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RESEARCH ARTICLE

ANTITUMOR ACTIVITY OF METHANOLIC EXTRACT OF *VERNONIACINEREA* LESS
BY ANIMAL MODEL

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ABSTRACT

Vernoniacineria (L.) Less a member of family compositae (Asteraceae), is an important medicinal plant has already been in used as antibacterial, Analgesic, antipyretic, anti-inflammatory preparation. In this study an attempt of the plant has been made to evaluate the anticancer activity of the methanolic extract of the plant, as stated in many hypothesis. The methanolic extract of leaves of *Vernoniacineria* L. Were screened for their anticancer activity by animal model, the various parameters such as hematological studies and protein estimation, solid tumor volume, median survival time (MST), life span (%LS). The changes in hematological parameters in mice altered significantly ($p > 0.01$) compare to the control. There was increase the %LS (57.43), MST (36.40) and solid tumor volume was reduced significantly ($p > 0.01$) compare to the control. Our studies point the possibility of developing *Vernoniacineria* L. as a novel potential agent in the area of cancer chemotherapy. Further investigation has to be carried out in isolation and characterization of active constituents and the mechanism involving in antitumor and cytotoxic effect.

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INTRODUCTION

Over 60% of currently used anticancer agents are derived in one way or another from natural sources. Scientific literature is the collection of research information and as such, serves as the reservoir of knowledge about a subject [1][2]. As the scientific literature of information should occur, helping to advance the science of these plants. [3][5][6][7] Hartwell, in his review of plants used against cancer, lists more than 3000 plant species that have reportedly been used in the treatment of cancer. The search for anti-cancer agents from plant sources started in earnest in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins [6][7][13][14]. *Vernoniacineria* (L.) Less a member of family compositae (Asteraceae), is an important medicinal plant found throughout the Indo-Malaysia region, tropical Africa, Australia and New Zealand. It is the one of the most common weeds in India, found throughout the country to an altitude of 2500m in the Himalayas Iwalewa E O, *et al.*, [15] Has reported an analgesic, anti-inflammatory, antipyretic and behavioral activity from the chloroform, methanolic and ether extracts of *Vernoniacineria* Less leaf. Mazumder U K, *et al.*, [16] has reported anti-inflammatory activity from the methanolic extract of the whole plant of *Vernoniacineria* Less. Malaya Gupta, *et al.*, [17] has reported antibacterial activity from the benzene extract of *Vernoniacineria* Less. Mary Latha R, *et al.*, [4] has reported the anti-inflammatory effect of an alcoholic extract from the flower of *Vernoniacineria* Less was tested in adjuvant arthritic rats. Mamta Tandon, *et al.*, [18] Insect Antifeedant Principles from *Vernoniacineria* Less. Adeboye J O, *et al.*, [19] proposed a study on diuretic and anti-diuretic

potency of the leaf extracts of *Vernoniacineria* in albino rats. Amritpal Sing, *et al.*, [21] has reported anti-inflammatory activity from the alcoholic extract of the flower of *Vernoniacineria* Less. Sotheara Hout, *et al.*, [22] The plant extracts were tested for in vitro Antiplasmodial activity against a chloroquine resistant *Plasmodium falciparum* strain. Yuan-Chuen W, *et al.*, [23] has reported that plant possess anti-Helicobacter pylori activity in *Vernoniacineria* Less. Cheeptham N, *et al.*, [25] has reported Antimicrobial activity from the leaf of *Vernoniacineria* Less shows Light-mediated activity tested against *Bacillus subtilis*, *Staphylococcus aureus* K147 methicillin-sensitive (Ms), *Escherichia coli* DC10, *E. coli* (wild), *Pseudomonas aeruginosa* 187 (wild), *Candida albicans* and *Aspergillus fumigates*. Henrik T S, *et al.*, [26] has reported the ethanol extract of the whole plant of *Vernoniacineria* Less. Shown antiplasmodial activity by *invitro* method. Valsaraj R, *et al.*, [27] has reported Antimicrobial screening of *Vernoniacineria* Less of Different concentrations of 80% ethanol extracts were tested, using the agar dilution method, against four bacteria. Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues), and sometimes metastasis (spread to other locations in the body via lymph or blood). Cancer may affect people at all ages, causes about 13% of all human deaths. According to the American Cancer Society, 7.6 million people died from cancer in the world during 2007. The Main objective of this studies to find out the possibility of developing *Vernoniacineria* L. as a novel potential agent in the area of cancer chemotherapy.

MATERIAL AND METHODS

Collection and Authentication of Plant Material

The leaves of *Vernoniacinerea* Less has been procured from Kanyakumari district, Tamilnadu. The plant material was identified by botanist, Prof. Dr.S. Jayaraman, plant anatomy Research centre (PARC) Chennai.

Preparation of plant extracts

Freshly collected leaves were washed, shade dried under room temperature for a period of three weeks. The dried plant material was made into a coarse powder. A weighed quantity of the powder (750g) was passed into sieve number 40 and subjected to hot solvent extraction in a Soxhlet apparatus using methanol at a temperature range of 40-80°C before and after every extraction the marc was completely dried and weighed. The filtrate was evaporated to dryness at 40°C under reduced pressure in a rotary vacuum evaporator.

Experimental animals

Inbred adult Swiss albino mice (25-30g) of male sex was used for this study. The animal was maintained in well-ventilated room with 12:12 hour light /dark cycle in polypropylene cages. Standard feed and tap water was provided throughout the experimentation period.

Anti Cancer studies Animal Model

Induction of cancer in experimental mice

Adult Swiss albino mice (24 numbers) of male sex overnight fasted, was subjected to a single intra peritoneal injection of Dalton's Ascitic Lymphoma cells (1×10^6 cells). After injection, the animal had free access to food and water. Complete induction of cancer was required for 6 days.

Collection of blood sample and determination of hematological parameters

Blood sample was collected by retro-orbital plexus method and hematological parameter was evaluated during the course of the study.

Grouping of Animals

The animal was divided into four groups of 6 animals; each group separation was as follows

- Group 1 – DAL cells injected to animals (1×10^6 cells/mice) i.p.
- Group 2 – Animals receiving 5-FU (20mg/kg) i.p.
- Group 3 – Animal receiving MEVC (200mg/kg) p.o.
- Group 4 – Animal receiving MEVC (400mg/kg) p.o.

The drug treatment started from 1st day of tumor transplantation and was carried out for 9 days. Blood was collected by retro orbital puncture and used for hematological studies. The blood was drawn gently and a study was performed.

The animal was divided into three groups of 3 animals each, group separation was follow

DAL cells injected to right hind limbs of all animals (1×10^6 cells/mice).

Group 1 – Tumor control

Group 2 – Animals receiving MEVC (200mg/kg) orally for 5 alternate days.

Group 2 – Animals receiving MEVC (400mg/kg) orally for 5 alternate days
(MEVC-Methanolic Extract of *Vernoniacinerea* Less)

The parameters that are evaluated during this study are changes in body weight, Life span of the animal, RBC count, WBC count, Estimation of hemoglobin content Protein estimation, Cancer cell count

In vivo method

Percentage increase life span (ILS %)

Recording the mortality monitored the effect of MEVC on tumor growth and percentage increase in life span (ILS %) was calculated

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

Mean survival time = $\frac{\text{Day of 1}^{\text{st}} \text{ death} + \text{Day of last Death}}{2}$

Effect of MEVC on Median Survival time (MST)

Animal of group I, II, III, IV was inoculated with 1×10^6 mice on day 0. The MEVC 200mg/kg and 400mg/kg b.w. was administered orally to group III and group IV respectively from the 1st day of cancer induction continued for 9 days. The MST of each group consisting of 5 mice was noted and mortality was calculated

Determination of tumor volume

The mice were 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 determined by centrifuging at 1000 rpm for 5 min.

Determination of tumor cell count

The ascitic fluid was taken in a RBC pipette and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the number of cells in 64 small squares was counted.

Hematological parameter

The effect of MEVC on peripheral blood was investigated. The drug treatment was started from the 1st day of tumor transplantation for group III and group IV. Group II was injected with a standard drug of 20mg/kg b.w. the appropriate dose was given continuously for a period of 9 days. Blood was gently withdrawn from retro-orbital puncture on the 14th day and used for hematological studies.

Enumeration of erythrocytes (RBC)

The blood was collected from the animals through the retro-orbital plexus. Blood was then drawn up to the mark 0.5 in the RBC pipette and diluting fluid was mixed by sucking up to mark 101, content in the RBC pipette was mixed by rolling between the palms. The first few drops were discarded and the rest was gently dropped onto Neubauer chamber. The total RBC was calculated as number of cells per cubic millimeter in counting area.

Enumeration of Leukocytes (WBC)

The blood was collected from the animals through the retro-orbital plexus, 1 in 20 dilution of the blood was made by adding 20 μ l of blood to 0.38ml of dilution fluid in a 75x10mm glass or plastic tube. Tube was fixed and the contents was

mixed for 1min by rolling between the palms and keep aside for 5 min. The first few drops discarded and then the rest was placed onto the Neubauer Chamber using a Pasteur pipette. The leukocytes, stained violet-black color, was counted using 4nm objective with a 2x6 eye-piece or 16mm objective with x6 or x10 eye-piece, in a 1mm or 2mm area.

Estimation of hemoglobin content

The blood was collected from the animals through the retro-orbital plexus. The diluting pipette of the hemoglobinometer was filled with N/10 HCl up to the mark of 2gm till the micropipette touches the level of acid in the tube. Blood was added to the mark 20µl and immediately deposited at the bottom of the graduated tube, mixed with N/10 HCl with the help of a stirrer and then allowed to stand for 15min, so that haemoglobin was converted to "Acid hematin". It was diluted drop by drop with water and stirred well till it exactly matches with the standard glass tube. Note the reading in the scale at the side of diluting tube

Estimation of Total Serum Protein

Total Protein

6ml of sulphate-sulphite solution was pipette into a centrifuge tube & 0.4ml of plasma layer on it. Mixed, by inversion & 2ml of the mixture was removed and to it 50ml of Biuret reagent was added immediately.

Albumin

To the rest of the plasma, sulphate-sulphite mixture and 3ml of ether was added, stopper and shaken 40 times. The tubes was capped and centrifuged till a firm globulin layer was formed. After centrifugation, the tube was tilted and 1ml of clear solution below the Globulin layer was pipetteout into a tube and 5ml of Biuret reagent was added.

Biuret Blank

Biuret blank is prepared by adding 2ml of sulphate-sulphite solution to 5ml of Biuret reagent.

Standard

0.4ml of standard solution was pipette into 6ml of sulphate-sulphite solution as above and 2ml of the mixture was transformed into 5ml of Biuret reagent. The tube was heated in water bath at 37°C for 10min and the color developed was read at 570nm.

Effect of Mevc on Solid Tumor

Mice divided into three groups (n=3). Tumor cells (1x10⁶ cells/mice) were injected into the right limb of all the animals' intra muscularly. Mice of group I were tumor control group II and III received MEVC orally for 5 alternative days. Tumor mass was measured from 11th day of tumor induction and repeated every 5th day for a period of 30 days. The volume of tumor was calculated by using the formula $v=4/3\pi r^3$ where r was the mean of r₁ and r₂ which are two independent radii of the tumor mass.

RESULTS

Antitumor Animal model studies

Any potential anticancer drug is expected to increase the mean survival time and thus increasing life expectancy. Mice transplanted with DAL (control) in our studies have MST of 23 days, which was increased to 29 days and 36days by MEVC of 200mg/kg and 400mg/kg respectively. These results are almost comparable to that of 5-FU, the standard drug for which the MST was 41 days. (Table 1) The increase in the life span of tumor bearing mice treated with MEVC (400mg/kg p.o.) and 5-FU was found to be 57.43% and 78.20% respectively (p > 0.01) as compared to the control group. (Table 1)

Table 1 Effect of methanolic extract of vernoniacinerea l. on the mean survival time in DAL bearing mice

Treatment	MST	Life span (%)
Tumor control	23.12±1.20	-
5-FU	41.20±1.36**	78.20
MEVC 200mg/kg	29.16±1.72*	26.12
MEVC 400mg/kg	36.40±1.32**	57.43

N=6 animals; Drug treatment-9 days; ** p< 0.001 Vs control; * p< 0.05 Vs control; Data were analyzed by using one way ANOVA following by Dunnett's test.

Hematological parameters of (Table 1) tumor bearing mice on day 14 were found to be significantly altered from test group. The total WBC count, protein and PCV were found to be increased with a reduction of the haemoglobin content of RBC. In a differential count of WBC, the percent of neutrophils increased while the lymphocyte count decreased (control). At the same time interval, MEVC (200mg/kg, 400mg/kg p.o.) treatment could change those altered parameters to near normal (Table 2).

Table 2 Effect of methanolic extract of Vernoniacinerea L. on hematological Parameter in DAL bearing mice

Treatment	Hb (g %)	RBC (Millions/mm ³)	WBC 10 ⁶ cells/mm ³	Protein (g %)	PCV (mm)	Differential count %		
						Lymphocytes	Neutrophils	Monocytes
DAL control 1x10 ⁶ cells	8.0±0.34	3.8±0.07	15.2±1.15	14.5±1.2	28.1±0.61	30.1±0.64	67.1±0.2	2.8±0.5
DAL+5-flu (20mg/kg)	14.1±0.43**	6.0±0.21**	7.2±0.2 **	8.3±0.56**	17.5±0.4 **	70.5±0.91 **	28.4±0.13**	1.0±0.8 **
DAL(1x10 ⁶ cells) + MEVC 200mg/kg	9.8±0.52 *	4.8±0.03**	11.1±1.6 **	11.5±0.4 **	21.3±0.17**	53.6±0.24 **	45.2±0.41**	1.2±0.2 **
DAL(1x10 ⁶ cells) + MEVC 400mg/kg	11.5±0.61**	5.5±0.51**	9.3±0.7 **	9.7±0.3 **	19.0±0.23**	68.1±0.12 **	30.9±0.72**	1.0±0.2 **

N= 6 animals in each group; Values expressed as mean± SEM; Statistical significance (P) calculated by one way ANOVA followed by Dunnett's test. **P < 0.01; *p < 0.05 Vs control.

Table 3 Effect of methanolic extract of Vernoniacinerea L. on solid tumor volume in DAL bearing mice

Treatment	Dose mg/kg	Solid tumor volume (ml)			
		15 th day	20 th day	25 th day	30 th day
Tumor control	Normal saline	3.5±0.06	4.1±0.03	5.7±0.06	6.6±0.1
Test-I	MEVC 200mg/kg	3.0±0.05*	3.6±0.09**	4.07±0.03**	4.7±0.09**
Test-II	MEVC 400mg/kg	2.6±0.12**	3.2±0.06**	3.6±0.09**	4.1±0.07**

N= 3 animals in each group; Values expressed as mean± SEM; **P < 0.01; *p < 0.05 Vs control. Data were analyzed by using one way ANOVA followed by Dunnett's test.

Estimation of solid tumor volume is a direct method of evaluation of anticancer activity. It is indeed a suitable method, which does not involve sacrificing the animal. In the study, the tumor mass was directly measured after implantation intramuscularly. The solid tumor volume increase by 6.6 ± 0.1 ml. DAL bearing mice, treatment with MEVC decreased significantly ($P < 0.01$, $p < 0.05$), the tumor volume to 4.1 ± 0.07 ml on dose dependant manner at the end of 30 days (Table 3).

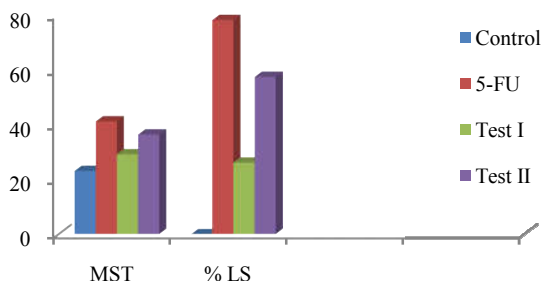


Figure 1 Effect of methanolic extract of *vernoniacinerea L.* on the Mean survival time in DAL bearing mice.

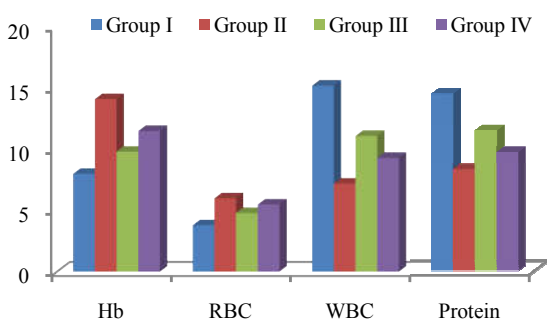


Figure 2 Effect of methanolic extract of *Vernoniacinerea L.* on hematological Parameter in DAL bearing mice

Group I = Tumor control
 Group II = 5-FU
 Group III = MEVC 200mg/kg
 Group IV = MEVC 400mg/kg

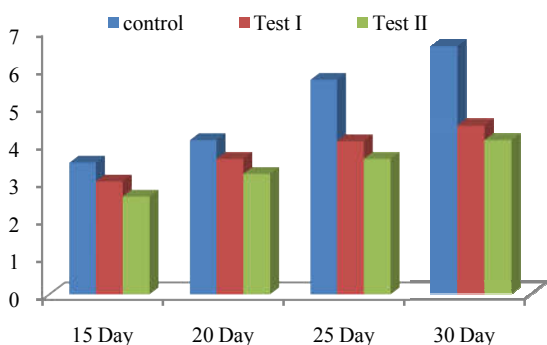


Figure 3 Effect of methanolic extract of *Vernoniacinerea L.* on solid tumor volume in DAL bearing mice.

DISCUSSION

Cancer is basically a disease of cells characterized by a shift in the control mechanism that governs cell proliferation and differentiation. Cells that have undergone neoplastic transformation usually express cell surface antigens that appear to be normal foetal type and have other sign of apparent “immaturity” and may exhibit qualitative or quantitative chromosomal abnormalities, including various translocations and the appearance of amplified gene sequence.

Such cells proliferate excessively and form local tumors that can compress or invade adjacent normal structures. Ideal characteristic of anticancer drug should eradicate cancer cells without harming normal tissues. Unfortunately, no current drug available agents meet this criterion and clinical use of this drug involves a weighing of benefits against toxicity in a search for favorable therapeutic index. Hence, the use of natural products now has been contempt of exceptional value in the control of cancer and its eradication programmer.

The results of the present study clearly demonstrate the tumor inhibitory activity of MEVC against DAL strain. The reliable criteria for evaluating an anticancer drug are prolongation of lifespan of the animal and decrease in WBC count of blood. Our results show an increase in lifespan accompanied by a reduction in WBC count in MEVC treated mice. It had significant effect in increasing the life span of ascitiestumour bearing animals and also found to reduce the solid tumour in animal models. These results clearly demonstrate the antitumour effect of MEVC against DAL. In cancer chemotherapy the major problems are of myelosuppression and anaemia [46][47] the anemia encountered in tumor bearing mice is mainly due to reduction in RBC and HB% and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. [48] The reliable criterion for judging the value of any anticancer drug is the prolongation of lifespan of the animal [49] and decreasing the WBC from blood. [50] The above results demonstrated the antitumour effect of MEVC against DAL in Swiss albino mice. A significant ($P > 0.01$) enhancement of MST and peritoneal cell count was observed.

Analysis of the hematological parameters showed minimum toxic effects in the mice which were treated with MEVC. After 14 days of transplantaion, MEVC treated groups were able to reverse the changes in the hematological parameters consequent to tumourinoculation. All these data point to the possibility of developing methanolic extract of *Vernoniacinerea L.* as a novel, potential agent in the area of cancer chemotherapy. Preliminary phytochemical screening indicated the presence of alkaloids and flavanoids in MEVC. Flavanoids, which have been shown to possess antimutagenic and anticarcinogenic activity. [51][52][53] Moreover, flavanoids have a chemo preventive role in cancer through their effects on signal transduction in cell proliferation [54] and angiogenesis [55] The cytotoxic and antitumor properties of the extract may be due to these compounds.

CONCLUSION

The present study points to the potential anticancer activity of *Vernoniacinerea L.* A further study to characterize the active principles and elucidate the mechanism of the action of MEVC is suggested.

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