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Antimitotic, apoptotic and antineoplastic potential of leaf extract of *Eupatorium ayapana*

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Abstract

Eupatorium ayapana of Asteraceae family was found to possess useful therapeutic properties like hypoglycaemic, antimicrobial, hematoprotective, hepatoprotective, and antioxidant activities. The present study was aimed to evaluate the antimitotic, cytotoxic and apoptotic potential of the ethanolic and water extracts of *Eupatorium ayapana* leaf on Ehrlich's ascites carcinoma.

Antimitotic study was done by using *Allium cepa* root tips model. *In vitro* cytotoxicity was determined by MTT assay, apoptosis was determined by fluorescence microscopy, and cell cycle progression was analysed using flow cytometry; *in vivo* antitumor activity was performed in Ehrlich's ascites carcinoma bearing mice.

Ethanolic and water extracts of *Eupatorium ayapana* leaf showed significant decrease in mitotic index. The IC_{50} value of ethanolic, and water extracts were 100.6 g/mL, 112.7 g/ml respectively in Ehrlich's ascites carcinoma cells. Fluorescence microscopy analysis showed significant increase in apoptotic cell death which was further confirmed through the flow cytometry analysis, which showed that the ethanolic extract arrests the cells in G_0/G_1 phase of the cell cycle. The *in vivo* study illustrated significant increase in the survival time and reduction in the tumor induced angiogenesis in Ehrlich's ascites carcinoma bearing mice after the treatment of the extracts.

Of this work the ethanolic and water extracts of *Eupatorium ayapana* leaf reveal antimitotic, cytotoxic and apoptotic potential on the Ehrlich's ascites carcinoma.

Keywords: *Eupatorium ayapana;* Ehrlich Ascites Carcinoma; Antimitotic; Apoptosis; Angiogenesis.

Introduction

Cancer, a deadly disease, is increasing worldwide and it is one of the leading causes of death [1]. Besides chemotherapy, radiotherapy and surgery in the treatment of cancer, development of anticancer drug having no side effect, is a challenge to the modern science [2]. However, due to the side effects of drugs used in the chemotherapy for different cancers, traditional herbal medicine, complementary and alternative medicines are becoming increasingly effective and popular among cancer patients in the developed countries. [3]. Herbal medicine has been used since ancient times to treat malignancies [4]. Systematic characterization of active phytochemicals in medicinal herbs and elucidation of their mechanisms of action are important to establish their efficacy [5] and it is also important to transform herbal practices into evidence-based medicine [6]. Plant-derived natural 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 properties *in vitro*, *in vivo*, or in both [7,5]. India is a rich source of medicinal plants. The rich and diverse plant sources of India are likely to provide effective cancer therapeutic agents.

Eupatorium ayapana is an ornamental perennial herb with aromatic leaves of family Asteraceae. Ayapana is native to South America and can be found in the Amazon region of Brazil, Ecuador, Peru, and the three Guyanas. It has been introduced into the U.S. and can be found in Puerto Rico and Hawaii and it has naturalized in 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, tonic, antitumorous, anticoagulant, depurative, cicatrizant, astringent, febrifuge, diaphoretic and emollient properties [8].

Ayapana is a rich source of naturally occurring coumarins. Coumarin has clinical value as the precursor for several anticoagulant drugs; most notably, one widely prescribed drug is warfarin. Ayapanin and ayapin are two coumarins from *Eupatorium ayapana.* These chemicals were reported to have pronounced blood-thinning or anticoagulant actions [9]. Ayapana also contains a coumarin named hernarin (7-methoxycoumarin) which exhibits antitumor property. This chemical was toxic to multi-drug resistant cancer cells [10] and leukemic cells [11]. Eupalinin A, a natural phytoalexin from *Eupatorium* chinense L., exhibited a marked inhibitory effect on cell growth in HL60 cells by induction of caspases, Bcl-2 family proteins, MAP kinase, and PI3K/Akt, and

mitochondrial membrane potential [12]. *Eupatorium ayapana* showed antimicrobial activity against different bacteria [13]. The water and ethanolic extracts of *Eupatorium ayapana* leaf exhibited antioxidant activities in Ehrlich's ascites carcinoma-bearing Swiss albino mice [14]. Natural agent 'thymoquinone' which is an important component of *Eupatorium ayapana* [10] is reported to inhibit the growth of many different types of tumor cells [15]. However, the antineoplastic effects of *Eupatorium ayapana* on Ehrlich's ascites carcinoma cells (EAC) are not explored so far.

Realizing these phytomedicinal properties of *Eupatorium ayapana*, the present study was carried out to evaluate the antimitotic, antineoplastic, and antiangiogenic activities of leaf extracts of *Eupatorium ayapana* against Ehrlich's ascites carcinoma (EAC) cell in both *in vitro* and in *vivo*.

Materials and Methods

Chemicals

Distilled water, Normal saline, PBS, MTT :(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium), dimethylsulfoxide(DMSO), propidiumiodide(PI) , Proteinase-K, RNase- A, and agarose (Sigma–Aldrich), Fetal bovine serum (Hyclone), and RPMI-1640 media(Invitrogen, India), Tris–HCl, HCl, acetic acid, orceine, EDTA, ETBR, PI, Triton X- 100.

Collection and preparation of plant materials

Plant materials

Leaves of *Eupatorium ayapana* was collected from different places of Purba and Paschim Medinipur district of West Bengal, India and authenticated (A voucher specimen No.BOT/VU/3, Collection date 15.03.2011) by Department of Botany, Vidyasagar University, Midnapore, West Bengal, India.

Extraction

At first, leaves of *Eupatorium ayapana* was shade dried at room temperature. Then it was allowed to crush in an electric grinder. 250 gm of pulverized powder was suspended in 450 ml of solvent (ethanol and water) for 48 hours in room temperature by frequent stirring. The extract was then filtered through filter paper separately. The filtrates were concentrated with a rotary evaporator under reduced pressure at 40^0C. The concentrated filtrates were then poured in petridishes and were incubated at 37^0C for drying to afford crude water (WEEAL) and ethanolic (EEEAL) extracts of *Eupatorium ayapana* leaf and stored in airtight container at 4^0C [14].

Animals

Male Swiss albino mice weighing between 20-25 g were used for the present study. 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. The mice were allowed free access to standard dry pellet diet and water *ad libitum*, and were acclimatized to laboratory conditions for 10 days before the beginning of the experiment. All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics committee.

Tumor cells

Ehrlich's ascites carcinoma (EAC) cells were collected from Dept. of Biotechnology, Indian Institute of Technology (IIT), Kharagpur. Cells were maintained by weekly intraperitoneal transplantation in the abovesaid mice at the concentration of 2 x 10^6/cells/mouse. EAC cells were harvested from mouse peritoneum after 7-10 days of inoculation. The washed and viable cells free of contaminating RBC were taken in 0.9gm% NaCl solution for transplantation. By trypan blue exclusion assay, 99% viability of EAC cells was found.

Study of mitotic index

The red variety of *Allium cepa* was collected from local market. The bulbs of *Allium cepa* were sprouted in water saturated sand tub for 3 to 4 day at room temperature. The roots thus developed were treated by dipping these in the EEEAL and WEEAL solution at the concentration of 100mg/ml for 24 hours. Treatment of roots with distilled water served as control. The roots tips treated with above solutions were cut and transferred to fixing solution, 1:3 v/v acetoalcohol for 1 hour in room temperature. Then the root tips were taken out and preserve in 70% alcohol in refrigerator. At the time of staining the root tips were taken out from 70% alcohol and immerge in 2% aceto-orceine and 1N hydrochloric acid and boiled at the smearing point. Then the root tips were placed on greeze free slide and 2% acetic acid was added with it and covered with cover slip. The slide was then squashed to prepare smear and observed under microscope. The numbers of cells in each stage of cell division were counted in four fields for each group (16).

$$\text{Mitotic index} = \frac{\text{Number of dividing cells}}{\text{Number of Total cells}} \times 100$$

In vitro assay for cytotoxic activity (MTT assay)

Cytotoxicity of the extracts were evaluated by using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide dye) assay in Ehrlich's ascites carcinoma (EAC) and normal mice lymphocyte cells (MLC). EAC cells and mouse lymphocyte cells were isolated from EAC-bearing mice and peripheral blood of a normal adult

mouse respectively. EAC cells and mice lymphocyte cells (MLC) were seeded at the concentration of 2.5 10^4 cells/ml in RPMI 1640 medium (10% FBS and antibiotic solution) in culture plates with various concentrations (25, 50, 75, 100 and 125 μg/ml) of WEEAL, EEEAL. The culture plates were incubated for 24 hours at 370C and under 5% CO2. After incubation, 10 μL of MTT (5mg/ml in PBS) was added to each well and it was incubated for additional two hours at 370C.The formazan crystals formed were dissolved in 100 μL of DMSO and incubated for 30 minutes at 370C [17]. The absorbance (abs) of the solution was read at 570 nm using a microplate reader (Microscan (MS5608A), India). Three independent experiments were done. The inhibitory concentration (IC_{50} value) was obtained by the formula mentioned below:

$$\% \text{ inhibition} = \frac{\text{Control abs}}{\text{Control abs}} \quad 100$$

Morphological analysis by polarizing microscopy

Ehrlich's ascites carcinoma (EAC) cells were treated with EEEAL, WEEAL at the IC_{50} concentrations in FBS-RPMI media for 24hr. After incubation changes in cell morphology were analyzed under polarizing microscope (Leica, Germany) [18].

Cell cycle analysis (FCM)

Cell cycle was analyzed by flow cytometry (FCM). The washed and viable cells free of contaminating RBC were taken and subjected to cell culture media containing 8% FBS, 1.3gm% DMEM under 5% CO2 and humidified environment. Each well contains 4 104 cells and cells were treated with EEEAL and WEEAL at the concentration of 100µg/ml for 24 h. The EAC cells were harvested and fixed in 70% chilled ethanol (stored at 200C). Then, the cells were washed with ice cold PBS (10mM, pH 7.4) and resuspended in 200µl of PBS followed by incubation with 20µl DNAse free RNAse (10mg/ml) and 20µl of DNA intercalating dye propidium iodide (PI) (1 mg/ml) at 37^0C for 1 h in dark. Apoptotic cells were determined by their hypochromic sub-diploid staining profiles. The distribution of cells in the different phases of cell-cycle was analyzed from the DNA histogram using Becton-Dickinson FACS Caliber flow cytometer and Cell Quest software [19].

Nuclear analysis by fluorescence microscopy by propidium iodide

Nuclear analysis was performed according to the method of Kim M.J. et al. 2006 (20). In brief, after the treatment(at IC_{50} concentrations of EEEAL and WEEAL), EAC cells were washed with PBS and smears were made on clean glass slides, cells were fixed with 70% chilled ethanol for 3h in -20^0C, washed with chilled PBS and stained with 1mg/ml propidium iodide-RNAse mixture for 45min at 37^0C. The cells were then washed with PBS and examined by fluorescence microscopy (Leica, Germany). Apoptotic cells were identified on the basis of chromatin condensation

Tumor growth inhibition experiment in vivo

To study the tumor growth inhibition experiment in vivo, 54 male mice were taken. Thirty mice were used to study the body weight change and angiogenesis, another 24 mice were used for the assay of mean survival time and increase in life span.

Change in Body weight

Thirty male mice were divided into five groups (n=6). All the mice were inoculated with EAC cells ($2x10^6$ cells/mouse) intraperitoneally except the mice of the control group and this was taken as day of zero. After 24 h of tumor cell inoculation, EEEAL and WEEAL, 5-fluorouracil and normal saline were injected intraperitoneally in Group 3, Group 4 and Group 5, group1 respectively for subsequent 14 days. All the extracts were injected at the dose of 150mg/kg body weight of mice and the standard drug was given at 20 mg/kg body weight, normal saline was given at the dose of 5 ml/kg body weight to the saline control group. Tumor growth was monitored by daily body weight change from the day of zero to the day of sacrifice [21].

Studies on host survival time

The survival time of host mouse was assayed [22] by recording the mortality daily for 6 weeks using the following equations.
MST= (Day of first death + Day of last death)/2

Preliminary study on angiogenesis

After the treatment for 15 days, mice of all groups were sacrificed and peritoneums were collected. Mouse peritoneum was washed three times by PBS and spreading was done by using ball pin on the dissecting tray. Angiogenesis of blood vessels was minutely observed by eye and photographs were taken.

Statistical Analysis

Statistical analyses were performed using Student's *t*-test. Results are given as mean ± SEM or expressed as relative percentage mean. Statistical analysis was done by Analysis of variance (ANOVA) followed by Student's t-test. The difference was considered significant when $p<0.05$.

Result

Mitotic index

Table-1: Mitotic index of *Alium cepa* root tips treated with ethanolic and water extracts (EEEAL and WEEAL) of *Eupatorium ayapana.*

Treatment	Total No. of cells in a microscopic field (A)	Dividing stages				Total No. of dividing cells (B)	Mitotic Index(%) B/A x 100
		Prophase	Metaphase	Anaphase	Telophase		
Control(Water)	40	6	4	7	6	23	57.5
EEEAL	40	11	3	1	1	16	40*
WEEAL	40	12	3	1	3	19	47.5*

Values are expressed as mean ± S.D. of three readings; *p<0.05, compared with control group

Mitotic index of root tips of *Allium cepa* treated with EEEAL and WEEAL at concentration 100mg/ml was observed to be 40 and 47.5 respectively (Table 1). The mitotic index of groups treated with EEEAL and WEEAL showed significantly lower mitotic index compared to control group. The maximum percentage of extracts treated root tips cells were observed to be in prophase.

Inhibition of growth and proliferation by EEEAL and WEEAL

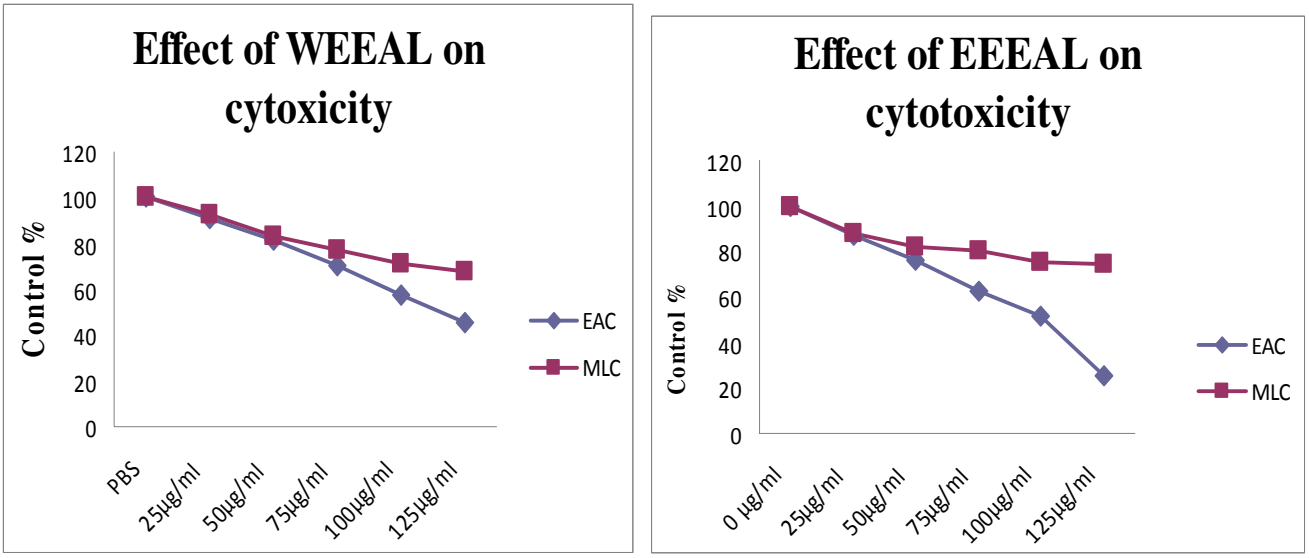


Figure.1: Dose-dependent growth inhibition of EAC cells by EEEAL and WEEAL.

The EEEAL and WEEAL were screened for their cytotoxicity by using MTT assay at different concentrations (25-125 μg/ml) to determine the IC_{50} value in EAC cells. The EEEAL, WEEAL induced cytotoxic response in a dose-dependent manner with the half maximal inhibitory concentration (IC50) were of 100.6 and 112.7 μg/ml respectively against EAC cells (Figure 1). In the case for normal mouse lymphocyte cells, EEEAL and WEEAL exhibited no cytotoxicity even at their higher dose of 125µg/mL.

Morphological analysis

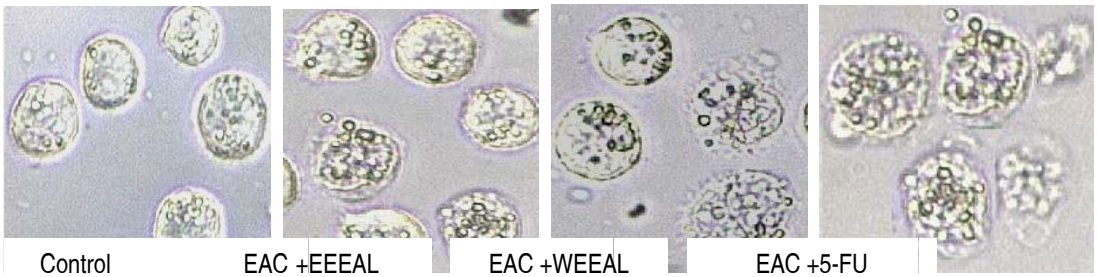
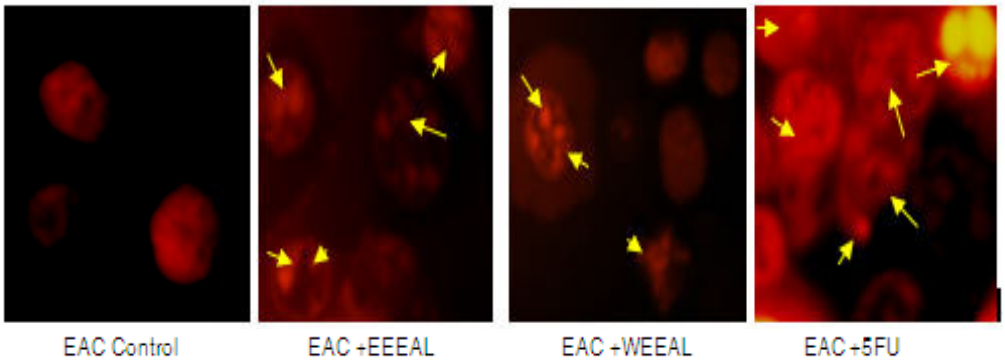


Figure.2: Polarizing microscopic photomicrographs (Leica 46) of EAC cells treated with EEEAL and WEEAL for 24 hours.

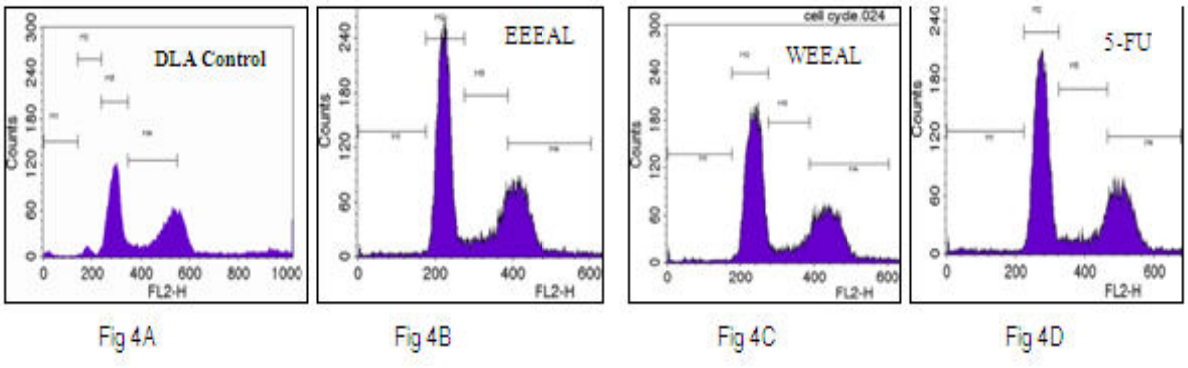
Phase-contrast microscopic analysis of EEEAL- and WEEAL-treated EAC cells revealed significant morphological changes in EAC cells treated with different concentrations of each drug as compared to respective controls. These changes included formation of prominent apoptotic blebs on their cell membrane. In contrast, the EAC-control cells showed normal morphology.

Chromatin condensation



Fluorescence microscopic study was done to observe the chromatin condensation of EEEAL and WEEAL treated and PI stained cells. The control EAC cells showed no condensation of nuclear chromatin but the EEEAL, WEEAL and 5-FU treated EAC cells showed prominent chromatin condensation.

Study on cell cycle analysis



Figire 4: Flow cytometry-based study of apoptotic cell death in EAC cells treated with EEEAL and WEEAL.

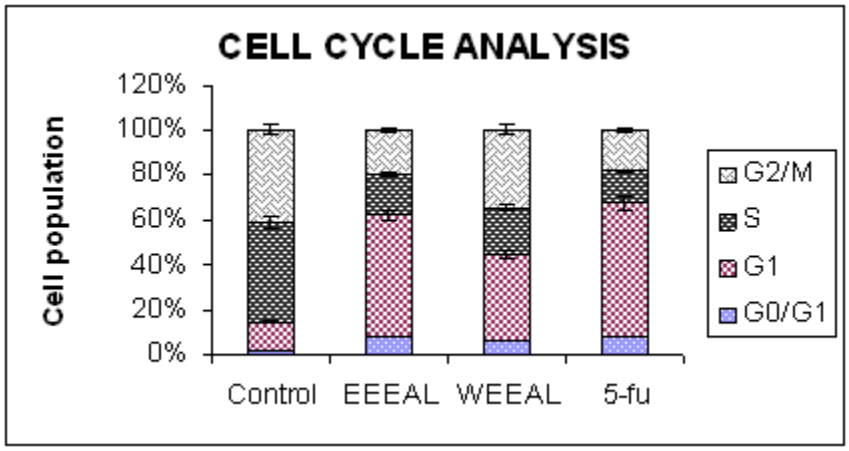


Figure 5 shows the comparative cell cycle analysis in EEEAL and WEEAL-treated EAC cells.

Flow cytometry-based study of apoptotic cell death was done to observe the cell distributions among sub-G_0, G_0/G_1, S and G2/M phases. Cells were treated with the IC_{50} concentrations of EEEAL and WEEAL for 24 hours. In case of EAC-control, maximum cell population was found in 's' phase. EEEAL and WEEAL treated cells were found to be arrested in 'G_1'phase of cell cycle. Accumulation of cells at G1 phase was significantly greater in cells treated with either drug than in EAC-control corroborating the results of the MTT cell proliferation assay.

Angiogenesis

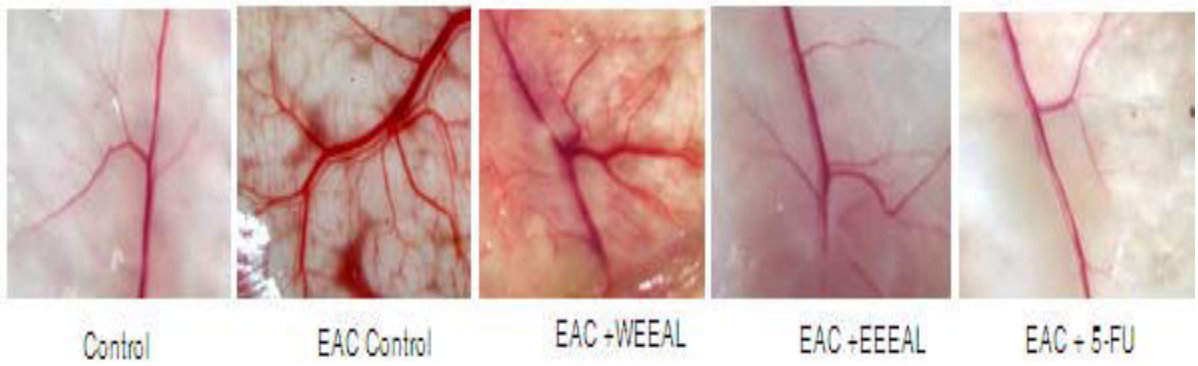


Figure.6: The effect of EEEAL and WEEAL on blood vessel regression in EAC bearing mice.

The distribution of blood vessels in mouse peritoneum was observed by simple angiogenesis study. The peritoneum of untreated EAC bearing mice contained maximum number of blood vessels. Due to this the peritoneum appeared in predominant red colour. But significantly less number of blood vessels was found in the peritoneum of EEEAL, WEEAL and standard treated mice.

Body weight

Body weight of the mice of tumor control group increases significantly compared to saline control group, but in extract-treated groups, body weight decreases significantly.

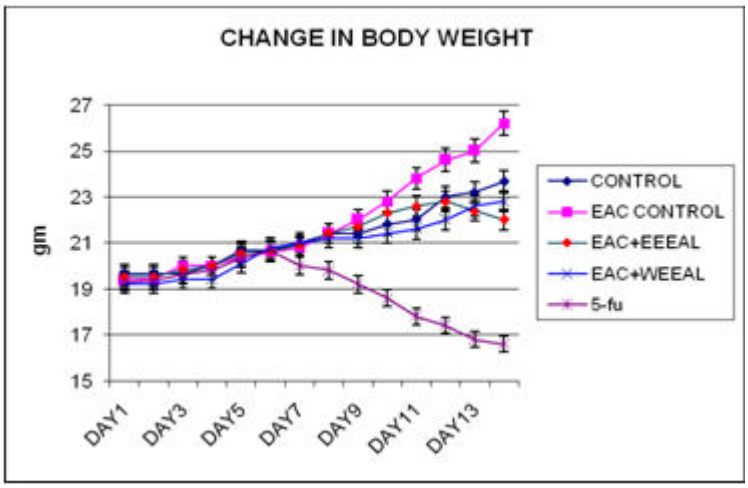


Figure.7 shows the antiproliferative effects of EEEAL, and WEEAL in EAC bearing mice. Data are expressed as Mean ± SEM.

Effect on mean survival time

The mice of the EAC-control group survived for a period of 25.75 ±1.03days. Treatment with EEEAL and WEEAL at the dose of 150mg/kg body weight increased the mean survival time (MST) by 47.25±1.89, 39.42 ±1.6, 50.26±2.01 days (Figure 8), respectively. The EEEAL was found to be the more potent in inhibiting the proliferation of EAC.

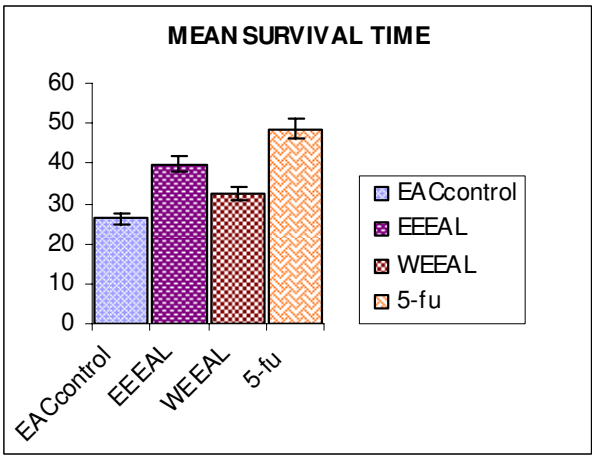


Figure.8 shows the effect of EEEAL and WEEAL on mean survival time (MST) of EAC bearing mice. Data are expressed as Mean ± SEM.;'t' statistically significant versus EAC control group".

Discussion

Retarded apoptosis and accelerated angiogenesis are important features of tumor development [23]. Induction of apoptosis in cancer cells and inhibition of tumor angiogenesis may halt tumor growth and decrease the metastatic potential of tumors, and together they may be one of the useful strategies for anticancer drug development [24]. In the present study EEEAL and WEEAL were treated against Ehrlich's ascites carcinoma (EAC) cells in vitro and in vivo to find out the apoptotic and antiangiogenic properties of leaves of *Eupatorium ayapana* extracts.

Phase specific cell division study was carried out to evaluate the antimitotic properties of EEEAL and WEEAL. Mitotic index of root tips of *Allium cepa* treated with EEEAL and WEEAL showed significantly less mitotic index compared to distilled water treated group (Table-1). Between the two extracts, EEEAL showed the highest degree of suppression in cell division. The maximum percentage of root tip cells was observed to be in prophase, indicating inhibition of transition from prophase to metaphase and to subsequent phases. *Eupatorium* contains various flavonoids, alkaloids which may suppress the cell division of *Allium cepa* and may be responsible for antimitotic property.

By *in vitro* cell viability assay, it was observed that EEEAL, and WEEAL decreased the viability of Ehrlich's ascites carcinoma cells in a dose dependent manner without affecting the normal mouse lymphocyte cells. The ethanolic extract showed prominent cytoxicity towards the EAC cells but did not induce cytotoxicity in a nonmalignant cell line even at higher concentration (Figure.1).

Previous studies have shown that anticancer agents exert anticancer property by the process of apoptosis [25]. Apoptosis is the best described path way of programmed cell death, whereas necroses invoke allergen reaction and damage the neighboring cell [26]. During apoptotic cell death, there is activation of endogenous nuclease(s) which cleaves DNA into oligonucleosomal fragments. This phenomenon is associated with the appearance of dense chromatin aggregates and ultimately leads to the fragmentation of nucleus into dense granular particles [27]. Membrane blebbing, chromatin condensation are the common characteristics of apoptosis [28]. Prominent apoptotic blebs were seen in treated groups, whereas control cells maintained its typical morphology (Figure. 2). The propidium iodide (PI) staining of EEEAL- and WEEAL-treated EAC cells showed typical apoptotic morphology with brightly red, condensed nuclei (intact or fragmented) and formation of apoptotic bodies compared to the EAC control cells

with round intact red nucleus (Figure-3). Increase in apoptotic bodies were seen in EEEAL-treated group compared to other. When the process of apoptosis proceeded chromatin undergoes a phase change from a heterogeneous, genetically active network to a highly condensed genetically inactive form which is subsequently fragmented and packaged into apoptotic bodies [29]. Morphology of EAC cells in treated groups exhibited apoptotic bodies, nuclear condensation, and inter-nucleosomal fragmentation.

Further, apoptosis was confirmed from apoptotic phase in cell-cycle analysis. In case of cell cycle analysis significant number of cells was found to be arrested at G_0/G_1 phase, when treated with EEEAL and WEEAL. In control group most of the cells were found in the S phase. This observation revealed that EEEAL and WEEAL arrested the cell cycle prior to the DNA synthesis and EEEAL exhibited the highest efficacy. The role of different proteins involved in the induction of G_0/G_1 phase arrest in EAC cells still needs further investigation.

EAC bearing mice were treated with EEEAL and WEEAL to investigate the change in body weight, tumor induced angiogenesis and mean survival time. Peritoneal fluid is the direct nutritional source for ascites tumor cells and a rapid increase in peritoneal fluid also increase the tumor growth that indicates the nutritional requirement of tumor cells from peritoneal fluid [30]. Body weight increased rapidly in case of tumor bearing mice. This is due to rapid increase in peritoneal fluid volume along with tumor cells. In EEEAL- and WEEAL-treated mice, body weight decreased significantly (Figure-7). EEEAL- and WEEAL-treated mice survived a significantly longer life span compared to tumor-bearing mice. Development of tumor is not only the excessive proliferation of tumor cell but it is the summative increase of tumor cell, blood cell, tissue fluid and formation of blood vessel. Tumor angiogenesis is a critical component of tumor growth and metastasis. [31]. Massive increase in number of blood vessels in the peritoneum of EAC control mice compared with extracts-treated mice (Figure 6). This may be due to the antiangiogenic potential present in EEEAL and WEEAL.

The phytochemical study reported that *Eupatorium ayapana* leaf contains flavonoids, coumarin thymoquinone, daphnetin, alpha-terineol [10]. Flavonoids have been shown to possess antimutagenic and antimalignant effects. Further, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation [32] and angiogenesis. [33]. Thus, in our study, antitumor effects produced by EEEAL and WEEAL may be due to the presence of flavonoids and thymoquinone in *Eupatorium ayapana*.

Conclusion

From the present study it can be concluded that the EEEAL and WEEAL are effective against EAC cell in both in *vitro*& *in vivo*. The extracts exhibited antimitotic, cytotoxic and antiproliferative property. Beside this, they have also an observable antiangiogenic property, which is one of the most essential properties of any anticancer chemotherapeutic agent.

So Eupatorium ayapana leaf is a potent antitumor natural remedy against Ehrlich's ascites carcinoma (EAC), and viewing the overall result it can be concluded that among the two extracts, EEEAL is more potent than WEEAL.

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