

Evaluation of Anticancer activity of *Aerva Sanguinolenta* (L.) (Amaranthaceae) on Ehrlich's Ascites cell induced Swiss Mice

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Abstract

The aim of this study is to investigate the effects of both ethanolic and aqueous extracts of *Aerva sanguinolenta* (Family: Amaranthaceae) by intraperitoneal route to Ehrlich ascites tumor bearing mice. After determination of acute toxicity [LD₅₀ = 2250mg/kg body weight which was given through i.p. route] of the plant extract on Swiss albino mice. The protocol started with tumor inoculation of 2x10⁶ cells i.p. After 24 hrs of tumor inoculation, ethanolic, aqueous extracts and vinblastine (standard drug) were administered intraperitoneally at a dose of 200 and 400mg/kg body weight of extracts and 1mg/kg body weight of vinblastine. After treatment of nine consecutive days and after 24-hours of last dose and 18-hours fasting, half of the animals in each group were weighed and sacrificed on the 10th day. i.p. fluid was collected for viable (cancer) and nonviable (dead) cell count, intraperitoneal fluid volume and packed cell volume and blood was collected by heart puncturing for determination of hemoglobin concentration, RBC count, and WBC count respectively. Half of the remaining animal groups were kept for survival time (days) determination. Ethanolic extract produced remarkable reduction in number of viable cells ($p < 0.01$) and increased the percentage of life span. From the result it was found out that the ethanolic extract of the plant has potent dose dependent antitumor activity and that is comparable to that of vinblastine.

Key words:

Aerva sanguinolenta, EAC cell line, Intraperitoneal fluid volume, Vinblastine, Survival time

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Introduction

The plant *Aerva sanguinolenta* family-
Amaranthaceae, is a perennial herb. The plant is
available in tropical countries of Asia such as
Bhutan, India, Nepal, Pakistan and also in China,

Malaysia and Indo-China regions. In China it is known as Bai-hua-mi, in Maharashtra (India) as Burval, in Uttarakhand (India) as Sufedphulia and in Assam (India) as Soru-araksan.

In folklore medication leaf and flower of the plant were used as wound healing and anti-inflammatory for injuries from falls, rheumatic arthritis and pain in muscles [1], the whole plant was used as diuretic and demulcent [2], tender shoot of the plant used as decoction form for galactogue to nursing mother [3] and decoction of whole plant was taken twice a day to expel intestinal worms [4].

Leaves and root of the plant have been used traditionally for body pain and the paste of leaf and root is applied to affected area [5]. The plant extract showed significant wound healing property [6]. Other plants of this family have shown antimicrobial [7] and pharmacological properties such as cytotoxic [7], hepatoprotective [8], nephroprotective [9][10] and hypoglycemic [11]. The aim of the present study is to investigate the anticancer property of the plant extracts. Both ethanolic and aqueous extracts were used to evaluate *in vivo* anticancer activity and ethanolic extract produced profound anticancer activity. Cancer is a disease in which there is uncontrolled proliferation and spread within the body of abnormal forms of the body's own cell [12].

In developed countries at least one in five of the population can expect to die of cancer. Few categories of medications are commonly used with a narrow therapeutic index and have a greater potential of causing severe side effects. As the synthetic antineoplastic drugs possess comparatively more adverse effects, so herbal drugs are being evaluated as these are comparatively safe or non-toxic to the host cell.

Material and Methods

Collection, identification and extraction of the plant material

Aerial part of *Aerva sanguinolenta* was collected in the month of September 2009 from different part of Midnapur district, West Bengal, India and botanically identified and authenticated by the Botanical survey of India, Botanical garden, Howrah, West Bengal, India (Vide Ref. no.-CNH/I-I/44/2009/Tech.II/124). Voucher specimen has been kept in our laboratory for future reference.

Thereafter the whole aerial parts were dried under shade and pulverized and extracted chronologically with menstrua in an order from low polarity to high polarity i.e. Petroleum spirit, Chloroform, Ethyl acetate, Ethanol and Water. Fatty or waxy materials and pigments present in the leaves are generally removed by Petroleum spirit [13]. Except aqueous extract, other extracts were dried under reduced pressure at temperature below 40°C through Eyla Rotary Evaporator (Japan) [14] and the dried extract were kept properly. The aqueous extract was evaporated by using Rotating vacuum evaporator. All solvents (Analytical grade) were purchased from Merck (Mumbai).

Chemicals

The chemicals used were sodium chloride, propylene glycol, trypan blue, methyl violet, sodium sulphate, methylene blue (MERCK Limited, Mumbai, India) and Cytoblastine (Vinblastine Sulphate) from Cipla Limited, Goa, India. All other chemicals and reagents used were of highest analytical grade.

Experimental animals

Male Swiss albino mice (20-25g) were grouped and housed in poly acrylic cages (38x23x10cm) 6animal/cage under standard laboratory condition (temperature 22-25°C, humidity 40-60% and dark/light cycle of 12 hours). The animals had free access to food (standard dry pellet diet, Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were allowed to acclimatize to laboratory condition for 7days before

commencement of the experiment. The animals were housed and used accordance with the guidelines of the **Institutional Animal Ethical Committee. Vide Reg. No. {(0367/01/C/CPCSEA) India}.**

Acute toxicity test

Male Swiss albino mice were divided into 10 groups of 10 animals each. The control group received 1ml/100g body weight of 0.9 % NaCl I.P. The other groups received 500,750, 1000, 1250, 1500, 1750, 2000, 2250 and finally 2500mg/kg body weight I.P. of ethanolic extract of *Aerva sanguinolenta*. At a dose of 2500mg/kg body weight all the animals were died within 48hrs but at the dose of 2250mg/kg body weight only half of animals group were died, so this dosage is determined as LD₅₀ of ethanolic extract [15].

Anticancer activity

The EAC cell line was maintained in the ascetic form by the sequential passages in Swiss mice, by means of every 10 days i.p transplantations of 2x10⁶ tumor cells. The animals were divided into seven groups of 20 animals in each and labeled accordingly. All the groups except the group-I (normal saline control) were inoculated with 2x10⁶ tumor cells suspended in 0.2 ml of phosphate buffered saline (PBS) solution (pH 7.2-7.4) i.p. The ascitic fluid was collected using a syringe and the tumor cell count was performed in a Neubauer Hemocytometer (MARIE field, Germany), using the Trypan Blue Dye exclusion method [16] [17]. This was considered as day '0'. Group II represent EAC control. From day '1' to day '9' standard drug Vinblastine (1mg/kg body weight), ethanolic extract 200 and 400mg/kg body weight and aqueous extract 200 and 400 mg/kg body weight were administered I.P. to standard group and test groups respectively for nine consecutive days. Half of the mice in each group were weighed and sacrificed on the 10th day after 24hrs of last dose administration and 18hrs

fasting by cervical dislocation [18]. Blood was collected directly by heart puncturing. Viable cell, non viable cell, i.p. fluid volume and packed cell volume were determined by I.P fluid collection. Haemoglobin, RBC and WBC counting were performed from blood [19] [20]. Mean survival time and increase in life span was determined by keeping half of animal groups with free access to food and water *ad libitum* [21] [8].

Tumor volume determination

The mice were dissected and ascitic fluid was collected from peritoneal cavity. With the help of graduated centrifuge tubes, I.P. fluid volume, packed cell volume was measured by centrifuging at 1000 rpm for 5min [19][20].

Tumor cell count

The ascitic fluid was diluted 100 times, 0.4% trypan blue stain was added, waited for 10 minutes and one drop was placed in Neubauer Hemocytometer (MARIE field. Germany) and the number of viable and non viable cells were counted. The non viable cells (shrinked and crenated) accept the stain and viable cells appear as colorless droplets [19][20]. Here, cell count = (Number of cells × dilution factor) / (Area × thickness of liquid film).

Percentage increase in life span

Mortality time was noted in standard, test (Ethanolic & aqueous extract) and EAC control groups. Percentage of increase in life span was calculated as follows [22] [23].

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

Mean survival time* = (first death + last death)/2

*time denoted by days.

Composition of phosphate buffer-saline (PBS)

Sodium chloride – 8.00 gm/L

Potassium chloride – 0.2 gm/L

Disodium hydrogen phosphate –1.44 gm/L
Potassium dihydrogen phosphate – 0.24 gm/L

Hemoglobin estimation

20µl of heparinized blood was transferred in Sahli's Hemoglobinometer, diluted and mixed with 0.1 (N) HCL till the color match with the standard color [24].

RBC count

Blood sample was diluted 1:100 with RBC diluting fluid, using Thoma pipette. It was mixed properly and a drop of the mixture was dropped gently under the cover slip of the Neubauer Hemocytometer and after the settlement of red corpuscles for three minutes, the number of RBC cells was counted in 5×16 small squares under binocular microscope, Magnus-ICON [19][20].

Composition of RBC diluting fluid (for 100ml distilled water)

Sodium chloride – 0.5 gm (maintain isotonicity)
Sodium sulfate – 2.5 gm (fix the corpuscles)
Mercuric per chloride – 0.25 gm (preservative)

WBC count

Blood sample was diluted 1:20 with WBC diluting fluid, using Thoma pipette. It was mixed properly and a drop of the mixture was added gently under the cover slip of the Neubauer Hemocytometer

and after the settlement of white corpuscles for three minutes, the number of WBC cells was counted in 4×16 large squares under binocular microscope [19][20].

Composition of WBC diluting fluid (for 100ml distilled water)

Glacial acetic acid – 2cc. (hemolysing RBC)
Trypan blue – 0.025 gm (staining the corpuscles)

Statistical analysis

All data are expressed as mean±S.E.M. (n = 10 mice per groups). Statistical significance (p) calculated by one-way ANOVA between the treated groups and the EAC control followed by Dunnett's Post Hoc Test of significance where p < 0.05 and p < 0.01 considered to be significant and highly significant, respectively.

Result

After treating of the Ehrlich ascites tumor bearing mice with ethanolic and aqueous extract of *Aerva sanguinolenta* at a dose of 200 and 400mg/kg body weight i.p, the effect of ethanolic extract was more prominent. Ascitic cell volume and packed cell volume are reduced. Viable cell count was reduced and non viable cell count was increased compared to that of control group. Life span was also increased comparable to that of the standard drug (Vinblastine) (Table-I).

Table-1: Representing the effect of ethanolic extract of *Aerva sanguinolenta* on I.P. fluid parameters and life span.

| Groups | I.P. fluid parameters | | | | Other parameters | |
|---|-------------------------|------------------------|------------------------------|-----------------------------|--------------------------|-------------------------|
| | Ascitic cell volume(ml) | Packed cell volume(ml) | Viable cell count/ml | Non viable cell count/ml | Mean survival time(days) | % increase in life span |
| EAC control | 2.8±0.05 | 2.2±0.04 | 6.42×10 ⁷ ±0.13 | 1.02×10 ⁷ ±0.005 | 20 | 0 |
| Ethanolic extract(200mg/kg body weight) | 1.36±0.016* | 0.7±0.005* | 2.68×10 ⁷ ±0.05* | 3.1×10 ⁷ ±0.08* | 28 | 40 |
| Ethanolic extract(400mg/kg body weight) | 0.94±0.036* | 0.41±0.015* | 1.72×10 ⁷ ±0.21* | 3.69×10 ⁷ ±0.05* | 35 | 75 |
| Vinblastine(1mg/kg body weight) | 0.48±0.007** | 0.24±0.014** | 1.3×10 ⁷ ±0.008** | 3.8×10 ⁷ ±0.03** | 38 | 90 |

All values are expressed as Mean±S.E.M. (n=10mice per groups).
*(p) < 0.01 while treated group compared to EAC control

** ($p < 0.01$) while treated group compared to EAC control

The ethanolic extract of *Aerva sanguinolenta* also restored the hematological parameters towards the saline control. The number of RBC count and hemoglobin content also increased (Fig.1 and 2) but the WBC count was decreased as compared to that of EAC control (Fig. 3).

Illustration of figures:

Figure(1) represent the effect of ethanolic extract of the plant *Aerva sanguinolenta* on RBC count (million/ μ l) of each group which highly reduced on EAC cell bearing group and comparatively increased on treated groups with ethanolic extract.

Figure(2) represent the effect of ethanolic extract of the plant *Aerva sanguinolenta* on hemoglobin parameter (g/dl) of each group which in extract treated groups were highly increased compared to that of EAC control group.

Figure(3) represent the effect of ethanolic extract of the plant *Aerva sanguinolenta* on WBC count (thousand/ μ l) of each group and it shows significant reduction in the number of WBC on extract treated groups compare to that of EAC control group.

FIGURE 1: RBC value (million/ μ l)

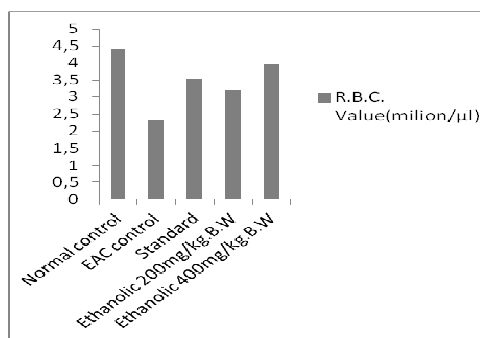


FIGURE 2: Hemoglobin (g/dl)

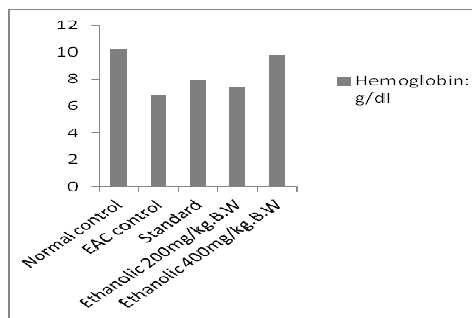
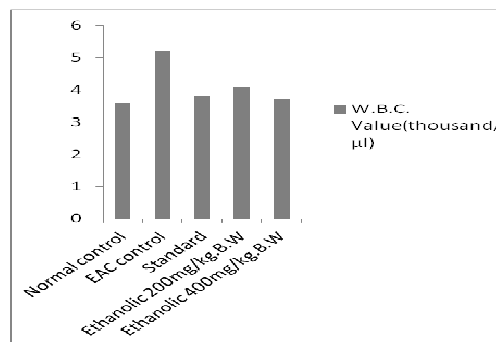


FIGURE 3: WBC value (thousand/ μ l)



Discussion:

The reliable criteria for judging the value of any anticancer drug are prolongation of life span and decrease the WBC [25]. Usually in cancer chemotherapy the major problem is anemia due to reduction in RBC. Present study indicated that ethanolic extract of the plant has significantly enhanced the erythrocyte count and hemoglobin level when compared to that of EAC control. Viable cell count was decreased and non viable cell count was increased in intraperitoneal fluid by ethanolic extract in a dose dependent manner. There is a complex relationship between the components normally present in the plant extracts. The active components of *A.sanguinolenta* are likely to be present in the ethanolic extract. [26].

Intraperitoneal fluid volume and packed cell volume was decreased. This indicated a toxic effect on these cells that resulted in cell death. It signifies that the drug was absorbed directly by the EAC cells in the peritoneal cavity and this herbal drug causes the lysis of the EAC cell by direct cytotoxic mechanism [27].

Ethanol extract normalized hematological parameters like RBC count, WBC count, and hemoglobin level. Thus ethanol extract possesses protective role in hematological parameters. I.P. fluid accumulation is comparatively less than that of the EAC control group. Preliminary phytochemical study indicated the presence of flavonoid, alkaloids and tannins in ethanol extract of *Aerva sanguinolenta*. Flavonoids have been shown to possess antimutagenic and antimalignant effect [28]. Furthermore, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis [29].

Conclusion:

It was found that the ethanol extract of the plant has potent dose dependent antitumor activity and that is comparable to that of vinblastine. The cytotoxicity and anticancer activity of ethanol extract of *Aerva sanguinolenta* is probably due to presence of flavonoids. All of the above data and findings supporting anticancer activity of the plant and increase life span of the tumor bearing object.

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