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Original Article

CYTOTOXIC ACTIVITY OF *CINNAMOMUM TAMALA LINN.* AGAINST EHRLICH ASCITES CARCINOMA (EAC) IN MICE

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ABSTRACT

This study was performed to explore the anticancer activity of acetone and ethanol extracts from the leaves of *Cinnamomum tamala* Linn. against Ehrlich Ascites Carcinoma (EAC) in mice. The activity was assessed using survival time, peritoneal cell count, hematological studies, solid tumor mass, histapathalogical studies and *in vitro* cytotoxicity. Results found that oral administration of both extracts increased the survival time and normal peritoneal cell count. Hematological parameters including protein and PCV, which were altered by tumor inoculation, were restored. Solid tumor volume was also significantly reduced. Both extracts exhibited significant cytotoxity activity at 200 µg/ml. The higher cytotoxic activity was found in ethanol extract of *Cinnamomum tamala*.

Keywords: Cytotoxic Activity, *Cinnamomum tamala,* Ehrlich Ascites Carcinoma (EAC)

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1.0 INTRODUCTION

It is well established that plants have been a useful source of clinically relevant antitumor 69 compounds. [1] Indeed there have been worldwide efforts to discover new anticancer agents from plants. There are different approaches for the selection of plants that may contain new biologically active compounds. [2] One of the approaches used is the ethnomedical data approach, in which the selection of a plant is based on the prior information on the folk medicinal use of the plant. It is generally known that ethnomedical data provides substantially increased chance of finding active plants relative to random approach. [3] However, as for cancer, the disease is complicated and heterogeneous, which makes it difficult to be well diagnosed, especially by traditional healers. The ethnomedical information obtained for a plant extract that is used to treat cancer might therefore not be reliable. [1] Traditional Indian and Chinese medicinal herbs have been used in the treatment of different diseases in the country for centuries. There have been claims that some traditional healers can successfully treat cancer using herbal drugs. Indeed, some traditional healers who were interviewed recently in the country stressed that they have successfully treated patients presented with cancer or cancer related diseases.

Cinnamomum tamala commonly known as Tejpata belongimg to family Lauraceae. It is a moderate sized evergreen tree attaining a height of 8 m, and a girth of 150 cm. Its bark produces mucilage. Leaves lanceolate, glabrous; alternately placed, opposite and short stalked. 3-nerved from the base.[4,5] *Cinnamomum tamala* is found in tropical and sub-tropical Himalayas, Khasi and Jaintia hills and in eastern Bengal, India.

In the essential oil from the leaves, mostly monoterpenoides were found. Linalool (50%) is the major compound, whereas a-pinene, p-cymene, ß-pinene and limonene range around 5 to 10% each. Phenylpropanoids appear only in traces. Latest reports founds 1% cinnamic aldehyde. [6]

The leaves are used extensively in northern India as a spice - Tejpat. In Kashmir they are used as a substitute for paan (betel leaves). The essential oil is used in perfuming soap and in medicine. Leaves of *C. tamala* are used in colic and diarrhoeal preparations. *C. tamala* leaf extracts produce a hypoglycaemic effect in experimental rats. [7] Plant parts are used in many Ayurvedic preparations e.g. sudarshan choorna and chander prabhavati and other products. It is useful in the treatment of circulation, muscles and joints complications and relieves arthritis, inflamed joints, muscular pains, rheumatism and sprains. Benefits the digestive system and helps improve

appetite and control infections. [8] The present studies is to investigate the cytotoxic activity of acetone and ethanol extracts of leaves of *Cinnamomum tamala.*

2.0 METHOD AND MATERIALS

Collection and Authentification of the Plant

The leaves of *Cinnamoamum tamala* were collected from Garden of National Botanical Research Institute, Lucknow, India in month of July 2009. The plant material was authenticated by Dr. Sayeeda Khatoon, chemotaxonomist and the voucher specimens (NAB 180023) were deposited in the departmental herbarium for future reference.

2.1 Preparation of extracts of Cinnamoamum tamala

The powdered plant material (1000g) were sequentially extracted using petroleum ether, chloroform, acetone, ethanol and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation. [9]

2.2 Preliminary phytochemical screening

Extracts of *Cinnamoamum tamala* was subjected to qualitative tests for the identification of various active constituents viz. carbohydrate, glycoside, alkaloid, amino acids, flavanoids, fixed oil, tannins, gum and mucilage, phytosterols etc. The phytoconstituents were identified by chemical tests, which showed the presence of various constituents in the different extracts. [9]

2.3 Cytotoxic activity

2.3.1 Animals

Adult Swiss male albino mice (20-25 g) were procured from Institute of Animal Health and Vetarnary Biological, Mhow, Indore, MP and used throughout the study. They were housed in microlon boxes in a controlled environment (temperature 25 ± 2^0C and 12 h dark/ light cycle) with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining Institutional animal ethical committee clearance.

2.3.2 Cells

EAC cells were obtained through the courtesy of Amala Cancer Research Center, Thrissur. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse. [10]

2.4 Effect of extracts on survival time [11]

Animals were inoculated with 1 X 10^6 cells/mouse on day '0' and treatment with both extracts started 24 h after inoculation, at a dose of 500 and 625 mg/kg/day, p.o. for ethanol and acetone extract respectively. The dose was selected by OECD 423 guidelines, which showed no toxicity up to 6.3 g/kg (p.o.) for acetone and 5 g/kg (p.o.) for ethanol extracts. The control group was treated with the same volume of 0.9%

sodium chloride solution. All the treatments were given for nine days. The median survival time (MST) of each group, consisting of six mice was noted. The antitumor efficacy of acetone and ethanol extracts from the leaves of *Cinnamoamum tamala* was compared with that of 5- fluorouracil (Dabur Pharmaceutical Ltd, India; 5-FU, 20 mg/ kg/day, i.p. for 9 days). The MST of the treated groups was compared with that of the control group using the following calculation:

Increase in lifespan = ((T - C)/C) X 100

Where T = number of days the treated animals survived

C = number of days control animals survived.

2.5 Effect of extracts on normal peritoneal cells [11]

To evaluate whether acetone and ethanolic extracts treatment indirectly inhibited tumour cell growth, the effect was determined on the peritoneal exudate cells of normal mice. Five groups of normal mice (n= 5) were used for the study. First two groups were treated with 500 and 625 mg/kg/day, p.o. for ethanol and acetone extracts respectively only once for a single day and other two groups received the same treatment for two consecutive days. The untreated group was used as control. Peritoneal exudate cells were collected after 24 h treatment by repeated intraperitoneal wash with normal saline and counted in each of the treated groups and compared with those of the untreated group.

2.6 Effect of extracts on hematological parameters [11]

In order to determine the influence of extracts on the haematological status of EAC-bearing mice, a comparison was made among four groups (n= 5) of mice on the 14th day after inoculation. The groups comprised of (1) tumor bearing mice, (2) tumor bearing mice treated with acetone extract (625 mg/kg/day, p.o. for the first 9 days), (3) tumor bearing mice treated with ethanol extract (500 mg/kg/day, p.o. for the first 9 days), and (4) control mice (normal). Blood was drawn from each mouse by the retro orbital plexus method and the white blood cell count (WBC), red blood cell count (RBC), hemoglobin, protein and packed cell volume (PCV) were determined. [12]

2.7 Effect of extracts on solid tumor [13]

Mice were divided into three groups (n=6). Tumor cells (1 X 10^6 cells/mice) were injected into the right hind limb (thigh) of all the animals intramuscularly. The mice of Group I were tumor control. Group II received acetone extract (625 mg/kg), Group III received ethanol extract (500 mg/kg) orally for 5 alternate days. Tumor mass was measured from the 15th day of tumor induction. The measurement was carried out

every 5th day for a period of 30 days. The volume of tumor mass was calculated using the formula

$$V = 4/3\pi r^2$$

where r is the mean of r_1 and r_2 which are two independent radii of the tumor mass. [14]

2.8 In Vitro Cytotoxicity

Short-term Cytotoxicity was assessed by incubating 1 X 10^6 EAC cells in 1 ml phosphate buffer saline with varying concentrations of the extracts at 37^0C for 3 hrs in CO_2 atmosphere ensured using a McIntosh field jar. The viability of the cells was determined by the trypan blue exclusion method. [15,16]

2.9 Histopathological studies [17]

A portion of liver and kidney of animals in all groups were stored in container for 12 hours in 10% formalin solution and subjected to histopathological studies

2.10 Statistical analysis

All values were expressed as mean ± SEM. The data were statistically analyzed by one-way ANOVA followed by Dunnett's test, the data of haematological parameters were analyzed using ANOVA followed by Tukey multiple comparison test and data of solid tumour were analyzed using Student's' t ' test. P values <0.05 were considered significant.

3.0 RESULTS AND DISCUSSION

The effect of acetone and ethanol extracts on the survival of tumour-bearing mice is shown in Table 1. The MST for the tumour control group was 20.83 ± 0.47 days, whereas it was 35.16 ± 0.30 days for acetone (625 mg/kg/day p.o) and 36.5 ± 0.42 days for ethanol (500mg/kg/day p.o) extracts treated groups. The MST for acetone extract treated group was increased to 40.75% and of ethanolic treated group increased to 42.93%.

Table No 1. Effect of ethanol and acetone extract treatment on the survival of tumour bearing mice

S No	Treatment	Mean Survival Time (Days)	Increase in life span (%)
1	Tumour Control	20.83±0.47	-
2	Ethanol extract (500 mg/kg, p.o)	36.5 ± 0.42*	42.93
3	Acetone extract (500 mg/kg, p.o)	35.16 ± 0.30*	40.75
4	5- FU (20mg/kg, i.p)	44.33 ± 0.49*	53.01

n=6 animals in each group,
*P<0.01 Vs control.
Days of treatment = 9,

Values are expressed as mean ± SEM

The average number of peritoneal exudate cells per normal mouse was found to be 5.8 ± 0.01 x 10^6. Single treatment of acetone extract (625mg/kg,p.o) enhanced the number to 7.21 ± 0.6 x 10^6 (P<0.001) and consecutive treatment for two days increase it to 10.21 ± 0.06 x10^6 (P<0.001). Similarly single treatment with ethanol extract (500mg/kg/day,i.p) enhanced the count to 9.38 ± 0.12 x 10^6 (P<0.001) and consecutive treatment for two days increased it to 14.31±0.19 x 10^6 (P<0.001).The results are shown in Table 2.

Table No 2. Effect of ethanol and acetone extract treatment on Peritoneal Cell Count in normal mice

Group	Treatment	Peritoneal cell count(×$10^{6)}$
I	Normal control	5.8 ± 0.01
II	Acetone (625mg/kg/day p.o) treated once	7.21 ± 0.07*
III	Acetone (625mg/kg/day p.o) treated twice	10.21±0.06*
IV	Ethanolic (500mg/kg/dayp.o) treated once	9.38±0.12[8]
V	Ethanolic(500mg/kg/day p.o) treated twice	14.31±0.19*

n=5 animals in each group
* is P< 0.001 Vs Normal control
Values were expressed as mean± SEM

The total WBC count, proteins and PCV were found to increase with a reduction in the haemoglobin content of RBC. The differential count of WBC showed that the percentage of neutrophils increased while that of lymphocytes decreased. At the same time interval, ethanol and acetone extracts (500 and 625 mg/kg/day, p.o.) treatment could change these altered parameters to near normal values.

There was reduction in the tumor volume of mice treated with ethanol and acetone extract (P<0.001) shown in table 4. Tumor volume of control animals (30^{th} day) was 11.66±0.33 ml, whereas for the extract-treated group it was 7.5±0.34 ml and 8.1±0.25 ml for ethanol and acetone extracts, respectively.

Table No. 4 Effect of ethanol and acetone extract treatment on solid tumour volume

Design of treatment	Solid tumour volume (ml)			
	15th days	20th day	25th day	30th day
Tumor control	7.50±0.22	8.0±0.25	9.0±0.25	11.66±0.33
5-fluorouracil(20mg/kg)	4.50±0.22*	4.66±0.21*	4.33±0.21*	3.66±0.21*
Acet. (625 mg/kg/day, p.o)	6.10±0.25*	6.66±0.21*	7.50±0.22*	8.1±0.25*
Etha.(500 mg/kg/day, p.o)	5.50±0.22*	6.33±0.21*	7.33±0.21*	7.5±0.34*

n=5 in each group.

* P<0.001 compared with tumor control

Values were expressed as mean±SEM,

The results of *In Vitro* Cytotoxic test were shown in Table 5 and 6. The ethanol extract shows remarkable cytotoxic activity against the tested cells, acetone extracts also showed cytotoxic activity against the tested cell line. At 200 µg/ml concentration, 98% (EAC) of activity was found for ethanol extract, whereas 84% (EAC) of activity for acetone extract.

Table No 5. *In-Vitro* Cytotoxicity activity of ethanol Extract

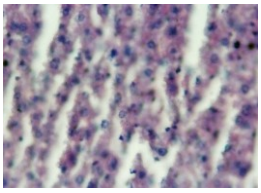
Sample	Cells 1×10^6	Concentration µg/ml	% Death
Ethanol Extract	Ehrlich ascitic carcinoma	200	98%
		100	94%
		50	89%
		20	29%
		10	7%
		control	1%

Table No. 6 *In-Vitro* Cytotoxicity activity of Acetone Extract

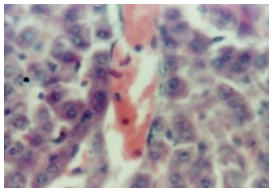
Sample	Cells 1×10^6	Concentration µg/ml	% Death
		200	84%
		100	71%
Acetone		50	64%
Extract	Ehrlich	20	21%
	ascitic	10	7%
	carcinoma	Control	4%

Microscopical examination of liver section of normal control group showed normal arrangement of hepatocytes, whereas the liver section of tumour control group showed various degrees of changes such as formation of steatoses, centrilobular fatty degeneration, cloudy swelling and necrosis of hepatic cells. The liver section of mice treated with ethanol extract (500 mg/kg, p.o) showed little dearrangement of hepatic cells, fatty degeneration. Remarkable improvement was noted in acetone extract (625 mg/kg, p.o) treated groups. It showed little damage to liver cells with centrilobular fatty degeneration and reduced degree of vascularisation. The result was shown in fig. no 1.

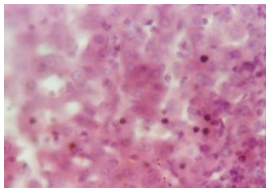
Figure No.1 Histopathological studies of liver



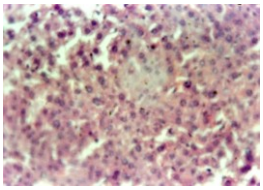
Normal liver



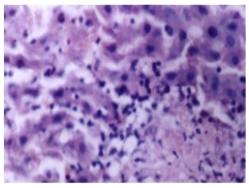
Tumour control



Tumour + 5-FU treatment



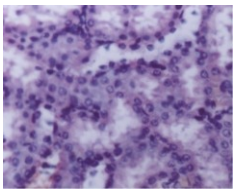
Tumour + Acetone extract



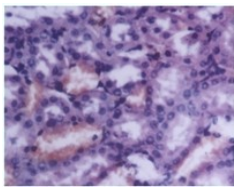
Tumour + Ethanolic extract

Microscopical examination of kidneys of normal mice showed normal morphological features of cells. The sections of tumour control group showed abnormal nuclei. The group treated with 5-FU (20mg/kg, i.p) showed almost normal kidney section. The groups treated with acetone (625 mg/kg, p.o) and ethanol (500mg/kg, p.o) extracts showed decreased damage to the kidneys as shown in fig no 2.

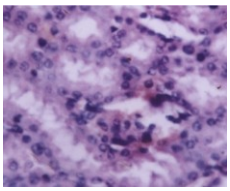
Figure No. 2 Histopathological studies of kidneys



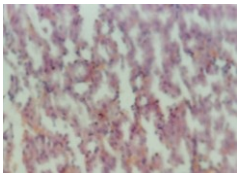
kidneyTumour control



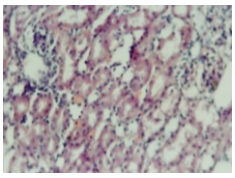
Tumour + 5-FU treatment



Normal



Tumour + Ethanol Extract Treatment



Tumour+ Acetone extract Treatment

CONCLUSION

The reliable criteria for judging the value of any anticancer drug are prolongation of lifespan and decrease of WBC from blood. [18,19] The results of the present study show an anticancer effect of ethanol and acetone extract against EAC in Swiss albino mice. A significant enhancement of MST and peritoneal cell count was observed.

The effect of ethanol and acetone extract treatment on the peritoneal exudate cells of normal mice is an indirect method of evaluating its inhibitory effect on tumor cell growth. Normally, a mouse contains about 5 X 10^6 peritoneal cells, 50% of which are macrophages. Ethanol and acetone extract treatment was found to enhance peritoneal cells count. These results demonstrate the indirect inhibitory effect of ethanol and acetone extract on EAC cells, which is probably mediated by the enhancement and activation of either macrophage or cytokine production.

The analysis of the hematological parameters showed minimum toxic effect in mice treated with ethanol and acetone extract. After 14 days of transplantation, ethanol and acetone extract was able to reverse the changes in the hematological parameters consequent to tumor inoculation. The present study reveals that the ethanol and acetone

extract was cytotoxic towards EAC. Preliminary phytochemical screening of ethanol and acetone extracts of *C. tamala* showed the presence of carbohydrate, alkaloid, amino acids, flavanoids, fixed oil, phytosterols and phenolic compound. Flavonoids have been shown to possess antimutagenic and antimalignant effects. [20,21] Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [22] and angiogenesis. [23] The cytotoxic and antitumor properties of the extract may be due to these compounds.

The present study points to the potential anticancer activity of ethanol and acetone extract. Further studies to characterize the active principles and elucidate the mechanism of the action of ethanol and acetone extract are in progress.

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