

Cytotoxic Activity of *Vitex negundo* against Ehrlich Ascites Carcinoma (EAC) in mice

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Abstract: Present study was performed to explore the cytotoxic activity of acetone and ethanol extracts from the leaves of *Vitex negundo* against Ehrlich Ascites Carcinoma (EAC) in mice. The activity was assessed using survival time, peritoneal cell count, hematological studies, solid tumor mass, histopathological 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. Tumor volume was also significantly reduced and both of extracts exhibited significant cytotoxicity activity at 200 µg/ml. The higher cytotoxic activity was found in ethanol extract of *Vitex negundo*.

Keywords: Cytotoxic Activity, *Vitex negundo*, Ehrlich Ascites Carcinoma (EAC).

Introduction

Plants have a great potential for producing new drugs for human benefit. Plants used in traditional medicine contain a vast array of substances that can be used to treat chronic and even infectious diseases. According to a report of World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs.¹

The demand for more and more drugs from plant sources is continuously increasing. It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments.

The increased interest in plant derived drugs is mainly because of the wide spread belief that 'herbal medicine' is safer than costly synthetic drugs which possesses side effects. Hence, there is need to screen medicinal plants for promising biological activity. Further, there is a continuous development of resistant strains which pose the need for search and development of new drug to cure diseases.²

Vitex negundo L. (Verbenaceae) commonly known as nirgundi chiefly occurring throughout India^{3,4} is widely distributed in Similipal Biosphere Reserve, Orissa. Though, almost all parts of *V. negundo* are used, the leaves and the barks are the most important in the field

of medicine.⁵ The decoction of leaves is considered as tonic, vermifuge and is given along with long pepper in catarrhal fever.⁵ Water extract of mature fresh leaves exhibited anti-inflammatory, analgesic and antihistamine properties.⁶

Leaves of this plant have been shown mosquito repellent effects⁷ as well as antiulcerogenic⁸, antiparasitic⁹, antimicrobial¹⁰ and hepatoprotective¹¹ potentials. The methanolic root extract possessed potent snake venom (*Viper russellii* and *Naja kaouthia*) neutralizing capacity.¹²

These works give little information on anticancer property of this plant. Hence, in the present experiment an attempt has been made to evaluate the cytotoxic activity of acetone and ethanol extracts against Ehrlich Ascites Carcinoma. Besides, phytochemical screening of the extracts were also carried out with view assess the presence of different phytochemicals in extracts.

Method and Materials

Collection and Authentication of the Plant

The leaves of *Vitex negundo* were collected from Sanjivini Botanical Garden, Bhopal, India in month of Nov 2009. The plant material was authenticated by Dr. Sayeeda Khatoon, chemotaxonomist and the voucher

specimens were deposited in the departmental herbarium for future reference.

Preparation of extracts of *Vitex negundo*

The powdered leaves (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.¹³

Preliminary phytochemical screening

Extracts of *Vitex negundo* 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.¹⁴

Cytotoxic activity

Animals

Adult Swiss male albino mice (20-25 g) were procured from Institute of Animal Health and Veterinary Biological, Mhow, Indore, MP and used throughout the study. They were housed in microcolon boxes in a controlled environment (temperature $25 \pm 2^{\circ}\text{C}$ 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.

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.¹⁵

Effect of extracts on survival time¹⁶

Animals were inoculated with 1×10^6 cells/mouse on day '0' and treatment with both extracts started 24 h after inoculation, at a dose of 500 mg/kg/day, p.o. for ethanol and acetone extracts. The dose was selected by OECD 423 guidelines, which showed no toxicity up to 5.4 g/kg (p.o.) for acetone and 7.8 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 *Vitex negundo* 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:

$$\text{Increase in lifespan} = ((T - C)/T) \times 100$$

Where T = number of days the treated animals survived

C = number of days control animals survived.

Effect of extracts on normal peritoneal cells¹⁶

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 mg/kg/day, p.o. for ethanol and acetone extracts 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.

Effect of extracts on hematological parameters¹⁶

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 (500 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.¹⁷

Effect of extracts on solid tumor¹⁸

Mice were divided into three groups (n=6). Tumor cells (1×10^6 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 (500 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.¹⁹

In Vitro Cytotoxicity

Short-term Cytotoxicity was assessed by incubating 1×10^6 EAC cells in 1 ml phosphate buffer saline with varying concentrations of the extracts at 37°C for 3 hrs in CO₂ atmosphere ensured using a McIntosh field jar. The viability of the cells was determined by the trypan blue exclusion method.^{20,21}

Histopathological studies²²

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

Statistical analysis

All values were expressed as mean \pm 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.

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 \pm 0.47 days, whereas it was 34.16 \pm 0.34 days for acetone (500 mg/kg/day p.o) and 37.5 \pm 0.47 days for ethanol (500mg/kg/day p.o) extracts treated groups. The MST for acetone extract treated group was increased to 39.02% and of ethanolic treated group increased to 44.45%.

The average number of peritoneal exudate cells per normal mouse was found to be 5.8 \pm 0.01 $\times 10^6$. Single treatment of acetone extract (500mg/kg,p.o) enhanced the number to 7.93 \pm 0.17 $\times 10^6$ (P<0.001) and consecutive treatment for two days increase it to 11.21 \pm 0.13 $\times 10^6$ (P<0.001). Similarly single treatment with ethanol extract (500mg/kg/day,i.p) enhanced the count to 9.78 \pm 0.24 $\times 10^6$ (P<0.001) and consecutive treatment for two days increased it to 14.95 \pm 0.19 $\times 10^6$ (P<0.001).The results are shown in Table 2.

Hematological parameters of tumor-bearing mice on Day 14 showed significant changes when compared with the normal mice shown in Table 3. 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 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 (30th day) was 11.66 \pm 0.33 ml, whereas for the extract-treated group it was 7.2 \pm 0.14 ml and 7.9 \pm 0.25 ml for ethanol and acetone extracts, respectively.

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 μ g/ml concentration, 97% (EAC) of activity was found for ethanol extract, whereas 88% (EAC) of activity for acetone extract.

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 (500 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.

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 and ethanol (500mg/kg, p.o) extracts showed decreased damage to the kidneys as shown in fig no 2.

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 \pm 0.47	-
2	Ethanol extract (500 mg/kg, p.o)	37.5 \pm 0.47*	44.45
3	Acetone extract (500 mg/kg, p.o)	34.16 \pm 0.34*	39.02
4	5- FU (20mg/kg, i.p)	44.33 \pm 0.49*	53.01

n=6 animals in each group, *P<0.01 Vs control.,Days of treatment = 9, Values are expressed as mean \pm SEM

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

Group	Treatment	Peritoneal cell count($\times 10^6$)
I	Normal control	5.8 \pm 0.01
II	Acetone (500 mg/kg/day p.o) treated once	7.93 \pm 0.17*
III	Acetone (500 mg/kg/day p.o) treated twice	11.21 \pm 0.13*
IV	Ethanol (500 mg/kg/day p.o) treated once	9.78 \pm 0.24*
V	Ethanol(500 mg/kg/day p.o) treated twice	14.95 \pm 0.29*

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

Table No 3. Effect of ethanol and acetone extract treatment on Haematological Parameters

Treatment /dose	Hb (g/dl)	RBC (million/mm ³)	WBC (million/mm ³)	Protein g %	PCV (mm)	Neutrophils %	Lymphocytes %	Monocytes %
Normal	14.3 \pm 0.10	4.68 \pm 0.06	7.48 \pm 0.03	8.21 \pm 0.06	16.5 \pm 0.42	30.83 \pm 0.60	68.5 \pm 0.42	1.16 \pm 0.16
Tumor Control	8.35 \pm 0.09 ^a	2.6 \pm 0.07 ^a	27.19 \pm 0.07 ^a	13.9 \pm 0.2 ^a	31.5 \pm 0.42 ^a	68.83 \pm 0.60 ^a	30 \pm 0.57 ^a	2.16 \pm 0.16 ^d
Tumour+5-FU (20 mg/kg)	14.0 \pm 0.05 ^{a,b}	4.11 \pm 0.04 ^{a,b}	8.23 \pm 0.02 ^{a,b}	8.65 \pm 0.04 ^{a,b}	19.5 \pm 0.42 ^{a,b}	31.83 \pm 0.47 ^{a,b}	64.66 \pm 0.42 ^{a,b}	1.33 \pm 0.21
Acetone (500 mg/kg)	12.0 \pm 0.10 ^{a,b}	3.13 \pm 0.06 ^{a,b}	9.52 \pm 0.02 ^{a,b}	9.6 \pm 0.05 ^{a,b}	24.5 \pm 0.42 ^{a,b}	42.16 \pm 0.60 ^{a,b}	54.0 \pm 0.68 ^{a,b}	1.5 \pm 0.22
Ethanol (500 mg/kg)	12.91 \pm 0.10 ^{a,b}	3.88 \pm 0.04 ^{a,b}	9.09 \pm 0.03 ^{a,b}	9.1 \pm 0.03 ^{a,b}	21.3 \pm 0.33 ^c	38 \pm 1.78 ^{a,b}	59.5 \pm 0.42 ^{a,b}	1.83 \pm 0.30

Values are expressed as Mean \pm SEM of 6 mice in each group

a $P < 0.001$ Vs Normal control, b $P < 0.001$ Vs Tumour control, c $P < 0.01$ Vs Normal Control,

d $P < 0.05$ Vs Normal Control

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

Design of treatment	Solid tumour volume (ml)			
	15 th days	20 th day	25 th day	30 th day
Tumor control	7.50 \pm 0.22	8.0 \pm 0.25	9.0 \pm 0.25	11.66 \pm 0.33
5-fluorouracil(20mg/kg)	4.50 \pm 0.22*	4.66 \pm 0.21*	4.33 \pm 0.21*	3.66 \pm 0.21*
Acet. (500 mg/kg/day, p.o)	6.30 \pm 0.15*	6.66 \pm 0.61*	7.58 \pm 0.12*	7.9 \pm 0.25*
Etha.(500 mg/kg/day, p.o)	5.54 \pm 0.52*	6.41 \pm 0.24*	7.23 \pm 0.11*	7.2 \pm 0.14*

n=5 in each group.

* $P < 0.001$ compared with tumor control

Values were expressed as mean \pm SEM,

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

Sample	Cells 1 x 10 ⁶	Concentration µg/ml	% Death
Ethanol Extract	Ehrlich ascitic carcinoma	200	97%
		100	92%
		50	88%
		20	24%
		10	9%
		Control	1%

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

Sample	Cells 1 x 10 ⁶	Concentration µg/ml	% Death
Acetone Extract	Ehrlich ascitic carcinoma	200	88%
		100	79%
		50	68%
		20	24%
		10	5%
		Control	2%

Figure No.1 Histopathological studies of liver

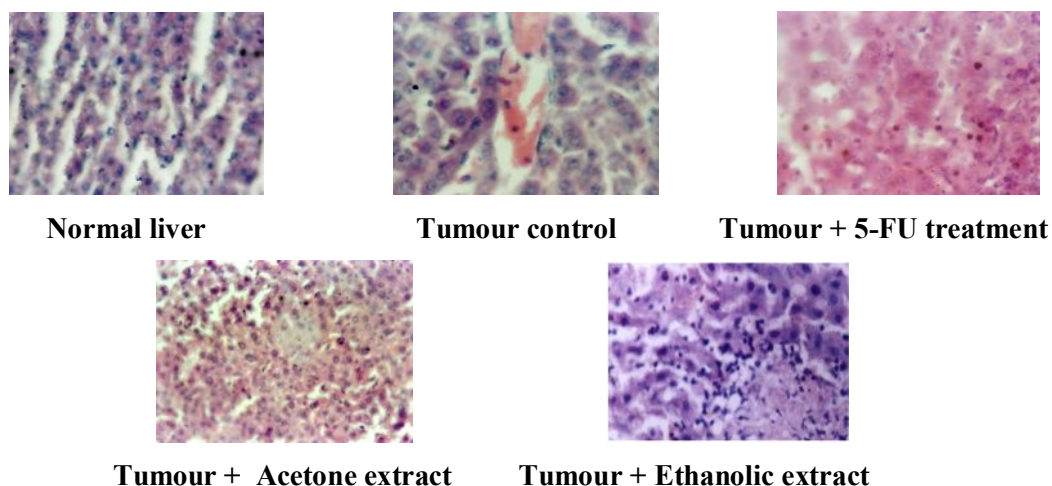
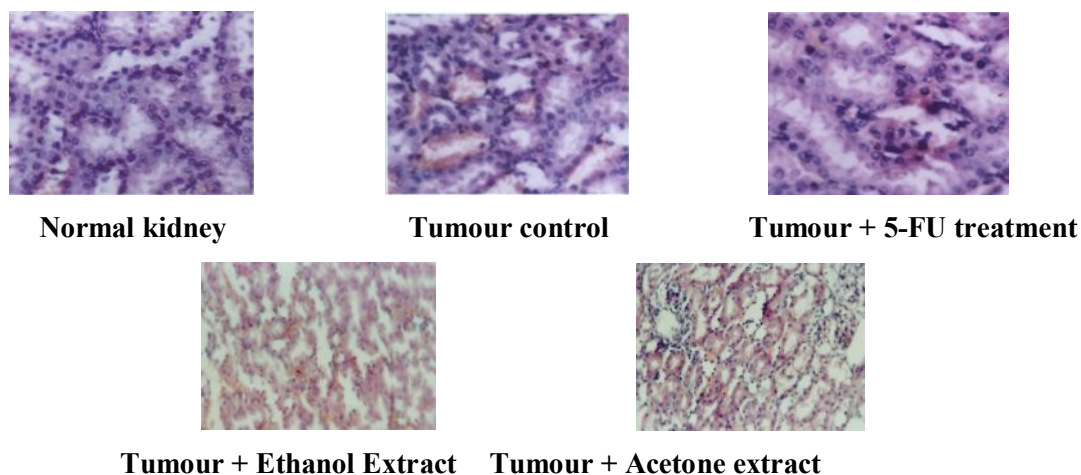


Figure No. 2 Histopathological studies of kidneys



Conclusion

The reliable criteria for judging the value of any anticancer drug are prolongation of lifespan and decrease of WBC from blood.^{23,24} 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 5×10^6 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 *V. negundo* 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.^{25,26} Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation²⁷ and angiogenesis.²⁸ 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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