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ANTITUMOR ACTIVITY OF POLYPHENOLIC EXTRACT OF *ICHNOCARPUS FRUTESCENS*

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Aim: **Phytochemical and dietary antioxidants are known to decrease the risk of many diseases such as cancer and cardiovascular diseases. In this study polyphenolic extract (PPE) of leaves of *Ichnocarpus frutescens* was evaluated for antitumor activity *in vivo*.** ***Materials and Methods:*** **Murine Ehrlich ascites carcinoma (EAC) model was used to assess PPE antitumor activity *in vivo*. PPE cytotoxicity was determined *in vitro* in U-937 monocytoid leukemia and K-562 erythroleukemia cell lines. PPE also have been assessed for the free radical scavenging activity against superoxide and nitric oxide radicals. Acute oral toxicity was performed by acute toxic classic method. The total phenolics content was quantified by the Folin-Ciocalteu method.** ***Results:*** **Results of *in vivo* study showed a significant decrease in tumor volume, viable tumor cell count and a significant increase of life span in the PPE treated group compared to untreated one: the life span of PPE treated animals increased by 53.41% (50 mg PPE/kg) and 73.95% (100 mg PPE/kg). PPE (5, 10 and 20 μg/mL) effectively inhibits *in vitro* proliferation of U-937 and K-562 cell lines. PPE exhibited pronounced radical scavenging activity with an inhibitory concentration (IC_{50}) value of 167.46 μg/mL and 158.52 μg/mL against superoxide and nitric oxide radicals, respectively.** ***Conclusion:*** **PPE of *Ichnocarpus frutescens* possesses strong free radical scavenging activity and anti-tumor activity *in vitro* and *in vivo*.**

Key Words: ***Ichnocarpus frutescens*****, polyphenolic extract, anti-tumor activity, Ehrlich ascites carcinoma, leukemia cell lines, free radical scavenging.**

Ayurveda, the Indian system of medicine mainly uses plant-based drugs or formulations to treat various ailments including cancer. Plant derived compounds, in particular have a special place in anti-cancer therapy, and some of the new chemotherapeutic agents currently available for use in a clinical setting include paclitaxel, vincristine, podophyllotoxin and camptothecin, a natural product precursor for water soluble derivatives [1–3]. Due to lack of effective drugs, cancer is a fatal disease rating the top three cause of death. Many of the chemotherapeutic agents sold for the treatment of cancer are highly expensive, mutagenic, carcinogenic and teratogenic and marrow inhibition limits their applications [4]. Therefore the quest for effective anti-cancer drug is an active research field. Efforts, therefore, are being made to identify naturally occurring anticarcinogens, which would prevent, slow/reverse cancer development [5].

Ichnocarpus frutescens (*Apocynaceae*), is a large evergreen, climbing, much branched shrub and ascending up to an altitude of 4000 ft, is found almost throughout India. Leaves are boiled in oil and applied in headaches and fevers; they are also applied to wounds [6]. The plant contains wide range of iridoid glycosides, triterpenoids [7] and polyphenolic compounds [8] such as simple phenolic acids and flavonoids, whereas it contains no alkaloids. The utilization of decoction of leaves of *I. frutescens* in the treatment of jaundice and diabetes is noteworthy [9]. Some of the constituents of the plant, such as triterpenoids and flavonoids were shown to present antitumor, antioxidant and related biological activities [10, 11]. Plants belonging to *Apocynaceae* are reported to have anticancer properties. Flavonoids have been shown to possess antimutagenic and anticarcinogenic activity [12-13]. Our earlier report reveals the antiinflammatory activity of hydroalcoholic extract of *I. frutescens* [14]. In spite of reported use, no systematic clinical experimental studies have been carried out to assess the antitumor activity of this species. Therefore *I. frutescens* leaves were investigated for their potential antitumor and free radical scavenging property in order to assess the ethnomedical uses.

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Abbreviations used: ANOVA – analysis of variance; Ara-C – cytarabine arabinoside; BSS – buffer solution saline; DMSO – dimethyl sulphoxide; EAC – Ehrlich ascites carcinoma; ELISA – enzyme-linked immunosorbent assay; FBS — fetal bovine serum; 5-FU – 5-fluorouracil; Hb – hemoglobin; ILS – increase in lifespan; IP – iIntraperitoneal; MST – median survival time; MTT – 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide; NADH – nicotinamide adenine dinucleotide (reduced form); NBT – nitroblue tetrazolium; OECD – Organization Economic Cooperation Development; PMS – phenazine methosulfate; PPE – polyphenolic extract; RBC – red blood cell; SEM – standard error mean; TLC – thin layer chromatography; WBC – white blood cell.

MATERIALS AMD METHODS

Chemicals. 5-Fluorouracil (5-FU) was obtained from Nicholas Piramal India Limited (Mumbai, India). Alpha tocopherol, quercetin, cytarabine arabinoside (Ara-C), fetal bovine serum (FBS), RPMI-1640 medium, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), gentamycin (40 μg/mL), penicillin (100 units/mL) and streptomycin (10 μg/mL) were purchased from Sigma Chemical Co (MO, USA). Sodium nitroprusside, Griess reagent, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS). All other

chemicals were of high purity and obtained from commercial sources.

Plant materials. The fresh leaves of *Ichnocarpus frutescens* were collected from Thiruchirappalli, India, in Febrary 2005, authenticated at Botanial Survey of India, Central National Herbarium, Howrah, India (Ref No: CNH/I-I/87/2005-TECH/1326). An authentic voucher specimen was deposited in the Herbarium of Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Preparation of polyphenolic extract (PPE). Dried leaves of *Ichnocarpus frutescens* (500 g) were finely powdered, mixed with 70% ethanol and kept at room temperature for 5 days. After 5 days solution was filtered and the solvent was evaporated. The residue was dissolved in water and the aqueous layer was washed with petroleum ether several times until a clear upper layer of petroleum ether was obtained. The lower layer was then treated with ethyl acetate containing glacial acetic acid (10 ml/l). Extraction of polyphenols was carried out for 36 h at room temperature and the combined ethyl acetate layer was concentrated [15]. The residue was lyophilized and stored at –70 °C. This yielded about 10–15 g per 100 g of leaf powder. The total polyphenolic and flavonoid content of the extract was assayed using the method of Slinkard and Singleton [16, 17].

Animals. Healthy male Swiss albino mice and Wistar albino rats, weighing about 20–25 g and 180–200 g, respectively were obtained from M/S Ghosh Enterprises, Kolkata, India and were used in the present study. Animals were collected from breeding colony and acclimatized to the laboratory condition for 2 weeks. The animals were fed with commercial diet from Hindustan Lever Ltd (Bangalore, India) and had free access to water during the experiments. Experiments were performed complied with the rulings of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi, India, and the study was permitted by the institutional ethical committee of the Jadavpur University, Kolkata, India.

Acute toxicity study. Swiss albino mice of either sex weighing 18–22 g were randomly distributed into six different groups (six animals per group). The animals were fasted overnight and the drug was administered orally at dose levels of 100, 200, 400, 800, 1600 and 3200 mg/kg body weight. The animals were closely monitored for the first 1 h for behavioral, neurological and autonomic profiles and for 72 h for mortality/lethality rate [18].

In vivo anti-tumor activity

Tumor cells. Ehrlich ascites carcinoma (EAC) described in this paper was hyperdiploid subline (obtained from Chitharanjan Cancer Research Institute (CCRI), Kolkata, India), which has been propagated in our laboratory by weekly intraperitoneal (IP) inoculation of about 2.5×10^6 cells/mouse. The ascites tumor cells, obtained from donor mouse, were diluted with buffer solution saline (BSS) and counted with hemocytometer.

Effect of PPE on EAC cell line. A total of 60 female mice were divided at random into 5 groups (12 animals per group). Group I (Normal) animals received orally normal saline solution for 9 days. Group II (Control) animals received orally normal saline solution for 9 days, and each mouse was inoculated (IP) with 2×10^6 EAC cells suspended in BSS on day 0. Group III (treated — 50 mg/kg) animals received orally 50 mg PPE/kg for 9 days, and each mouse was inoculated (IP) with 2×10^6 EAC cells suspended in BSS on day 0. Group IV (Treated — 100 mg/kg) animals received orally 100 mg PPE/kg for 9 days and each mouse was inoculated (IP) with 2×10^6 EAC cells suspended in BSS on day 0. Group V (5-FU treated — 20 mg/kg) animals received orally 20 mg/kg 5-FU for 9 days and were injected IP with 2×10^6 EAC-cells. After the administration of last dose of PPE followed by 18 h fasting 6 mice from each group were sacrificed for the study of antitumor activity, hematological and biochemical parameters. The remaining animals in each group were kept to check the median survival time (MST) of the tumor bearing hosts. At the end of each treatment period, survival time of each mouse was recorded and MST of each group was then calculated. Antitumor effect of PPE was assessed by observation of changes with respect to body weight, ascetics tumor volume, packed cell volume, viable & nonviable tumor cell count, MST and percentage increase in life span (ILS, %). MST of each group containing 6 mice was monitored by recording the mortality daily for 6 weeks and % ILS was calculated [20, 21].

Tumor volume, viable/non-viable tumor cell count. The mice were killed by cervical dislocation (acceptable method of euthanasia), dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube and packed cell volume was determined by centrifuging at 1000 rpm for 5 min. The viable tumor cell counts (trypan blue test) were carried out with Neubauer hemocytometer. The cells were then stained with trypan blue (0.4% in normal saline) dye. These viable and nonviable cells were counted.

$$\text{Cell count} = \frac{\text{Number of cells} \times \text{Dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

Percentage increase of life span (% ILS). The effect of PPE on tumor growth was monitored by recording the mortality daily for a period of 5 weeks and % ILS was calculated. The animals that survived 35 days were considered to be cured.

$$\%\ \text{ILS} = \left(\frac{\text{Mean survival of treated group}}{\text{Mean survival of control}} - 1\right) \times 100$$

Median Survival Time = (Day of 1 death + Day of last death) / 2

Hematological Parameters. At the end of the experimental period, all mice were killed the next day after an overnight fasting by decapitation. Blood was

collected from freely flowing tail vein and used for the estimation of hemoglobin (Hb) content, red blood cell count (RBC) and white blood cell count (WBC). WBC differential count was carried out from Leishman stained blood smears.

In vitro anti-tumor activity

Cell culture. U-937 monocytoid leukemia and K-562 erythroleukemia cell lines were obtained from National Facility for Animal Tissue & Cell culture, Pune (India). The growth of the cell lines was maintained in the Pharmacognosy and Phytotherapy Research Laboratory in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum and gentamycin (40 µg/mL), penicillin (100 units/ml) and streptomycin (10 µg/ml). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2, 95% air in a CO_2 incubator.

Cell viability study. Log phase cell suspension of U937 and K562 at a concentration of 10^5/ml in RPMI 1640 (with 10% FBS) was used for the experiment in 96 well microtitre sterile plate. To each well 100 µl of cell suspension was placed. The test drug was added at different concentration (5, 10 and15 µg/ml) against the standard drug Ara-C (20 µg/mL) and the viable count was done by Trypan blue exclusion method [22] after 24 h of treatment.

Cytotoxicity assay. Cell lines at exponential growth phase were washed, trypsinized and resuspended in RPMI 1640 medium. Cells were kept at a concentration of 10^5 cells/well in 96 well microtitre plate. The cells were treated with different concentration of test drug (5, 10 and 15 µg/ml) against the Ara-C (20 µg/mL), and the control which contained only the medium, and incubated for 24 h. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] solution was added to each well to make the final concentration of 400 µg/ml and further incubated at 37 °C in a CO_2 incubator for 3 h [23]. The reaction resulted in the reduction of MTT by the mitochondrial dehydrogenase of viable cells to a purple formazan product. The MTT-formazan product was dissolved in DMSO and estimated by measuring the absorbance at 570 nm in an ELISA plate reader.

Free radical scavenging activity. Scavenging activity of nitric oxide radical nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [24]. The reaction mixture (3 ml) containing 10 mM sodium nitroprusside in phosphate-buffered saline and PPE and the reference compound at different concentrations (50, 100, 150, 200 and 250 µg/ml) was incubated at 25 °C for 150 min. A 0.5 ml aliquot of the incubated sample was removed at 30 min intervals, and 0.5 ml Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was measured at 546 nm. All tests were performed in triplicate. Percent inhibition of the nitric oxide generated was measured by comparing the absorbance values of control and test preparations. Alpha tocopherol was used as a positive control.

Superoxide anion radical scavenging activity of PPE. The superoxide anion scavenging activity of PPE was determined by the method described by Nishimiki et al. [25], slightly modified. About 1 ml of NBT solution containing 156 µM NBT dissolved in 1.0 ml 100 mM phosphate buffer, pH 7.4, 1 ml NADH solution containing 468 µM NADH dissolved in 1 ml 100 mM phosphate buffer, pH 7.4, and 0.1 ml of various concentration of PPE and reference compounds (50, 100, 150, 200 and 250 µg/ml) were mixed and the reaction was started by adding 100 µl phenazine methosulfate solution containing 60 µM phenazine methosulfate in 100 mM phosphate buffer, pH 7.4. The reaction mixture was incubated at 25 °C for 5 min and absorbance at 560 nm was measured against control samples. All tests were performed in triplicate. Percent inhibition was calculated by comparing the results of control and test samples. Alpha tocopherol was used as reference compound.

Statistical analysis. Data were analyzed using Graphat Instat Software (San Diego, CA, USA). The experimental data were expressed as mean ± SEM. the significance of difference among the various treated groups and control group were analyzed by means of one-way ANOVA. The level of significance was set at $p < 0.05$. IC_{50} (inhibitory concentration which caused 50% inhibition) were estimated using linear regression method of plots of the percent of antiradical activity against the concentration of the tested compounds using Microsoft Excel Software Programme.

RESULTS

Preliminary phytochemical studies. The results of our present study are presented in Tables 1–4 and Fig. 1–6. Preliminary phytochemical screening of PPE was carried out for the detection of phytoconstituents, using standard chemical tests. Triterpenoids, flavonoids, simple phenolic acids, steroids and tannins were detected in PPE. Chromatography on silica gel 60 with chloroform, methanol as mobile phase, in a saturated chamber, allows baseline separation of the target compounds. The PPE profile can be visualized with Fast Blue Salt B reagent.

Table 1. Effect of PPE and 5-FU treatment on the survival of EAC (2×10^6 cells) treated mice

Group	Median survival time (Days)	Increase of life span (%)
1	–	–
2	19.66 ± 0.88	–
3	30.16 ± 0.60*	53.41%
4	34.20 ± 0.87*	73.95%
5	36.76 ± 0.72*	86.97%

Notes: n = 30; results are presented as mean ± SEM. Days of drug treatment: 9 days.*$p < 0.01$ compared to group 2. Data were analyzed by One-way ANOVA followed by Dunnett's test.

The polyphenolic extract of *Ichnocarpus frutescens* did not cause any mortality up to 2000 mg/kg and was considered as safe (OECD, 2002). Total phenolic content of PPE was expressed as mg of pyrocatechol equivalent per gram of dry weight of PPE extract. 1000 µg of PPE was used to determine the amount of total polyphenolic content. The level of total polyphenolic compounds was 100.51 mg of pyrocatechol equivalent per gram of PPE. The present study showed the flavonoid content determined by two independent colorimetric methods, one

for the determination of flavones and flavonols and other for determination of flavanones, as reported earlier. The contents of total flavonoids in the PPE of *I. frutescens* were expressed as the sum of two complementary methods for the determination of flavones, flavonols and flavononones and the results found to be 17.8 mg of quercetin and naringenin equivalent per gram of PPE. Major types of phenolic constituents identified in the leaves of *I. frutescens* are simple phenolic acids, flavonol, flavones, flavononones and flavonoid glycosides.

Acute toxicity study. In the acute toxicity study, PPE upto dose level of 3200 mg/kg of body weight did not exhibit any lethality or toxic symptoms. According to organization for Economic Co-operation and Development (OECD) guidelines for acute oral toxicity, an LD50 dose of 2000 mg/kg and above is characterized as unclassified and hence the drug is found safe. Acute toxicity study revealed that the extract was safe up to a dose level of 3200 mg/kg of body weight.

Effect on body weight changes. Tumor-bearing mice (group 2- positive control) showed a significant ($p < 0.01$) increase in body weight as compared with group 1 (negative control). Treatment with PPE (100 mg/kg) significantly reduced the increase in body weight of EAC bearing mice at 10, 15 and 20 days, whereas at the dose of 50 mg PPE/kg the retardation of increase in body weight was statistically significant ($p < 0.01$) at 15 and 20 days after tumor implantation. 5-Flurouracil treatment also significantly reduced the increase in body weight of tumor-bearing mice at 15 and 20 days after implantation. The effect of PPE treatment was comparable with that of 5-FU (Fig. 1).

Effect on survival time. In group 2, no animals survived after 22 days after EAC transplantation. The mean survival time in this group was 19.66 days. PPE treatment (100 mg/kg/day) increased the mean survival period to 32.40 days, with no animals survived after day 45. The increase in life span (ILS) of PPE treated (50 and 100 mg/kg) groups was 73.95%. 5-Fluorouracil treatment (Group 3) increased the mean survival period to 36.76 days. No animals survived after 40 days. ILS in groups treated with PPE (50 mg/kg/day) and 5-FU (20 mg/kg) was 53.41 % and 86.97% respectively. The percentage increase of lifespan and mean survival time of mice treated with PPE at higher dose was comparable that observed in the 5-FU treatment group (Fig. 2).

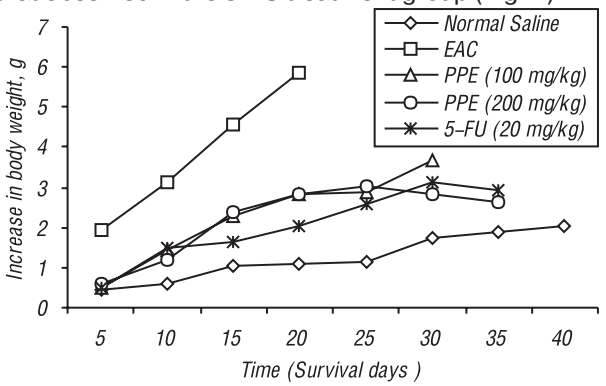

Fig. 1. Effect of polyphenolic extract (PPE) of I.frutescens and 5-FU treatment on body weight changes in mice treated with EAC cells. Each valuse represents the mean ± SEM of six animals.

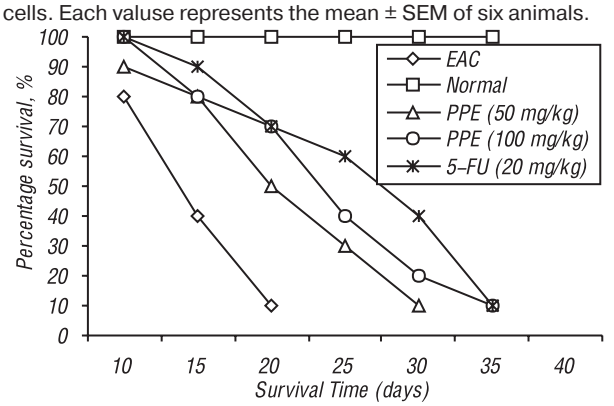

Fig. 2. Effect of polyphenolic extract (PPE) of I.frutescens and 5-FU on the percentage survival days of mice treated with EAC cells. Each values represents the mean ± SEM of six animals.

Effect of PPE on in vivo cytotoxicity and tumor cell viability. Table 2 shows the effect of PPE treatment on EAC growth in mice. The tumor volume

Table 2. Effect of polyphenolic extract (PPE) of *I. frutescens* and 5-FU treatment on tumor growth *in vivo*.

Parameters	Group 2	Group 3	Group 4	Group 5
Tumor volume (cm^3)	4.1 ± 0.159	2.73 ± 0.120	2.3 ± 0.15*	1.71 ± 0.213*
Change over control (%)	–	66.58	56.09	41.70
Viable tumor cell count × 10^6	8.78 ± 0.228	4.83 ± 0.270	3.41 ± 0.20*	2.86 ± 0.252*
Change over control (%)	–	55.01	38.83	32.57
Non-viable tumor cell count × 10^6	0.508 ± 0.030	0.653 ± 0.022	0.856 ± 0.02*	0.893 ± 0.02*
Change over control (%)	–	128.54	168.58	175.78

Notes: n = 30; results are presented as mean ± SEM. Days of drug treatment: 9 days; *$p < 0.01$ compared to group 2. Data were analyzed by One-way ANOVA followed by Dunnett's test.

Table 3. Effect of polyphenolic extract (PPE) of *I. frutescens* and 5-FU treatment on hematological indexes of experimental animals.

Hematological parameters	Group 1	Group 2	Group 3	Group 4	Group 5
Hemoglobin (%)	13.2 ± 1.18**	8.66 ± 0.198	10.10 ± 1.03**	11.58 ± 0.312**	12.16 ± 0.260**
Total RBC(cells/ml × 10^9)	6.31 ± 0.142**	4.36 ± 0.168	5.46 ± 0.229**	5.60 ± 0.169*	6.30 ± 0.152**
Total WBC(cells/ml × 10^9)	6.63 ± 0.180**	19.35 ± 0.366	12.50 ± 0.201**	9.48 ± 0.164**	6.75 ± 0.224**

Notes: n = 30; results are presented as mean ± SEM. Days of drug treatment: 9 days; *$p < 0.001$; **$p < 0.01$ compared to group 2. Data were analyzed by One-way ANOVA followed by Dunnett's test.

Table 4. Effect of PPE and 5-FU treatment on differential counts of Whit blood cells (WBC) of mice treated with Ehrlich Ascites Carcinoma (2 × 10^6 cells/mouse) cells.

Experimental groups	Neutrophil (%)	Eosinophil (%)	Lymphocytes (%)	Monocytes (%)
Vehicle	18.68 ± 1.07*	0.66 ± 0.019*	82.63 ± 1.38*	1.75 ± 0.048*
EAC control	62.66 ± 1.23	1.75 ± 0.041	32.23 ± 1.69	1.048 ± 0.050
PPE (50 mg/kg)	47.53 ± 2.38*	1.22 ± 0.048*	51.93 ± 2.16*	1.076 ± 0.043NS
PPE (100 mg/kg)	39.16 ± 1.34*	0.77 ± 0.026*	59.85 ± 1.89*	1.366 ± 0.059*
5-FU (20 mg/kg)	43.46 ± 1.15*	0.78 ± 0.038*	62.28 ± 1.86*	1.47 ± 0.363*

N = 30; Results are presented as mean ± SEM. Days of drug treatment 9 days. NS-Non significant, *$p < 0.01$ compared to EAC treated group. Data were analyzed by One-way ANOVA followed by Dunnett's test.

of the control group showed progressive elevation, whereas tumor volume of both PPE treated groups demonstrated significant decrease of tumor volume compared to control groups. The PPE extract at a dose of 100 mg/kg inhibited the tumor growth as effectively as the standard reference drug 5-FU. PPE treatment significantly reduced the total number of cells obtained from the positive control (Group 2). This treatment also, reduced the viability of EAC cells. The reduction in viability was statistically significant at the higher dose (100 mg/kg/day) and that it was comparable to that of 5-FU (Table 2). On the other hand, 5-FU treatment (group 3) caused significant diminution ($p < 0.01$) in the viability of cells.

Effect on hematological status. Hematological indexes of PPE treated EAC bearing mice are presented in Table 3. The total WBC count and proteins were found to increase along with a reduction in the Hb content of RBC in tumor-bearing mice. A fall in Hb has been observed in control group with respect to normal (untreated) mice, but in group 3 treated with PPE (100 mg/kg/day) Hb content has found to be close to normal. RBC content has shown a modest decrease in both PPE treated groups, but the WBC count is significantly enhanced. Treatment with PPE (100 mg/kg) caused differential effect on WBC count and those altered parameters returned to normal ones. However no beneficial effect has been observed in this aspect.

Effect of PPE on in vitro cytotoxicity and cell viability. The inhibition of viability and growth of cells treated with PPE and Ara-C is shown in Fig. 2 and 3. The proliferation of U-937 and K-562 cell lines was strongly inhibited by PPE and Ara-C.

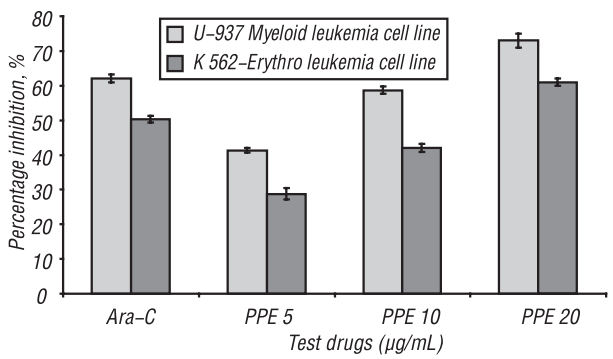

Fig. 3. Effect of *in vitro* inhibition of cell growth by polyphenolic extract (PPE) of and Cytarabine arabinoside (Ara-C) on two different leukemia cell lines. Values are expressed as mean ± SEM. *$p < 0.01$significant vs control.

Effect of PPE on free radical scavenging activities. The free radical scavenging activity of PPE and alpha tocopherol was studied in the presence of superoxide anion radicals and nitric oxide radicals (Fig. 5 and 6). The superoxide and nitric oxide free radical scavenging capacity of PPE was found to have IC_{50} of 159.90 μg/ml and 168.67 μg/ml respectively, with respect to reference compound alpha tocopherol 150.07 μg/ml and 143.03 μg/ml, used as positive control. The superoxide and nitric oxide free radicals scavenging activity of PPE extract was shown to be concentration dependent.

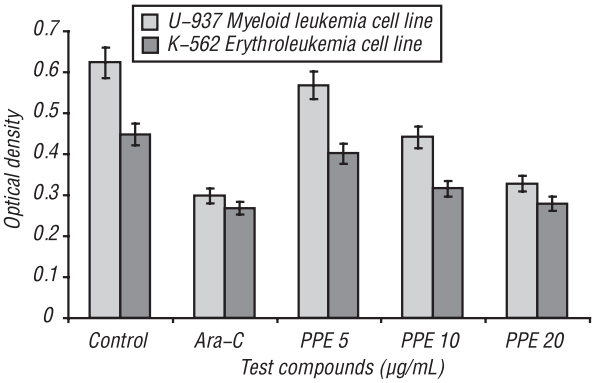

Fig. 4. Effect of *in vitro* inhibition of cell viability by polyphenolic extract (PPE) of I. frutescens on two different leukemia cell lines. values are expressed as mean ± SEM. *$p < 0.01$ significant vs control.

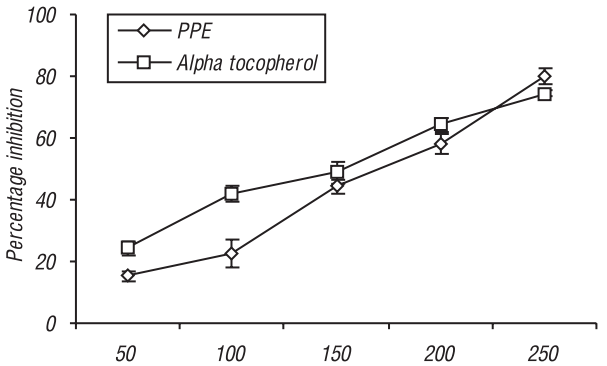

Fig. 5. Super oxide radical scavenging activity of polyphenolic extract (PPE) of I. frutescens and alpha tocopherol.
Each value represent the mean ± SEM of three experiments

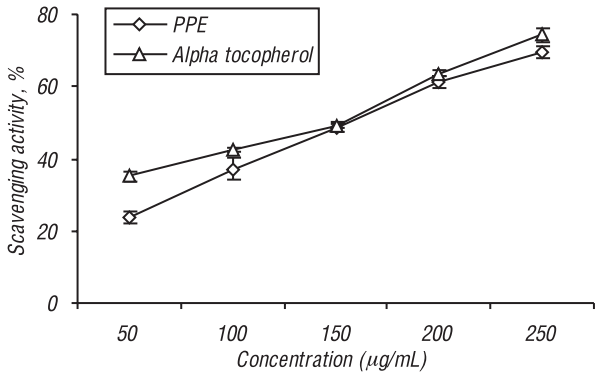

Fig 6. Nitric oxideradical scavenging activity of polyphenolic extract (PPE) of and alpha tocopherol.
Each value represent the mean ± SEM of three experiments

DISCUSSION

Cancer prevention using chemical compounds or natural products that revert or inhibit cell transformation and prevent invasion and metastasis would be less painful, more economical and rational approach for the cancer control. Use of natural and dietary agents is being increasingly utilized as an effective way for the treatment of many cancer treatments [26, 27]. Obviously natural products are extremely important as sources of medicinal plants.

In the present study, we have shown that polyphenolic extract of *I. frutescens* has significant antitumor effect *in vivo*. Various plant components and extracts from plants such as Taxol, curcumin, phenolic acids and flavonoids are reported to inhibit tumor growth in

many types of cancer [28, 29]. The administration of various doses of PPE caused a dose dependent retardation in the tumor development, as it is evident by the inhibition in body weight and ILS. A similar effect has been reported earlier in mice treated with the extract of *Phyllanthus amarus, Bahunia variegate, Coriander sativum* and *Tinosporia cordifolia* [30-32].

The results on *in vivo* cytotoxic potential of PPE that showed a significant reduction in the number of EAC cells and their viability support these data. However, our results on EAC cell count and viability after treatment with PPE were comparable to the effects of 5-FU.

Tumor growth is generally associated with marked changes in hematopoiesis and immune response, myelosuppression and anemia. Hematological studies have revealed that PPE at the dose of 100 m/kg has restored WBC differential count, Hb and RBC content close to normal levels.

I. frutescens is known to contain many kind of polyphenolic compounds and flavonoids of which several are reported to have antitumor activity [33, 34]. In this study, we have examined the cytotoxicity activity of PPE of *I. frutescens* on two different leukemia cell lines. Flavonoids are generally regarded to have antitumor activity, and many polyphenolic extracts containing flavonoids inhibited the growth leukemia cells to some extent [35–39]. Flavonoids included in almost all the plants we usually are consumpting may therefore be considered as tumor preventing compounds, but the present results suggest that the mechanism of PPE action on tumor cells should to be elucidated in detailed manner.

Superoxide radical is known to be very harmful to cellular components as precursor of more reactive oxygen species. Polyphenolic extract was found to act as scavenger of superoxide radical generated *in vitro*. Superoxide anion radicals (O_2^-) formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils, and the production of O_2^- is an important factor in killing of bacteria by phagocytes. In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen from the coupling of PMS-NADH, reduces NBT.

H_2O_2 is rather inert at low concentrations. Biologically H_2O_2 is converted to oxygen and water by catalase. In the presence of antioxidants the oxidation is inhibited. The nature of the inhibition is an ambiguous because there are several potential inhibition pathways. Therefore, it is difficult to explain the actual chemical meaning of cell death. The hydrogen peroxide scavenging activity of PPE is not documented. Nevertheless, Duh et al. [40] reported similar results for *Chrysanthemum morifolium* with high relationship between their phenolic content and scavenging activity of the PPE. This ability to scavenge H_2O_2 could be an efficient assessment method to evaluate antioxidant property of *I. frutescens*. The gain of superoxide and hydrogen peroxide radical scavenging activities was also associated to the increased total phenolic and flavonoid content of the extracts.

Preliminary phytochemical investigation of the polyphenolic extract of *I. frutescens* revealed the presence of flavonoids and novel pentacyclic triterpenoids. Four phenolic acids were isolated from the leaves and identified. Those natural antioxidative substances have a phenolic moiety in their molecular structure. Polyphenols were reported to have an important role in stabilizing lipid peroxidation and are associated with a wide range of biological activities including antioxidant properties [41–42]. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups. The anticancer and free radical scavenging activity of PPE can be attributed to the presence of phenolic acids and flavonoids.

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REFERENCE

1. **Kinghorn AD, Balandrin MF.** Human Medical Agents from Plants. Am Chem Soc Symp Series 534. Washington, DC: Am Chem Soc, 1993; 80–95.

2. **Gerzon K.** Anticancer Agents Based on Natural Product Models. Cassady JM, Douros JD, eds. New York: Academic Press, 1980; 271–317.

3. **Wall ME, Wani MC.** Human Medicinal Agents from Plants. Kinghorn AD, Balandrin MF, eds. Am Chem Soc Symp Series 534. Washington, DC: Am Chem Soc, 1993; 149–69.

4. **Mascarenhas M.** Structure-activity characterization, a quick method to screen mushrooms for the presence of antitumor glucans. Mushroom Res 1994; **3**: 77–80.

5. **Chuang SE, Kuo, ML, Hsu CH, Chen CR, Lin JK, Lai M, Hsieh GY, Cheng AL. C**arcinogenesis 2000; **21**: 331–5.

6. **Anonymous.** Wealth of India. Raw Materials, Council of Scientific and Industrial Research (CSIR), CSIR, New Delhi, vol. V. 1976: 162–63.

7. **Lakshmi DKM, Venkata Rao E, Venkata Rao D.** Triterpenoid constituents of *Ichnocarpus frutescens*. Indian Drugs 1985; **22**: 552–3.

8. **Singh RP and Singh RP.** Flavonoids of the flowers of *Ichnocarpus frutescens*. J Indian Chem Soc 1987; **LXIV**: 715–56.

9. **Parinitha M, Harish GU, Vivek NC, Mahesh T, Shivanna MB.** Ethno-botanical wealth of Bhadra wild life sanctuary in Karnataka. Ind J Trad Knowledge 2004; **31**: 37–50.

10. **Ferguson PJ, Kurowska E, Freeman DJ, Chambers AF, Koropatnick DJ.** A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. J Nutr 2004; **134**: 1529–35.

11. **Hudson, EA, Dinh PA, Kokubun, T, Simmonds MSJ, Gescher A.** Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. Cancer Epidemiol Biomarkers Prev 2000; **9**: 1163–70.

12. **Babu BH, Jayram HN, Nair MG, Ajaikumar KB, Padikkala J.** Free radical scavenging, antitumor and anticarcinogenic activity of gossypin. J Exp Clin Cancer Res 2003; **22**: 581–90.

13. **Kuroda Y, Hara Y.** Antimutagenic and anticarcinogenic activity of tea polyphenols. Mutat Res 1999; **436**: 69–97.

14. **Kumarappan CT, Chandra R, Mandal SC.** Anti-inflammatory activity of *Ichnocarpus frutescens*. Pharmacology Online 2006; **3**: 201–16.

15. **Xia J, Allenbrand B, Sun GY.** Dietary supplementation of grape polyphenols and chronic ethanol administration on LDL oxidation and platelet function in rats. Life Sci 1998; **63**: 383–90.

16. **Singleton VL, Orthofer RM, Ramuela-Raventos RM.** Analysis of total phenols and antioxidants and other substrates by means of Folin – Ciocalteu reagent. Methods. Enzymol 1999; **299**: 152–78.

17. **Chang, CC, Yang MH, Wen HM, Chern JC.** Estimation of total flavonoid content propolis by two complementary colorimetric methods. J. Food Drug Anal 2002; **10**: 178–82.

18. OECD (Organization for Economic Co-operation and Development). OECD Guidelines for the Testing of Chemicals / Section 4: Health Effects Test No. 423: Acute Oral Toxicity — Acute Toxic Class Method. Paris: OECD, 2002.

19. **D'Armour FE, Blood FR, Belden DA.** The manual for laboratory work in mammalian physiology. 3rd ed. Chicago, USA: The University of Chicago Press, 1965; 4–6.

20. **Wintrobe MM, Lee GR, Boggs DR, Bithel TC, Athens JW, Foerester ??.** Clinical Hematology 5th ed. Philadelphia, USA, 1961; 326.

21. **Sur P, Chatterjee SP, Roy P, Sur B.** 5-Nitrofuran derivatives of fatty acid hydrazides induce differentiation in human myeloid leukemic cell lines. Cancer Lett 1965; **94**: 27–32.

22. **Kawada M, Amemiya M, Ishizuka M, Takeuchi M.** Differential induction of apoptosis in B16 melanoma E1–4 lymphoma cells by cytostatin and bactobolin. Jpn J Cancer Res 1999; **90**: 219–25.

23. **Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR.** Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem 1982; **126**; 131–8.

24. **Nishimiki M, Rao NS, Yagi K.** The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun 1972; **46**: 849–53.

25. **Miyoshi N, Kakamura Y, Ueda Y, Abe M, Ozawa Y, Uchida K, Osawa T.** Dietary ginger constituents, galanals A and B, are potent apoptosis inducers in human T lymphoma Jurkat cells. Cancer Lett 2003; **199**: 113–9.

26. **Wenzel U, Kuntz S, Brendel MD, Danial H.** Dietary flavone is a potent apoptosis inducer in human colon carcinoma cells. Cancer Res 2000; **60**: 3823–31.

27. **Lee NK, Kim HJ, Yang SJ, Kim YS, Choi HH, Shim MJ, Awh OD, Kim TU.** The anticancer mechanism of taxol-diethylenetriamine pentaacetate conjugate in HT29 human colorectal cancer cells. J Biochem Mol Biol 2001; **34**: 237–43.

28. **Sartippour MR, Liu C, Shao ZM, Go VL, Heber D, Nguyen M.** *Livistona* extract inhibits angiogenesis and cancer growth. Oncol Rep 2001; **8**: 1355–7.

29. **Rajesh Kumar NV, Joy, KL, Kuttan, G, Ramsewak RS, Nair MG, Kuttan R.** Antitumor and anti-carcinogenic activity of *Phyllanthus amarus* extract. J Ethnopharmacol 2002; **81**: 17–22.

30. **Jagetia GC, Rao SK.** Evaluation of the antineoplastic activity of Guduchi (*Tinospora cordifolia*) in Ehrlich ascites carcinoma bearing mice. Biol Pharm Bull 2006; **29**: 460–6.

31. **Rajkapoor B, Jayakar B, Murugesh N.** Antitumor activity of *Bauhini variegata* against Ehrlich ascites carcinoma induced mice. Pharm Biol 2003; **41**: 604–7.

32. **Chen D, Daniel KG, Chen MS, Kuhn DJ, Landis-Piwowar KR, Dou QP.** Dietary flavonoids as proteasome inhibitors and apoptosis inducers in human leukemia cells. Biochem Pharmacol 2005; **69**: 1421–32.

33. **Czyz J, Madeja Z, Irmer U, Korohoda W, Hulser DF.** Flavonoid apigenin inhibits motility and invasiveness of carcinoma cells *in vitro*. Int J Cancer 2005; **114**: 12–8.

34. **Zheng PW, Chiang LC, Lin CC.** Apigenin induced apoptosis through p53-dependent pathway in human cervical cancer cells**.** Life Sci 2005; **76**: 1367–79.

35. **Mitrocotsa D, Bosch S, Mitaku S, Dimas C, Skaltsounis AL, Harvala C, Briand G, Roussakis C.** Cytotoxicity against human leukemic cell lines, and the activity on the expression of resistance genes of flavonoids from *Platanus orientalis*. Anticancer Res 1999; **19**: 2085–8.

36. **Chen J, Kang JH.** Quercetin and trichostatin A cooperatively kill human leukemia cells. Pharmazie 2005; **60**: 856–60.

37. **Horvathova K, Novotny L, Vachalkova A.** The free radical scavenging activity of four flavonoids determined by the comet assay. Neoplasma 2003; **50**: 291–5.

38. **Ferguson PJ, Kurowska, E, Freeman DJ, Chambers AF, Koropatnick DJ.** A flavonoid fraction from granberry extract inhibits proliferation of human tumor cell lines. J Nutr 2004; **134**: 1529–35.

39. **Duh PD, Tu YY, Yen GC.** Antioxidant activity of water extracts of Harng Jyur (C*hrisanthemun morifolium* Ramat). Lebensm Wiss Technol 1999; **32**: 269–77.

40. **López-Lázaro M.** Flavonoids as anticancer agents: structure-activity relationship study. Curr Med Chem 2002; **2**: 691–714.

41. **Horvathova K, Novotny L, Tothova D, Vachalkova A.** Determination of free radical scavenging activity of quercetin, rutin, luteolin and apigenin in H2O2-treated human ML cells K562. Neoplasma 2004; **51**: 395–9.

ПРОТИВООПУХОЛЕВАЯ АКТИВНОСТЬ ЭКСТРАКТА ПОЛИФЕНОЛОВ ИЗ *ICHNOCARPUS FRUTESCENS*

Цель: **исследовать противоопухолевую активность экстракта полифенолов (PPE) из листьев *Ichnocarpus frutescens* на модели *in vivo*. *Материалы и методы:* для анализа противопухолевой активности PPE *in vivo* использовали модель асцитной карциномы Эрлиха мыши. Цитотоксичность PPE *in vitro* изучали на клетках моноцитоидной лейкемии линии U-937 и эритроидной лейкемии линии K-562. Также была проанализирована способность PPE к элиминации свободных радикалов. Острую токсичность препарата проверяли классическим методом. Общее содержание фенолов определяли методом Фолина — Чикалто. *Результаты:* результаты исследования *in vivo* продемонстрировали значительное уменьшение объема опухоли, количества живых опухолевых клеток и значительное увеличение продолжительности жизни у животных, получавших лечение PPE: продолжительность жизни в группах, получавших 50 и 100 мг PPE/кг, повысилась на 53,41% и 73,95% соответственно. PPE в концентрациях 5, 10 и 20 μг/мл ингибировал *in vitro* пролиферацию клеток линий U-937 и K-562. PPE проявил высокую активность к элиминации свободных радикалов с величиной IC_{50} 167,46 μг/мл и 158,52 μг/мл по отношению к супероксид-аниону и оксиду азота. *Выводы:* PPE из *Ichnocarpus frutescens* обладает высокой противоопухолевой активностью *in vitro* и *in vivo*.**

Ключевые слова: ***Ichnocarpus frutescens*****, экстракт полифенолов, противоопухолевая активность, асцитная карцинома Эрлиха, клетки лейкемии, элиминация свободных радикалов.**