 24 h. Test material was then added to the wells and cells were further allowed to grow for another 48 h.

The anti-proliferative SRB assay[25] was performed to assess growth inhibition which estimates cell number indirectly by staining total cellular protein with the dye SRB. In brief, the cell growth was stopped by gently layering 50 µl of 50% (ice cold) trichloroacetic acid on the top of growth medium in all the wells. The plates were incubated at 4°C for 1 h to fix the cells attached to the bottom of the wells. Liquid of all the wells was then gently pipetted out and discarded. The plates were washed five times with distilled water and air-dried. SRB 100 µl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min. The unbound SRB was quickly removed by washing the cells five-times with 1% acetic acid. Plates were air-dried, tris buffer (100 µl, 0.01 M, pH 10.4) was added to all the wells to solubilize the dye and then plates were gently stirred for 5 min on a mechanical stirrer. The optical density (OD) was recorded on ELISA reader at 540 nm. Suitable blanks (growth medium and DMSO) and positive controls (prepared in DMSO and distilled water) were also included. Each test was done in triplicate and the values reported were mean values of three experiments.

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

OD change in presence of control = Mean OD of control – Mean OD of blank

OD change in presence of test sample = Mean OD of test sample – Mean OD of blank

% Growth in presence of control = 100/OD change in presence of control

% Growth in presence of test sample = % Growth in presence of control × OD change in presence of test sample

% Inhibition by test sample = 100 – % Growth in presence of test sample

The growth inhibition of 70% or above was considered active while testing extracts, but in testing of active ingredients at different molar concentrations, the growth inhibition of 50% or above was the criteria of activity.

In vivo studies

Non-inbred Swiss albino mice from an in-house colony were used in the present study. The experimental animals were housed in a standard size polycarbonate cages providing internationally recommended space for each animal. Animals were fed balanced mice feed supplied by M/s Ashirwad Industries, Chandigarh (India) and autoclaved water was available *ad libitum*. Animals were housed in controlled conditions of temperature (23 ± 2°C), humidity (50-60) and 12:12 of light: dark cycle. The experimental protocol was approved by the Institutional Animal Ethics Committee.

Ehrlich ascites carcinoma (EAC) and Sarcoma 180 (solid) tumor models

Two different models were used to evaluate the samples[26]. For both models animals of the same sex weighing 20 ± 3 g were injected 1×10^7 cells collected from the peritoneal cavity of non-inbread Swiss mice with 8-10 days old ascitic tumor.

Cells were then transplanted in the peritoneal cavity for ascites carcinoma and right thigh for Sarcoma 180 model of fresh non-inbred Swiss mice on day 0. The next day, animals were randomized and divided into test groups with 7 animals each and one control group (15 animals). Test material (0.2 ml) was administered intraperitoneally (i.p) to test groups as suspension in 1% gum acacia (prepared in normal saline) for nine consecutive days. The control group was similarly administered 1% gum acacia (0.2 ml, i.p).

In case of EAC, the percent tumor growth inhibition in test groups was calculated on day 12 by sacrificing the animal by cervical dislocation. Ascitic fluid was collected from peritoneal cavity and percent tumor growth inhibition was calculated. 5-Flurouracil (22 mg/kg, i.p) was used as positive control for both models.

$$\text{Percent tumor growth inhibition} = \text{Average tumor wt. of control group} - \frac{\text{Average no. of cells in control group}}{\text{Average no. of cells in treated group}} \times 100$$

For Sarcoma 180 (solid) tumor model, the percent tumor growth inhibition in test groups was calculated on day 13 by measuring the dimension of tumor.

$$\text{Tumor wt. (mg)} = \frac{\text{Length (mm)} \times [\text{width (mm)}]^2}{2}$$

$$\text{Percent tumor growth inhibition} = \text{Average tumor wt. of control group} - \frac{\text{Average tumor wt. of treated group}}{\text{Average tumor wt. of control group}} \times 100$$

Statistical analysis

The values were expressed as mean ± S.D, unless otherwise indicated. Comparisons were made between control and treated groups by analysis of variance (ANOVA) and the Student's *t*-test. P values < 0.05 were considered significant.

Results and Discussion

Phytochemical screening

The phytochemical screening revealed the presence of flavonoids, saponins and tannins in all the six extracts and anthraquinones and cardiac glycosides in four extracts, except methanolic and aqueous (Table 1). Phytochemicals, such as flavonoids, phenolic compounds and tannins have been shown to possess cytotoxicity towards tumor cells and antitumor activity in experimental animal models and are known to be effective against cancer cells that are resistant to conventional chemotherapeutic agents[27-30].

Cytotoxicity

The extracts were evaluated for *in vitro* cytotoxic potential each at the concentrations of 10, 30 and 100 μg/ml against seven different human cancer cells from seven different origins, *viz.*, SW-620 (colon), HEP-2 (liver), OVCAR-5 (ovary), PC-3 (prostate), HeLa (cervical), IMR-32 (neuroblastoma) and HOP-62 (lung) with appropriate positive controls. Negative controls (DMSO and complete medium) demonstrated no effect on the growth of cancer cells. The extracts exhibited cytotoxic activity in a dose-depende-nt manner and were cell line-specific.

Ethyl acetate extract suppressed the proliferation of all the human cancer cells lines showing significant growth inhibition (71-92%) at 100 μg/ml. Chloroform extract was active against the human cancer cell lines, except OVCAR-5 and IMR-32 and the growth inhibition ranged between 70-91% at 100 μg/ml. Methanolic extract (50%) showed cytotoxic effect against four human cancer cell lines, namely SW-620, OVCAR-5, PC-3, IMR-32 at 100 μg/ml and the growth inhibition was from 71-83%. Aqueous extract was cytotoxic against three human cancer cell lines OVCAR-5, PC-3, HOP-62 at the concentration of 100 μg/ml and range of 72-85%. OVCAR-5 (72%) and HeLa (76%) were the only two human cancer cell lines suppressed by *n*-hexane extract at the concentration of 100 μg/ml (Table 2).

Table 1—Phytochemical analysis of *E. citriodora* extracts

Secondary metabolites	*n*-Hexane extract	Chloroform extract	Ethyl acetate extract	Methanolic extract	Methanolic extract (50%)	Aqueous extract
Anthraquinones	+	+	+	-	+	-
Cardiac glycosides	+	+	+	-	+	-
Flavonoids	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Tannins	+	+	+	+	+	+

+ = Present, - = Absent

Table 2—Growth inhibitory effect of different extracts from *E. citriodora* leaves against seven human cancer cell lines from different tissues along with appropriate positive controls

[Growth inhibition above 50% (at 10 and 30 µg/ml**)** and above 70% (at 100 µg/ml**)** indicated in bold numbers. (-) indicates that the particular human cancer cell line was not treated with that particular positive control]

Extracts	Conc. (µg/ml)	Colon (SW-620)	Liver (HEP-2)	Ovary (OVCAR-5)	Prostate (PC-3)	Cervix (HeLa)	Neuroblastoma (IMR-32)	Lung (HOP-62)
		Growth inhibition (%)						
n-Hexane	10	11	33	19	5	7	31	37
	30	38	41	27	11	16	40	46
	100	53	60	**72**	64	**76**	64	56
Chloroform	10	27	24	3	11	19	36	15
	30	**65**	**53**	11	**58**	36	47	45
	100	**91**	**70**	62	**82**	**84**	68	**74**
Ethyl acetate	10	11	26	**53**	30	19	28	18
	30	**54**	48	**65**	**56**	40	**55**	38
	100	**80**	**78**	**89**	**71**	**74**	**92**	**78**
Methanolic	10	2	20	9	22	36	11	30
	30	9	22	19	33	48	17	45
	100	45	31	53	64	65	23	50
50% Methanolic	10	5	22	**54**	**54**	34	19	49
	30	37	34	**72**	**81**	39	35	54
	100	**80**	41	**78**	**83**	68	**71**	60
Aqueous	10	11	23	13	26	23	20	48
	30	33	39	**61**	**64**	28	37	**72**
	100	53	44	**72**	**76**	43	46	**85**
Positive controls	Conc. (Molar)							
5-Fluorouracil	1×10^{-5}	50	-	-	-	54	-	-
Mitomycin C	1×10^{-6}	-	48	-	56	-	-	-
Paclitaxel	1×10^{-6}	-	-	45	-	-	-	52
Adriamycin	1×10^{-6}	-	-	-	-	-	54	-

The maximum activity was observed at 100 µg/ml for all extracts, except methanolic extract which did not exhibit any cytotoxic effect against any of the seven human cancer cell lines at any concentration. In comparison to positive controls used in the present study, the cytotoxicity exhibited by ethyl acetate extract was significant as compared to other extracts that showed moderate activity.

Phytochemicals isolated from *Eucalyptus* spp. are known to possess cytotoxic and antitumor activities[31-35]. Essential oils from *E. sideroxylon* and *E. torquata* are have shown cytotoxic activity against human hepatocellular carcinoma cell line (HEP-G2) and breast adenocarcinoma cell line (MCF-7)[36]. Extracts from *E. globules* and *E. camaldulensis* are known to possess antioxidant and cytotoxic potential[37,38].

In vivo studies

As the extracts exhibited *in vitro* cytotoxic efficiency, *in vivo* studies were carried out against EAC and Sarcoma 180 (solid) tumor models. In case of EAC, the groups treated with ethyl acetate extract (200 mg/kg of weight) showed significant tumor growth inhibition on day 9, whereas aqueous extract showed lower tumor growth inhibition. Positive control 5-fluorouracil showed highly significant activity and not much difference was observed between the different control groups (Table 3). On the other hand, the untreated mice group showed a fast progressive increase in tumor volume on day 9. Both the extracts exhibited optimal tumor growth inhibition in Sarcoma 180 (solid) tumor model (Table 4).

Cancer is the most common and fatal disease responsible for 2-3% deaths recorded worldwide annually. Medicinal plants can be a promising source of novel chemotherapeutic agents, including for cancer. Various compounds, including phenolics, terpenoids, alkaloids *etc*. are present in plants. Jointly or independently, they may contribute to a variety of

Table 3—*In vivo* anticancer potential of ethyl acetate and aqueous extract from *E. citriodora* leaves against EAC murine model

[Values represent mean ± S.D]

Sample	Dose (mg/kg, i.p.)	Tumor weight (g)	Tumor volume (ml)	Cell count (10^7)	Tumor growth inhibition (%)
#Control	NS	8.81 ± 0.50	9.22 0.58	179.72 ± 3.51	-
Ethyl acetate extract	200	6.18 ±0 .40	6.47 ±0.34	126.18 ± 6.21	29.79*
Aqueous extract	200	7.19 ± 0.61	7.52± 0.33	205.45 ± 41.28	18.48
5-Flurouracil (Standard)	20	0.40 ± 0.43	0.51± 0.12	6.03 ± 1.2	95**

*Significant (p = <0.05), **highly significant (p = <0.01), #Animals in control group were administered normal saline (0.85% w/v, 0.2 ml per animal, i/p.)

Table 4—*In vivo* anticancer potential of ethyl acetate and aqueous extract from *E. citriodora* leaves against Sarcoma 180 (solid) murine model

[Values represent mean ± S.D.]

Sample	Dose (mg/ kg, i.p.)	Animal/mortality	Body weight (g)	Tumor weight (mg)	Tumor growth inhibition (%)
#Control	NS	10/0	22 ± 0.84	1560.35 ± 129.60	-
Ethyl acetate extract	200	7/0	21.47 ± 0.50	1344.26 ± 133.81	13.86*
Aqueous extract	200	7/0	20.42 ± 0.52	1427.21 ± 133.77	8.57
5-Flurouracil (standard)	22	7/0	17.66 ± 0.88	675.91 ± 36.22	56.72**

*Significant (p = <0.05), **highly significant (p = <0.01), #Animals in control groups were administered normal saline (0.85% w/v, 0.2 ml per animal, i.p.).

biological activities[39]. It has been reported that flavonoids possess antimutagenic and anti-malignant effects[40,41]. Also, many polyphenolic compounds, including flavonoids inhibit the growth of leukemia cells to some extent[42,43]. Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis[44]. Although use of phytochemicals for prevention and treatment of cancer is well documented, their importance in modern medicine remains under-estimated.

In the present study, results from six different fresh extracts from leaves of *E. citriodora* revealed that all the extracts, except methanolic significantly inhibited the proliferation of human cancer cells. Non-polar extracts were found to be more effective and displayed strong anticancer effect on a range of human cancer cell lines in a dose-dependant and cell line-specific manner, indicating that active principle might be non-polar in nature.

In conclusion, the present study demonstrated significant *in vitro* and *in vivo* antitumor potential of ethyl acetate extract from *E. citriodora* leaves against various human cancer cells derived from different origins, as well as against murine solid tumors from different etiologies. The ethyl acetate extract demonstrated a significant effect on three human tumor cell lines (IMR-32, SW-620, OVCAR-5). Further studies are required to characterize the bioactive constituents in the ethyl acetate extract for designing novel anticancer drugs that may find use for the treatment of human malignancies.

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