Research Article

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ANTI CARCINOGENIC ACTIVITY OF PARTIALLY PURIFIED BETULINIC ACID ON EAC INDUCED MICE

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ABSTRACT

Diospyros melanoxylon has the wonder compound betulinic acid, a pentacyclic triterpenic acid. Betulinic acid has been found to selectively kill human melanoma cells while leaving healthy cells alive. *In vitro* study of PPBA (partially purified betulinic acid) is cytotoxic towards the tumour cells in 3h assay (Tryphan blue dye exclusion method). *In vivo* antitumor studies revealed that PPBA significantly increase the life span of Ehrlich ascites carcinoma (EAC) bearing mice dose dependently. Moreover, it significantly reduced the tumor development in mice. The tumor reduction was high in animals treated simultaneously with PPBA thereby showing decrease in body weight. The decrease in cancer cell number observed in the PPBA (100 and 200 mg/kg) treated mice indicates that the test drug is having significant inhibitory effect on the tumor cell proliferation. An abnormal enlargement of peritoneal cavity was observed in tumor-induced mice. Treatment with PPBA reduced the tumor weight and hence increased the life span. From the hematological studies it was observed that there was significant rise in WBC in cancer control. As the progression of cancer was brought under control by PPBA, the WBC count got reduced and there was a significant increase in the levels of RBC and Hb in treated mice. The packed cell volume percentage also reduced to normal levels. It is clearly evident that the characteristic features of PPBA are remarkable in the study of carcinogenicity.

Keywords: Betulinic acid, EAC, cytotoxic.

INTRODUCTION:

Natural products are excellent source of lead compounds in the search of medicaments for treatment of various diseases. Presently huge amounts of sources of such materials lie in tropical and subtropical regions of the world. They offer a rich and relatively untrapped source for the discovery of new drugs from the natural products. India, a tropical Asian country, also has a long history of traditional medicine systems.

Betulinic acid is a naturally occurring pentacyclic triterpenoids and has been shown to exhibit a variety of biological activities including inhibition of human immunodeficiency virus (HIV), antibacterial, antimalarial, anti-inflammatory, anthelmintic, antioxidant and most effectively anticancer activities (Yogeeswari and Sriram, 2005). The growth inhibitory effect of betulinic acid was attributed to a prominent induction of apoptosis in tumor cells independent of death receptor ligation and wild-type p53 via a mitochondrial pathway that could be inhibited by overexpression of Bcl-2 and Bcl-x_L.

In the present study the activity against EAC is evaluated by Isolation of partially purified fraction of betulinic acid (PPBA) from *Diospyros melanoxylon* Roxb. (Ebenaceae). Evaluation of In *vitro* cytotoxicity and *in vivo* antitumor activity of PPBA against Ehrlich's Ascites Carcinoma (EAC) cell line induced mice.

MATERIALS AND METHODS:

Collection and authentication

The bark of *Diospyros melanoxylon* Roxb have been collected from Srinivasamangapuram near Tirupathi, Andhra Pradhesh, during the month of May 2007 and dried under shade. The plant was identified and authentified by Dr. N. Nagaraju, Reader in Botany, S. G. S Arts and Science College, Tirupathi. The voucher specimen is available in the herbarium file of our department.

Drugs and chemicals

Thiobarbituric acid, trichloro acetic acid, butylated hydroxyl toluene, oxidized glutathione; epinephrine and DTNB were

obtained from Sisco Research Laboratories Pvt., Ltd., Mumbai. 2 - 2'dipyridyl and *O*-dianisidine were obtained from Himedia Laboratories Ltd., Mumbai. All other drugs and chemicals used in the study were obtained commercially and were of analytical grade.

Instruments

REMI Motors Centrifuge, Eppendorf Minispin, Jasco V – 530 UV/VIS spectrophotometer, Elco L1127, pH meter, INCO Homogenizer, Jasco FT/IR 410, Shimadzu class LC – 10 A HPLC system.

Source of cell lines

Ehrlich Ascites Carcinoma (EAC) was obtained with courtesy from Amala Cancer Research Institute, Thrissur, Kerala.

Experimental animals

Male *Swiss* albino mice of either sex weighing between 25-30 g were used for the study. The animals were housed in polypropylene cages inside a well ventilated room. The room temperature was maintained at 23 ± 2°C with a 12 h light/dark cycle. The animals were fed with commercial food pellets and provided with drinking water *ad libitum*. All animal procedures have been approved by Ethical Committee in accordance with animal experimentation and care (CPCSEA).

Isolation of betulinic acid

Bark of the plant was dried under shade at room temperature, powdered and sieved through No. 22 mesh sieve. About 1.5 kg of the bark powder was soaked in toluene and the mixture was stirred approximately at 90-95°C for 12 h. The semi concentrated extracts chilled between 0-10°C for 16-24 h and the insoluble material found to contain betulinic acid was then separated by centrifugation. After centrifugation the remaining mother liquor was discarded. The solid so obtained was washed with toluene and it was dissolved in hot methanol and refluxed with activated charcoal and filtered. The crude betulinic acid subjected to solvent partition. The solvents used were n-hexane: ethyl acetate: methanol: water in 10:5:2.5:1 ratio. The methanolic portion was collected and dried to obtain partially purified betulinic acid (PPBA) which was found to be 220 mg. The PPBA was characterized using TLC, IR, NMR and HPLC (Enwerem *et al*., 2001; Pinzaru *et al*., 2002; Zhao *et al*., 2007).

Maintanance of cell culture

Ehrlich Ascites Carcinoma (EAC) cell line was propagated in the peritoneal cavity of mice. After propagation, the ascitic fluid (1 ml) was withdrawn using 18 guage needle in a sterile syringe (on 10^{th} day after induction). The tumor cells were washed with (0.9%) normal saline, centrifuged and the supernatant was discarded. Washing was done thrice and the deposited cells were resuspended in sterile normal saline.

***In vitro* cytotoxicity**

Tryphan blue dye exclusion method (Shylesh and Paddiakala, 2000)

Aspirate tumour cells from the peritoneal cavity of mice and add to test tube containing PBS and wash the cells with PBS and centrifuge 3 times. Cells were then suspended in 1ml 'PBS' (0.1ml + 0.9 ml) and adjust the cell number to 10 million i.e. 10×10^6 cells/ml. Check the cell viability using tryphan blue stain (0.1 ml cell sample + 0.8 ml PBS + 0.1 ml tryphan blue (1%). Count the cells in haemocytometer. The cell count should be 100 in the four large sized quadrants. If the cell count is below 100 add extra cells make it 100 or above and 100 dilute with PBS respectively . If the cell count is 100: No. of cells in the diluted sample is 1 million (1×10^6)/ml. No. of cells in the stock is 10 million (1×10^5)/ml. Add (0.1 ml) different concentrations of PPBA (10, 20, 50, 100 and 200 µg/ml) with 1×10^6 (0.1ml) tumour cells. Make up the volume of mixture to 1.0 ml using PBS and incubate at 37°C for 3h. After incubation, add 0.1 ml tryphan blue and determine the number of dead cells using haemocytometer.

$$\text{Percentage cytotoxicity} = \frac{\text{No.of dead cells}}{\text{No. of viable cells + No.of dead cells}} \times 100$$

***In vivo* anticancer activity (Christina *et al*., 2004; Babu *et al*., 1995).**

The tumor cell count was done using tryphan blue dye exclusion method in a Haemocytometer. The cell suspension was diluted to get 10^6 cells/ml. Cancer was induced by *i.p* inoculation of 10^6 cells/mouse. The body

weight of mice were noted on the day of tumor inoculation and then on alternate days up to the 10th day. The mice weighing 18-25 g were divided into 5 groups of twelve animals in each, 6 animals from each group was retained for survival period and the other (6) for drug treatment

Group I – served as normal control which received 10 ml/ kg normal saline and was not induced with cancer cell line.

Group II – served as cancer control which received 10 ml/ kg normal saline

Group III – received Standard drug, 5-flurouracil, 20 mg/kg.

Group IV – received 100 mg/kg partially purified betulinic acid (PPBA).

Group V – received 200 mg/kg PPBA.

Cancer (EAC cell line) was induced intraperitorneally to mice in Groups II-V.

The drug treatment was started after 24 h of induction of cancer and treatment was continued upto 10 days. On day 11 ascitic fluid was withdrawn and suspended in PBS and adjusted to $1x10^6$ cells/ ml and the following parameters were observed:

1. Cancer cell count
2. Packed cell volume (PCV)
3. Body weight
4. Increase in life span.

Cancer cell count - Ascitic fluid (0.1ml) was withdrawn from the peritoneal cavity of each mouse using sterile syringe and cell suspension was diluted with 0.8 ml of PBS corresponding approximately to $1x10^6$ cells/ml. To this add 0.1 ml of tryphan blue (0.1 mg/kg) and preserved in an icebath for 10 min. The total number of the living cells were counted using haemocytometer under high power objective lens in compound microscope (Babu et al., 2002).

Packed cell volume (Christina *et al*., 2004)

Sacrifice the animal, using light ether anesthesia and open peritoneal cavity and transfer tumor cells into a clean beaker. Transfer 1 ml of fluid into the Wintrobe tube and centrifuge at 3000 rpm for 30 min. The packed cell volume (PCV) was calculated using the formula:

$$\% \text{ Tumor inhibition} = \frac{\text{Test PCV}}{\text{Control PCV}}$$

Body weight (Christina *et al*., 2004)

The body weights of each mice were estimated on each alternate day's upto 10th day of treatment and the change in the body weight was noted.

Increase in life span (Latha and Pannikar, 1998)

The percentage (%) increase in life span of control and treated groups were calculated using the formula

$\% \text{ ILS} = (T - C)/C \times 100$

Where T and C are mean survival of treated and control mice respectively

Determination of hematological parameters

The effect of PPBA at 100 and 200 mg/kg b.w on hematological parameters was studied. Blood was collected from the mice of all the groups by retro-orbital plexus method and WBC, RBC, Hb counts estimated.

RESULTS AND DISCUSSION:

In vitro cytotoxicty of PPBA was tested using EAC cell line. PPBA at concentration of 10 µg/ml exhibited 1% inhibition of activity and at dose of 20 µg/ml 2.3% inhibitions was noticied, while at concentration of 50 and 100, 8.6 and 27.3% inhibition was observed. The highest % inhibition was observed at 200 µg/ml and was found to be 98.3%. The % inhibition increased gradually upon increasing concentration in a dose dependent manner. The mean CTC_{50} value was found to be 188 µg/ml for PPBA. There was a significant ($P<0.01$) increase in the body weight of cancer control mice. Animals treated simultaneously with the partially purified betulinic acid (PPBA) showed a decrease in body weight when compared to the cancer control.

Animals induced with cancer showed a significant ($P<0.01$) increase in the levels of cancer cell count and packed cell volume (PCV). However in animals treated with PPBA there was decrease in cancer cell count and PCV of the peritoneal fluid, which are shown to be significant ($P<0.01$). Similarly animals induced with cancer exhibited a significant ($P<0.01$) increase in the levels of WBC and decrease in the levels of RBC and Hb counts respectively. However in animals treated with PPBA there was decrease in WBC and increase

in RBC and Hb counts and the results were found to be significant (P<0.01). After cancer induction the cancerous mice and the treated mice were kept for the survival period to check the increase in life span and there was an increase in life span of the treated group when compared to that of cancer control (P<0.01).

Table 1: *In vitro* cytotoxicity of PPBA on EAC cell line

Drug	Concentration (µg/ml)	% inhibition	Mean CTC_{50} (µg/ml)
PPBA	10	1 ± 0.1	188
	20	2.3 ± 0.3	
	50	8.6 ± 0.3	
	100	27.3 ± 0.3	
	200	98.3 ± 0.3	

Values are mean ± S.E.M, n = 3.

PPBA was found to inhibit 50% proliferation of EAC cells in short term Assay at a concentration of 188µg/ml.

Table 2: Effect of PPBA on body eight, cancer cell count, packed cell volume, mean survival period and hematological parameters.

Groups	Treatment	Body weight (g)		Cancer cell count($\times10^6$)	Packed cell volume (%)	Total WBC($\times10^3$)	Total RBC ($\times10^5$)	Hb count (g/ml)	Mean Survival time (days)
		Pre treatment	Post treatment						
I	Control (10 ml/kg saline)	18.6 ± 0.1	19.2 ± 0.4	-	-	4.88 ± 0.62	5.53 ± 0.02	13.56±0.15	>29
II	Cancer control (10 mg/kg saline)	20.2 ±0.4	28.6 ±0.2	1.43 ± 0.02	50.20 ±0.02	7.36 ± 0.03	3.28 ± 0.03	7.3±0.21	16 ± 1.02
III	PPBA (100 mg/kg)	19.8 ±0.6	23.1 ± 0.1	0.79 ± 1.26	25.66 ± 0.21	5.03 ± 0.03	4.46 ± 0.02	12.2±0.10	27 ± 1.24
IV	PPBA (200 mg/kg)	20.6 ± 0.2	21.6 ± 0.3	0.86 ±0.22	32.26 ± 0.15	5.85 ± 0.02	4.93 ± 0.03	9.41±0.2	23 ± 1.12
V	5-FU (20 mg/kg)	19.5 ± 0.5	20.1 ± 0.3	0.89 ±0.18	30.16 ± 0.20	5.25 ± 0.02	5.13 ± 0.02	10.58±0.27	27 ± 1.31

Values are mean ± S.E.M, n = 6 animals in each group. [a]P < 0.01, [b]P < 0.05. Group II was compared with group I. Group III, IV and V were compared with group II.

CONCULSION:

All these results suggest that PPBA has an anticancer effect on EAC induced mice both *in vitro* and *in vivo.*

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