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Inhibition of Ehrlich's Ascites Carcinoma by the Leaf Extracts of *Eupatorium ayapana* in Swiss Albino Mice.

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ABSTRACT

Eupatorium ayapana, belonging to the family Asteraceae, is therapeutically used as an antiseptic, antidysenteric, antibacterial and haematostatic agent. The objective of the present study is to explore the antitumour activity of the ethanolic and water extracts of the *Eupatorium ayapana* in Ehrlich Ascites Carcinoma (EAC) bearing Swiss albino mice. To evaluate the antitumour activity, ethanolic and water extracts of *E. ayapana* were administered at the dose level of 150 mg/kg body weight intraperitonally for 14 consecutive days using 5-flurouracil as standard drug. After 24 h of the last dose, the antitumor effect of ethanolic and water extracts was assessed by evaluating tumor volume, viable cell count, increase in body weight, mean survival time and hematological parameters of the EAC bearing host. Both extracts caused significant decrease in tumor volume, viable tumor cell count, body weight and elevated the life span of EAC tumor bearing mice. Haematological profiles such as red blood cell (RBC), haemoglobin, and white blood cell (WBC) count restored more or less to normal level in extract-treated mice. The results demonstrated that the both extracts at 150 mg/kg body weight have potent antitumour activity and ethanolic extract is comparatively more potent than the water extract.

Keywords: *Eupatorium ayapana;* Ehrlich Ascites Carcinoma; antitumour activity; Hematological parameters; 5-flurouracil.

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INTRODUCTION

Natural products, especially plants have been used for the treatment of various diseases for thousands of years. India is a rich source of such medicinal plants which are used to treat different diseases from ancient time. Plant derived natural products and their synthetic derivatives are expected to play an important role in the development of chemotherapeutic agents to inhibit the onset of cancer [1]. Different plant derived products such as flavonoids, terpenoids, and steroids, etc. have received considerable attention in recent years due to their diverse pharmacological properties, including antioxidant and antitumor property [2]. Cancer is increasing worldwide as the single most common cause of deaths in both developed and developing countries [3]. In modern medicine, there are various treatments i.e. chemotherapy, radiotherapy etc. available for cancer treatment [4]. However, chemotherapeutic drugs have various side effects. So researches are going on throughout the world to develop modern anticancer drugs from less toxic plant products with proven medicinal properties [5]. One of the best ways in the search for anticancer agents from plant resources is the selection of plants based on traditional uses and testing the selected plant's efficacy and safety in light of modern science.

Eupatorium ayapana is belonging to the family Asteraceae. It is an aromatic, erect perennial herb, and has long been naturalized in India and other tropical countries as well. Ayapana has three different Latin names (*Ayapana triplinervis, Eupatorium ayapana, and E. triplinerve*) but all three names refer to the same plant.It has stomachic, antiseptic, antitussive, antiulcerous, hemostatic, hepatoprotective, antitumorous, anticoagulant, astringen, febrifugal, diaphoretic and emollient properties [6]. Chemical constitutions like 7-ethoxy coumarin (ayapanin), 6,7-dimethoxy coumarin (ayapin); carotene, vitamin-C and stigmasterol have been isolated from its leaves [7]. Five additional coumarins, viz. hydrangetin, daphnetin, daphnetin-7-methyl ether dimethyl ether, and umbelliferone have also been isolated [8]. Ayapana also contains hernarin (7-methoxycoumarin), a coumarin which may help to explain why the plant is used in herbal medicine as an anti-tumor remedy. From recent studies it is reported that hernarin was toxic to multi-drug resistant cancer cells [9] and leukemic cancer cells [10].

Considering much traditional informations, various experimental evidences and the medicinal activities of *Eupatorium ayapana*, the present study was carried out to evaluate the chemotherapeutic potential of alcoholic and water extracts of *Eupatorium ayapana* leaves by analyzing its antiproliferative properties in Ehrlich's Ascites Carcinoma (EAC) bearing Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Distilled water, Normal saline, Absolute alcohol, Ether, Drabkin's diluent solution, RBC dilution fluid, WBC dilution fluid, Leishman stain, Trypan blue, Ethylenediamine tetraacetic acid (EDTA), 5 –flurouracil (5-FU), Potassium dihydrogen phosphate(KH_2PO_4) were purchased from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals used were analytical grade and obtained from Merck Ltd., SRL Pvt. Ltd., Mumbai, India.

Collection and preparation of plant materials

Eupatorium ayapana leaf collected from the district of Purba and Paschim Midnapore, West Bengal and authenticated (A voucher specimen No.BOT/VU/3, Collection date 15.03.2011) by the Department of Botany, Vidyasagar University, West Bengal, India. The leaves were shade dried at room temperature. Then it was allowed to crush in an electric grinder and powdered. In 450 ml of solvent (ethanol or water) 250 gm of powder was suspended for 48 hours in room temperature. The extracts were then filtered through filter paper separately. The filtrates were concentrated with a rotary evaporator at 400C under low pressure. The concentrated filtrates were then poured in petridishes and were incubated at 370C for drying to afford crude water (WEEAL) and ethanolic (EEEAL) extracts of *Eupatorium ayapana* leaf [11].

Animals

Male Swiss albino mice (20-25 g) were collected. The mice were grouped and housed in polyacrylic cages (38 x 23 x 10 cm) with six animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C; humidity 55 ± 5%) with 12 hrs dark/light cycle. They were acclimatized to laboratory conditions for 10 days before the beginning of the experiment. The study was approved by the Institution's animal ethical committee.

Tumor cells

Ehrlich's Ascites Carcinoma (EAC) cells were collected from Dept. of Biotechnology, Indian Institute of Technology (IIT), Kharagpur were maintained by weekly intraperitoneal transplantation in the abovesaid mice at the concentration of 2 x 106/cells/mouse. The EAC cells were harvested after 7-10 days. The washed and viable cells free of contaminating RBC were taken in 0.9gm% NaCl solution for transplantation. EAC cells were found to be 99% viable by the trypan blue exclusion assay.

Study of the in vivo antitumour activity [12]

Sixty male mice were divided into five groups (n=12). Except the control group, all the groups were inoculated with Ehrlich's Ascites Carcinoma (2 x 10^6cells/mouse**)** intraperitoneally and this was taken as day of zero. From the second day, ethanolic and water extract of *Eupatorium ayapana,* 5-fluorouracil and normal saline were injected intraperitoneally in Group 3, Group 4, Group 5 and Group 1 respectively for subsequent 14 days. All the extracts were injected at the dose of 150mg/kg body weight of mice and the standard drug (5-FU) was given at the dose of 20 mg/kg body weight. After the last dose and 24 hr fasting, six mice from each group were sacrificed for the study of antitumor activities and hematological parameters. The rest of the animals of all groups were kept for study of the host survival time and tumor growth response.

Studies on body weight (13) and host survival time [12,14]

Tumor growth was monitored by daily body weight change and the survival time of host mouse was assayed by recording the mortality daily for 6 weeks and increase in life span (ILS %) was calculated, using the following equations.

MST= (Day of first death + Day of last death)/2

ILS %= [(Mean survival time of treated group / Mean survival time of tumor control group)-1] x 100

Determination of tumor volume [15]

At the time of sacrifice, 2ml of normal saline was injected into intraperitoneal cavity of each mouse. Then the peritoneal fluid was aspirated aseptically from the mice peritoneal cavity and the tumor volume was calculated the by the following formula:

Tumor volume = Volume of mixture (tumor cells and saline) in ml– Volume of saline in ml.

Then each of the calculated volume was compared with the calculated tumor volume of tumor control group.

Determination of Tumor cell count [16]

Aseptically drawn EAC cells from the intraperitoneal cavity of each mouse, it was diluted to 100 times with phosphate buffered saline. Then a drop of the diluted cell suspension was charged on the haemocytometer chamber and the numbers of tumor cells in the 64 small squares were counted.

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Determination of Viable tumor cell count [16]

Viable tumor cells are counted by the trypan blue exclusion method. Cells which did not take up the dye were counted and the percentage was determined. The results of treated group were compared with the results of tumor control group.

Study of Hematological parameters

Total red blood cell (RBC), white blood cell (WBC) counts and haemoglobin content were measured from freely flowing tail vein blood of every mouse [17,18]**.** Differential leucocyte count of WBC was done from Leishman-stained blood smears of normal, EAC control, extracts-treated groups.

Statistical Analysis

The experimental results were expressed as the Mean ± Standard error of mean (SEM). Statistical analysis of the collected data was done by Analysis of variance (ANOVA) followed by Student's t-test. The difference was considered significant when $p<0.05$.

RESULTS

Antitumour activity of EEEAL and WEEAL against tumour bearing mice was assessed by tumour volume, body weight increase,tumour cell count, viable tumor cell count, mean survival time and % increase in life span.

Change in Body weight

Body weight of the mice increases significantly in EAC treated group compared to saline control group, but in EEEAL and WEEAL treated groups body weight decreases significantly (Fig.1).

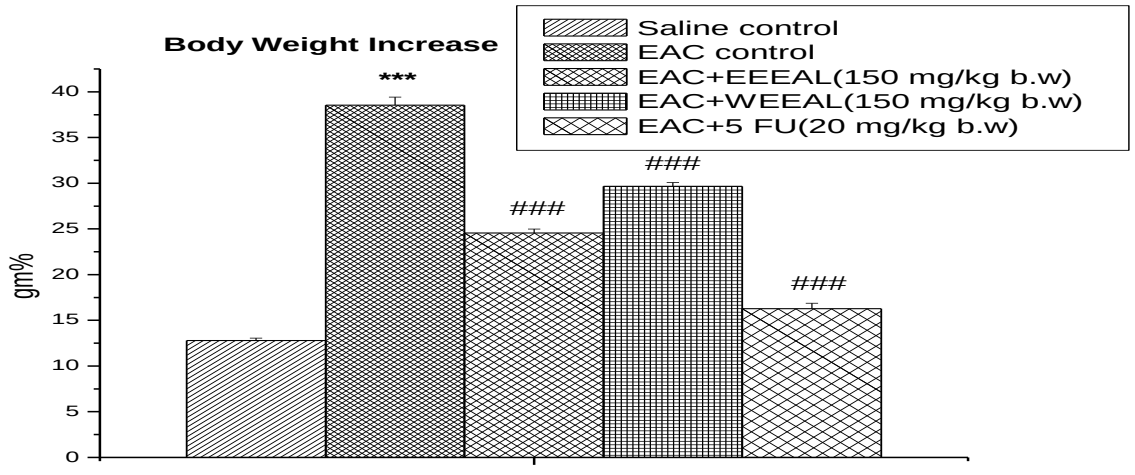

Figure 1: The effect of EEEAL, and WEEAL on change in body weight in EAC bearing mice.

Data are expressed as Mean± SEM (n=6). *** & ### indicate significant difference at $p < 0.001$; EAC Control is compared to Saline control; Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

Mean Survival Time (MST) and Increase in Life Span (ILS %)

Mean Survival Time of the EAC control group was 23.75 ±1.2days whereas in EEEAL and WEEAL treated groups MST was increased by 45.23±2.2, 36.42±1.6 days, respectively. The EEEAL was found to be the

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most potent in inhibiting the proliferation of EAC with a percentage increase in life span (ILS) of 90.44 %, whereas in case of WEEAL, it is 53.35% (Fig.2).

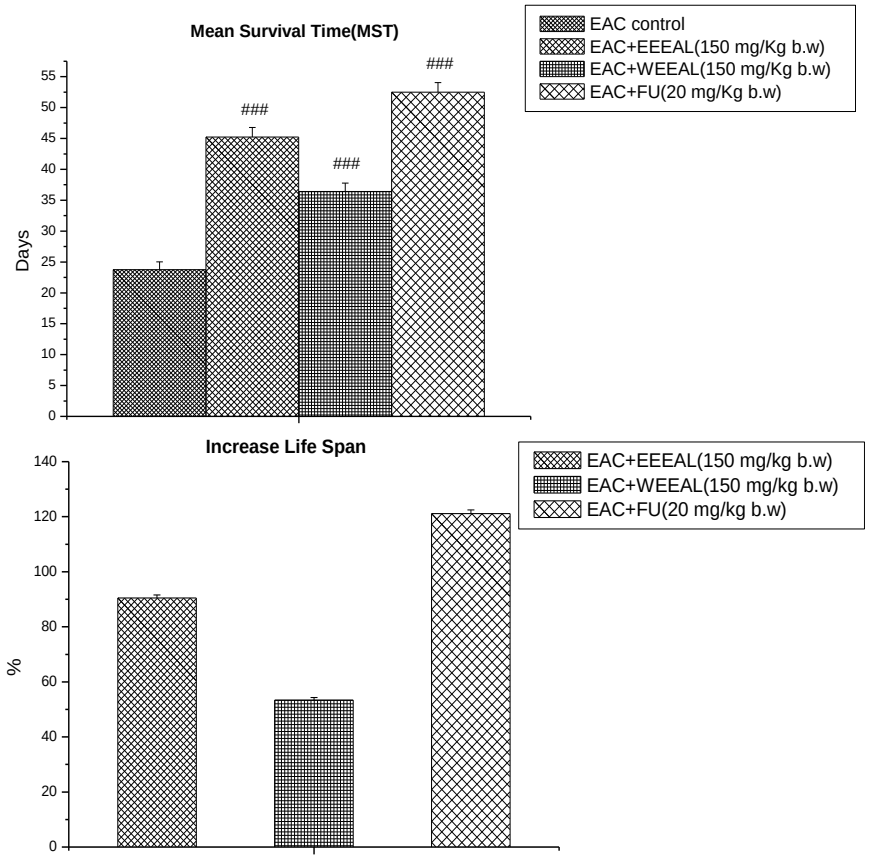

Figure 2: The effect of EEEAL, and WEEAL on mean survival time (MST) and increase life span in EAC bearing mice.

Data are expressed as Mean± SEM (n=6). ### indicate significant difference at $p < 0.001$. Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

Tumor volume

Tumor volume was decreased significantly in EEEAL, WEEAL and 5-FU treated groups compared to EAC control (Fig.3).

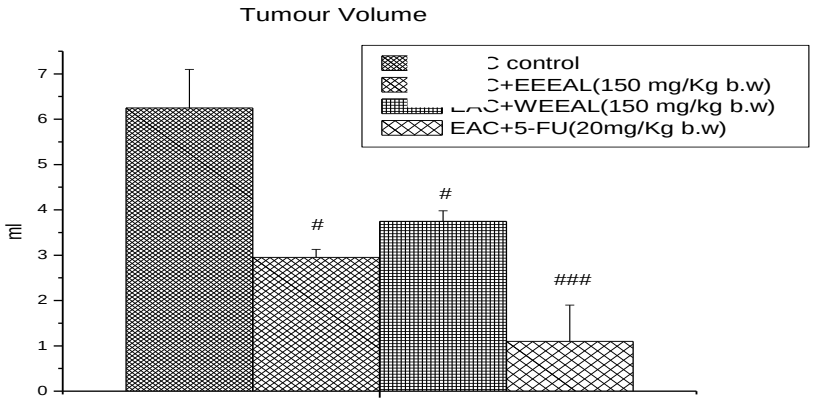

Figure 3: The effect of WEEAL and EEEAL on tumor volume in EAC bearing mice.

Data are expressed as Mean± SEM (n=6). '#'represents significant difference at $p<0.05$; '###'represents significant difference at $p<0.001$. Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

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Tumor cell count

Harvested tumor cells grow enormously in EAC inoculated mice (14.4 x 10^8cells). It was decreased to 8.2x10^8, 11.3x10^8 and 7.1x10^8 in EEEAL, WEEAL 5-FU treated groups respectively (Fig.4).

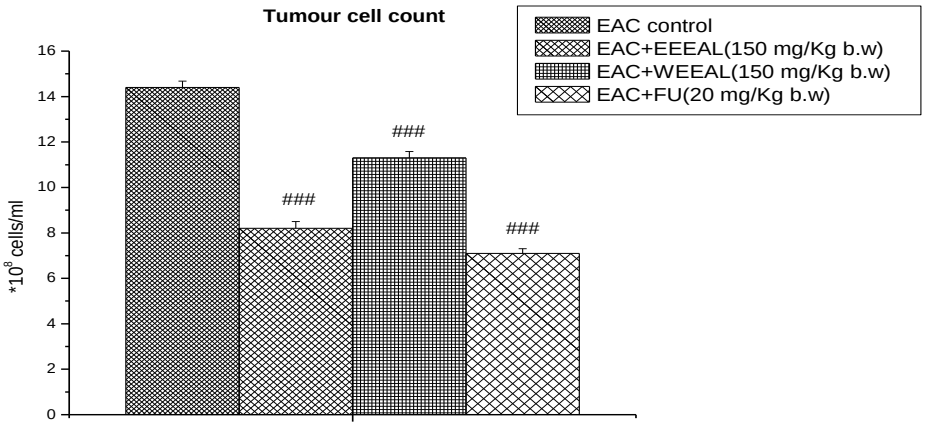

Figure 4: The effect of WEEAL and EEEAL on tumor cell count in EAC bearing mice.

Data are expressed as Mean± SEM (n=6). '###'represents significant difference at p<0.001. Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

Viable tumor cell count

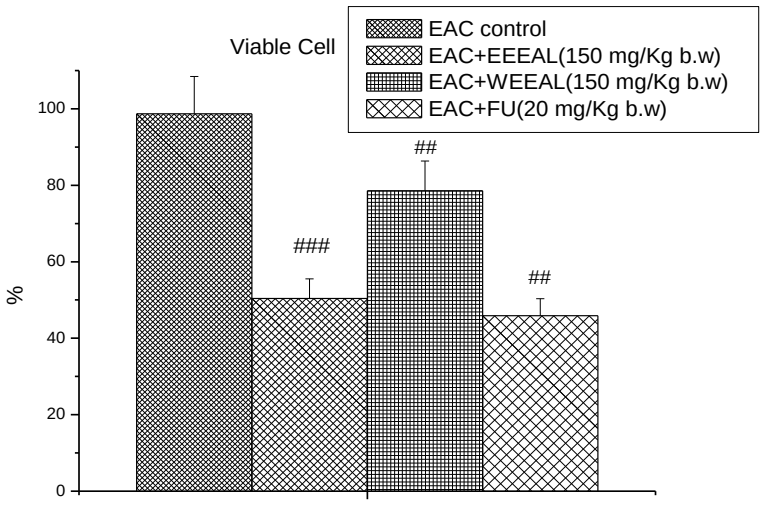

Figure 5: The effect of WEEAL and EEEAL on viable tumor cell count in EAC bearing mice.

Data are expressed as Mean± SEM (n=6). '##'represents significant difference at p<0.01; '###'represents significant difference at p<0.001. Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

Hematological parameters

Different Hematological parameters are shown in Table-1. Hemoglobin content and RBC count in the EAC control group were significantly decreased as compared to the saline control group. Treatment with EEEAL and WEEAL significantly increased the hemoglobin content and RBC count to more or less normal levels. The total count of WBC was found to be increased significantly in the EAC control group compared to the saline control group. Administration of EEEAL and WEEAL significantly reduced total WBC count compared with the EAC control group. In differential count of WBC, the percentage of neutrophil increased, while the lymphocyte count decreased

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in the EAC control group. Treatment with EEEAL changed these altered parameters towards more or less normal (Table.1).

Table 1: Effect of EEEAL and WEEAL on Hematological parameters in Ehrlich's Ascites Carcinoma (EAC) bearing mice.

PARAMETER	SALINE CONTROL	EAC CONTROL	EAC + 5-fu	EAC+EEEAL	EAC+WEEAL
Haemoglobin (g %)	12.86± .079	8.72±0.3**	11.34±0.214$^{###}$	11.08±0.652$^{#}$	10.35±0.274$^{##}$
RBC($\times10^6$ /mm^3)	8.44±0.079	4±0.091**	6.025±0.093 $^{###}$	5.77±0.315 $^{##}$	5.25±0.295 $^{##}$
Total WBC ($\times10^3$/mm^3)	6.18±0.079	17.98±0.485***	4.03±0.101 $^{###}$	12.65±0.554 $^{###}$	13.32±0.295 $^{###}$
Neutrophil (%)	17.9±0.201	67.22±0.923***	33.77±2.355 $^{###}$	43.15±3.491 $^{##}$	45.07±1.57 $^{###}$
Lymphocyte (%)	70.65± 0.926	33.5±0.273***	65.55±1.99 $^{###}$	55.67±3.664 $^{##}$	53.17±1.259 $^{###}$

Data are expressed as Mean± SEM (n=6). '**' and '##'represents significant difference at p<0.01; '***' and '###'represents significant difference at p<0.001. EAC Control is compared to Saline control; Treated groups are compared to EAC Control by one-way ANOVA followed by Student's t-test.

DISCUSSION

The present study was carried out to evaluate the antitumor potential of ethanolic and water extracts of *Eupatorium ayapana* leaf in Ehrlich's Ascites Carcinoma (EAC) bearing Swiss albino mice. The ethanol and water extracts treated animals at the dose of 150 mg/kg significantly inhibited the body weight increase, tumor volume, tumor cell count, viable tumor cell count and brought back the hematological parameters to more or less normal levels. These extracts also increase the mean survival time and increase in lifespan % in EAC bearing mice.

In EAC tumor bearing mice, a regular rapid increase in ascitic tumor volume was observed. Ascitic fluid is the direct nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth would be a means to fulfil the nutritional requirement of tumor cells [19] Treatment with ethanolic and water extracts of *E. ayapana* leaf inhibited the tumor volume, viable tumor cell count. These results could indicate either a direct cytotoxic effect of WEEAL and EEEAL on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition. Decrease of tumour volume may be the cause of reduction of body weight in treated tumour bearing mice and increased the life span which is one of the reliable criteria for judging the value of any anticancer drug [20]**.** It may be concluded that ethanolic and water extracts of *E ayapana* increase the life span of EAC bearing mice by decreasing the nutritional fluid volume and arresting the tumor growth. Thus ethanolic and water extract of Eupatorium ayapana has antitumor activity against EAC bearing mice.

In cancer chemotherapy myelosuppression and anemia are the major problems [21,22]**.** The anemia appeared in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions [22,23]**.**

Treatment with the ethanol and water extract of *Eupatorium ayapana* leaf brought back the hemoglobin (Hb) content, RBC and WBC count more or less to normal levels. This clearly indicates that *Eupatorium ayapana* leaf have protective activity on the hemopoietic system.

The phytochemical study revealed that different flavonoids, coumarin thymoquinone, daphnetin, alpha-terineol are present in the leaf of *Eupatorium ayapana* [9]. Flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [24] and angiogenesis [25]. Thus, in our study antitumour effects produced by the with EEEAL and WEEAL may be due to flavonoids and thymoquinone. Further studies are in progress to characterize the active principles involved in this antitumour activity.

CONCLUSION

From the present study it can be concluded that the EEEAL and WEEAL are effective against Ehrlich's Ascites Carcinoma (EAC) cell in *vivo*. However, more investigations have to be carried out to confirm the plant as a potent source of anticancer agent.

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