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In vitro and *In vivo* inhibitory effects of *Piper longum* fruit extracts on mouse Ehrlich ascites carcinoma

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ABSTRACT - In order to screen the In vitro and In vivo inhibitory effects of *Piper longum* fruit extracts on mouse Ehrlich ascites carcinoma., Crude extracts of Piper longum fruits, (petroleum ether (60°-80°), chloroform, ethanol 95%, and water extract) were tested for short term in vitro cytotoxic activity using Ehrlich ascites carcinoma cells, and In vivo cytotoxicity using the Swiss albino mice intraperitoneally inoculated with Ehrlich ascites carcinoma cells. Pet ether and chloroform extracts have shown the significant cytotoxic action on the EAC cell lines. Petroleum ether extract increased the life span of tumor bearing animals by 44.44%. Chloroform extract at a dose of 200 mg/kg body weight increased the life span of tumor bearing animals by 33.33%. Combination therapy of both the extracts increased the life span by 87.5%. These results clearly indicate the further detailed work on these extracts can provide us with an effective non-toxic anti tumor agents.

KEY WORDS - *Piper longum*, short-term *in vitro* cytotoxicity, Ehrlich ascites carcinoma.

INTRODUCTION

Piper longum Linn. is an important medicinal plant used traditionally in Indian medicine. Fruits of *Piper longum* are known as "Pippali" in Sanskrit. Fruits are used as Carminative, liver tonic, abortifacient and used in the treatment of joint pains. Decoction of the fruit is used extensively in acute and chronic bronchitis (1). *Piper longum* is one of the components of formulations used for the treatment of gonorrhea, menstrual pain, tuberculosis, respiratory tract infections and arthritic conditions (2). Alcoholic extract of *Piper longum* fruits has shown the promising immunomodulatory and antitumor activity (3), however no antitumor activity was reported on other extracts of piper longum fruits, hence it was decided to investigate the antitumor activities of petroleum ether and chloroform extracts of *Piper longum* fruits.

MATERIALS AND METHODS

Authenticated dried fruits of *P. longum* were obtained form M/s Indian Herb, herbal manufacturing unit, Bangalore. Ehrlich ascites carcinoma cells were initially procured form Department of Radiobiology, Kasturba Medical College, Manipal. It was propagated by serial transplantation into the intraperitonial cavity of female Swiss albino mice. Ascites fluid from the intraperitonial cavity of the donor animals was aseptically aspirated using hypodermic syringe7-8 days after tumor inoculation. The aspirate was diluted in Dulbeco's modified Eagle's medium to get a concentration of 5×10^6 cells/ml and 0.2 ml of this tumor cell suspension (1×10^6 cells) was injected in the intraperitonial cavity of mice (3).

Extraction

Fruits were course powdered and extracted with Petroleum ether (60°-80°C) Chloroform, Ethanol 95%, successively in Soxhlet apparatus and water extract was prepared by macerating the powder for 7 days in chloroform water. Solvents were distilled off under vacuum.

In Vitro Cytotoxic Studies. All the animal experiments were performed as per the OECD guidelines. The animals were maintained under standard conditions with food and water *ad libitum*.

100 mg of Petroleum ether and Chloroform extract were dissolved in 10 ml of dimethyl sulfoxide, where as alcohol extract was dissolved in distilled water. This stock solutions were diluted with phosphate buffered saline (pH 7.2), (small amount of DMSO does not have toxic effect on Ehrlich ascites carcinoma) (4).

The short-term *in vitro* cytotoxic studies were carried out using Ehrlich ascites carcinoma cells (5). Cells were aspirated form the intraperitonial cavity of Swiss albino mice and washed three times with normal saline, and cell count was done using neubeur's counter. Suspension containing approximately 1 x 10^6

cells was taken in each tube containing different concentration of extracts and the volume was made up to 1 ml with phosphate buffered saline. All the tubes were incubated at 37° for 3 h. The percentage of dead cells was determined by using trypan blue exclusion method (6).

In Vivo Studies

Petroleum ether and Chloroform extracts were prepared for i.p. injection according to NCI (National Cancer Institute) protocol (7). Petroleum ether and Chloroform extracts were prepared for i.p injection by resuspending them in 0.1% tween 80. Experimental animals were prepared by injecting $1x10^6$ cells of EAC into the intraperitonial cavity of female Swiss albino mice. Treatment was started 24 h. after the tumor transplantation.

Petroleum ether extract was administered at doses 50, 100 and 200 mg/kg body weight, i.p once daily for 10 days Chloroform extract was administered at the dose of 200 mg/kg body wt., i.p once daily for 10 days. Petroleum ether extract at a dose of 50 mg/kg body wt, was administered i.p. 1, 5 and 9^{th} day and Chloroform extract on remaining days for 10 days at a dose of 200 mg/kg body wt. Doses were selected based on the toxicity studies. All the control groups received 0.1% tween 80 in normal saline (Since all the test materials were prepared using 0.1% tween 80 as stated above). All the mice were weighed on the day of tumor inoculation and every day before treatment.

The tumor response was assessed on the basis of median survival time (MST) and % increase in life span (% ILS) . Activity was assessed on the basis of % T/C ('T' and 'C' represent the number of days that treated and control animals survived, respectively) (reproducible % T/C $\geq$ 125 confirms the activity against tumor.). Statistical analysis was done by student "t" test.

Toxicity Studies

Three groups of 6 Swiss albino mice each received 0.1% Tween 80 in normal saline, 30 mg/kg-body weight of Petroleum ether extract, and 200 mg/kg body weight of Chloroform extract everyday for 10 day. The hematological measurements were done on day 0, 2, 4, 6 8 and 10 by obtaining peripheral blood from retro-orbit puncture, using neubeur's chamber.

Hematological studies after treatment with pet ether extract and chloroform extracts to the normal animals were performed. Three groups of Swiss albino mice each comprising 6 animals were given with 0.1% Tween 80 (control group), 30 mg/kg body weight of pet ether extract and 200 mg / kg body weight of Chloroform extract. Peripheral blood (0.5 ml) was collected from all the three groups on day 0, 2, 4, 6, 8 and 10^{th} post treatment days. Total WBC count and differential count was determined at histopathology department at Institute of Animal Health and Veterinary Biologicals, Bangalore.

Statistical analysis

Results were expressed as the mean±standerd deviation. Statistical evaluation was done by Student's *t*-test and the difference was considered statistically significant at $P<0.001$.

Results

Short-term *in vitro* cytotoxicity activities of the extracts are given in table 1. Both Petroleum ether and Chloroform extracts have shown the significant cytotoxic action on the EAC cell lines. Whereas alcoholic extract did not show marked cytotoxic action. The same result has been proved in the animal studies also. Petroleum ether extract at a dose of 50 mg/kg body weight the life span of tumor bearing animals of 44.44% whereas the higher doses has shown toxic effects. Whereas chloroform extract at a dose of 200 mg/kg body weight increased the life span of tumor bearing animals of 33.33%.Interestingly, combination therapy of both the extracts has shown the synergistic effect by increasing the life span by 87.5%, but where as the increase in body weight was not significantly controlled. n the important observation the effective doses of both the extracts did not show any significant changes in total WBC count, and differential count.

Discussion

Among currently available drugs, synthetic drugs do have potential adverse reactions and which can be minimized to greater extent through natural compounds. (As supported by Vinca alkaloids and taxol), Still there are many natural drugs which are yet to be explored scientifically. Fruits of *P. longum* were used successfully in the treatment of asthma and bronchitis. Still many uses have been indicated in Ayurvedic literature. Treatment of tumors is one of them. All the parameters of our studies showed that both pet ether extract and chloroform extract at a doses of 50 mg/kg body weight and 200 mg/kg body weight respectively has got significant effect on growth of tumor ascites both *in vitro* and *in vivo*.

Both the extracts in the effective doses have significantly increased the life span of tumor bearing animals.

Table 1: Short term in vitro cytotoxic effect of Piper longum fruit extracts on Ehrlich ascites carcinoma cells.

Extracts	% Death*		
	10 ppm	100 ppm	1000 ppm
Petroleum ether	22.17	70.16	100.00
Chloroform	19.20	31.91	100.00
Alcohol	9.02	18.47	23.75

**Approx .1 x 10^6 EAC cells were incubated with the extract in 1ml PBS at 37°C for 3 hrs.*

Table 2: Effect of Piper longum fruit extracts on Ehrlich ascites carcinoma bearing animals.

Treatment	Dose (mg/kg body wt)	MST [a] (days)	% ILS[b]
Control	-	9±0.8	-
PPE	50	13±2.3	44.44
	100	11±1.9	22.22
Control	-	12±1.2	-
PCE	100	12±2.8	0.0
	200	16±3.1	33.33
Control	-	8±1.4	-
PPE + PCE	50 + 200	15±2.5	87.5

**Approx. 1 x 10^6 cells of EAC inoculated into the intraperitonial cavity of female Swiss albino mice. Average of 6 animals ± SEM, P<0.001. (Student "t" test), [a] Median survival time, [b] percentage increase in life span.*

Table 3: Effect of Piper longum fruit extracts on peripheral WBC count.

Days	Total WBC (cells / mm^3)*		
	Control	PPE (30mg/kg)	PCE (200mg/kg)
0	8400±0.24	8400±0.32	8500±0.28
2	8500±0.35	8000±0.30	8200±0.30
4	8500±0.23	8500±0.34	6500±0.32
6	8400±0.24	8700±0.32	6800±0.28
8	8400±0.30	8500±0.28	8400±0.32
10	8500±0.24	8600±0.30	8200±0.34

** Counts are average of 6 animals ± SEM, P<0.001. (Student "t" test)*

Table 4: Effect of P.longum fruit extracts on differential count of Swiss albino mice.

Days	% Of cells *					
	Control		PPE (30mg/kg)		PCE (200mg/kg)	
	L	N	L	N	L	N
0	69±0.35	31±0.45	69±0.42	30±0.38	70±0.58	30±0.23
2	70±0.56	30±0.56	65±0.75	34±0.55	69±0.45	30±0.45
4	70±0.23	30±0.26	70±0.46	30±0.26	70±0.64	29±0.75
6	68±0.47	31±0.45	75±0.25	24±0.35	71±0.68	29±0.56
8	69±0.89	30±0.69	73±0.69	27±0.24	70±0.75	29±0.45
10	69±0.56	31±0.58	72±0.45	28±0.48	69±0.63	31±0.35

** Counts are average of 6 animals ± SEM, P<0.001. (Student "t" test)*
L – Lymphocytes ; N – Neutrophils.

Combination of these two extracts has shown a synergistic effect by increasing the life span of animals by 87.5%. Moreover the hematological studies showed that the effective doses of these extracts have no effect on the normal blood cells.The antitumor activity observed against EAC cells may be due to its cytotoxic effect as observed in short term in vitro cytotoxicity studies. Previous studies have shown that many of the steroidal molecules have got the antitumor properties (9, 10), especially β sitostirol (11, 12). Some of the Triterpinoidal compounds from plant sources have shown the inhibitory action on cancer cell lines (13, 14). The phytochemical investigation of Piper longum fruit extracts shown the presence of steroids, β-sitostirol and triterpinoids in petroleum ether and chloroform extracts. Hence the observed antitumor property of Piper longum fruit extracts may be due to the presence of steroidal and triterpinoidal molecules in the extracts. These results clearly indicate the further detailed work on these extracts can provide us with an effective non-toxic anti tumor agents.

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