

Assessment of *in vitro* cytotoxicity and *in vivo* antitumor activity of *Sphaeranthus amaranthoides* burm.f

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ABSTRACT

Background: A number of plants have been used in Indian system of medicine such as ayurveda, unani and siddha, but most of these plants were not explored properly. *Sphaeranthus amaranthoides* (SA) Burm.f., is one such plant used as an energizer in siddha. **Objective:** To evaluate the anticancer effect of chloroform extract of the whole plant of SA Burm.f. against Ehrlich ascites carcinoma bearing Swiss albino mice. **Materials and Methods:** The anticancer effect of chloroform extract was investigated in Swiss albino mice bearing Ehrlich ascites carcinoma at two different dose levels. Acute toxicity studies were also performed to determine the safety of the extract. Mice injected with Ehrlich ascites carcinoma cells were treated with the extract of doses 200 and 400 mg/kg body weight and standard 5-fluorouracil 20 mg/kg body weight for 15 consecutive days. Animals were sacrificed on day 15 for determination of anticancer activity by evaluating tumor volume, nonviable and viable tumor cell count and hematological parameters. **Results:** Mice treated with the extract showed a significant decrease in tumor volume and viable cell count and an increase in nonviable cell count and mean survival time. The hematological parameters were also found to be restored to a normal level. **Conclusion:** The results indicate that the chloroform extract was producing anticancer activity comparable with that of the standard 5-fluorouracil.

Key words: Antitumor, ehrlich ascites carcinoma, *Sphaeranthus amaranthoides* Burm.f

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INTRODUCTION

Cancer is the leading cause of mortality next to cardiovascular disorders. It causes around 6 million death per year, and this number may increase up to 11.5 million by 2030 worldwide.^[1] More than 60% of currently used anti-cancer agents are obtained from natural sources, like plants, micro-organisms, and marine-organisms. Molecules obtained from natural sources have played and continue to play a main role in the invention of leads for the development of conventional drugs for the treatment of a number of human diseases.^[2]

Sphaeranthus amaranthoides (SA) Burm.f., (Tamil-Sivakaranthai) a small procumbent herb belonging to the family *Asteraceae*, was used to cure skin diseases, eczema, acne dermatitis,

and also removes kapha, vata and piles. The flowers of the plant produce cooling and tonic effect. Seeds and root are used as stomachic and anti-helminthic.^[3] Powdered leaf is used for the treatment of urethral discharges and jaundice. The plant was reported to have biological activities such as analgesic, sedative and anti-inflammatory activity.^[4] The plant was also active against a number of bacteria.^[5,6] To investigate the potential role of SA Burm.f. as an anticancer agent, in this study the chloroform extract of the whole plant was tested against Ehrlich's ascites carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

The entire plant of SA Burm. f. was collected from Thuthukudi District of Tamil Nadu in the month of September 2010 and was identified and authenticated by Mr. V. Chelladurai, Retired research officer, Botany, CCRAS, Government of India. A voucher specimen of the plant was preserved in the department for future studies. Air-dried plant material was extracted with

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solvents of increasing polarity such as petroleum ether, chloroform, ethyl acetate and methanol in succession using soxhlet extraction apparatus. After extraction, the solvents were removed under reduced pressure and stored in a vacuum desiccator until use. The chloroform extract was selected for *in vivo* anticancer studies as it showed a good anti-proliferative activity in MTT assay. Preliminary phytochemical studies showed the presence of flavonoids, tannins and alkaloids in the chloroform extract.

Acute toxicity study

Acute toxicity studies were performed as per OECD 423 guidelines. Swiss albino mice (18 ± 2 g) (male, $n = 3$), maintained at a temperature of $22^\circ\text{C} \pm 2^\circ\text{C}$, fed with standard pelleted feed and water *ad libitum* was used for the study. The animals were kept fasting for overnight provided only with water. Then, the extract was administered orally by gastric intubation and observed for 14 days for toxic symptoms like behavioral change, locomotion, convulsion, and mortality.

Anticancer studies

Ehrlich's ascites carcinoma cell line was procured from National Centre for Cell Science (Pune, India). Cells were grown in minimum essential medium supplemented with 15% Fetal Bovine serum and penicillin/streptomycin (100 IU, 100 mg/ml, respectively) at 37°C in 5% CO_2 . Experiment was performed in 60 mm petri plates with cells seeded at a density of 200,000 cells/plate and incubated at 37°C with humidified atmosphere till cells reached confluence.^[7]

In vitro cytotoxicity assay

Pre-confluent EAC cells were seeded in 96-well plates at a density of 8,000 cells/200 μL /well. Cells were treated with different concentrations of chloroform extract after 24 h following plating and incubated at 37°C for one day. At 20 h following drug exposure, the cells were incubated at 37°C with 5 mg/ml MTT for 4 h. At the end of the experiment, the medium was removed, and the insoluble formazan product was dissolved in dimethyl sulfoxide (200 μL) and kept at least 15 min in the dark. MTT reduction was quantified by measuring the absorbance at 570 nm and 630 nm in Thermo Scientific Multiscan ultraviolet spectrophotometer, USA. The assay was performed in triplicates.^[8]

Determination of Ehrlich's ascites carcinoma viability by trypan blue staining

Ehrlich's ascites carcinoma cells were harvested after reaching confluence and washed with PBS, followed by centrifugation at 2500 rpm for 5 min. The cell pellet was suspended in 1 ml of fresh culture medium. The cell suspension was mixed with an equal volume of trypan blue (4 mg/ml) in the ratio 1:1 and incubated for 5 min at

37°C . The estimation of the total number of viable cells was done using hemocytometer chamber.^[9] Percentage of viable cells were calculated by the formula,

$$\text{Percentage viable cells} = [1.00 - (\text{Number of trypan blue stained cells} / \text{Total cells})] \times 100$$

Tumor induction and test drug exposure

Briefly, 0.1 ml (irrespective of body weight) of EAC cells was implanted into the peritoneal cavity of each recipient mouse to develop tumor except the negative control for the development of ascites tumor.^[10] The entire process was approved by the Institutional Animal Ethical Committee IAEC/XXXIII/SRU/251/2013. Animals were distributed into five groups of ten mice of similar weight.

- Group 1: Negative control (no tumor) - received vehicle
- Group 2: Positive control (tumor) - received vehicle
- Group 3: Received 5-fluoro uracil 20 mg/kg body weight ip
- Group 4: Received extract at dose 200 mg/kg body weight per orally
- Group 5: Received extract at dose 400 mg/kg body weight per orally.

After 24 h following induction, vehicle/standard drug/test drug was administered for a period of 14 days. Body weight was recorded once in 3 days throughout the experimental period. Following treatment, blood was collected through retro-orbital plexus for analysis of hematological parameters. Further, half of the animals were sacrificed and liver and spleen tissues were collected for histopathology evaluation.^[11,12]

Tumor volume

After dissection of the mice, the ascetic fluid from the peritoneal cavity was collected in a measuring tube and the volume was noted. It was then centrifuged at 1000 rpm for 5 min and the packed cell volume was determined.

Viable/nonviable tumor cell count

The cells of the ascetic fluid were stained with trypan blue dye during which only the nonviable cell took up the dye. Therefore, the colored cells were nonviable, and the others were viable. Both the viable and nonviable cells were counted.

Increase in life span

All the animals were monitored carefully, and the time of death of each mouse was noted. Percentage increase in lifespan was calculated using the formula:

$$\% \text{ increase in life span} =$$

$$\left\{ \left[\frac{\text{MST of drug treated group}}{\text{MST of control group}} \right] - 1 \right\} \times 100$$

Where MST is mean survival time, which can be calculated by adding the time in the day of the first death and final death and dividing by two.

Effect on body weight

All the animals were weighed on the day of inoculation and then during the postinoculation period once in 3 days. The percentage increase in weight was calculated using the formula:

$$\% \text{ Increase in weight} = \left[\frac{(\text{animal weight on respective day} / \text{animal weight on day-0}) - 1}{1} \right] \times 100$$

Hematological studies

Comparison was made between groups of mice on the fourteenth day after transplantation, to find the effect of the extract on the hematological parameter of EAC-bearing mice. Blood was collected from the retro-orbital plexus of each mouse under ether anesthesia. Determination of total white blood cell (WBC) count, red blood cell (RBC) count, and hemoglobin content was done.

Statistical analysis

Statistical analysis was performed by one-way ANOVA followed by Tukey *post-hoc* analysis using SPSS computer package (SPSS version 16).

RESULTS AND DISCUSSION

In vitro cytotoxicity assay

In vitro cytotoxicity assay of chloroform extract of SA had shown that there was a significant increase in percentage inhibition with increase in concentration. More than 50% of the cells were inhibited by <3 µg of chloroform extract. By increasing the concentration of chloroform extract to 15 µg, around 82% of the cell were inhibited, showing the cytotoxic nature of the extract against EAC cell lines [Figure 1].

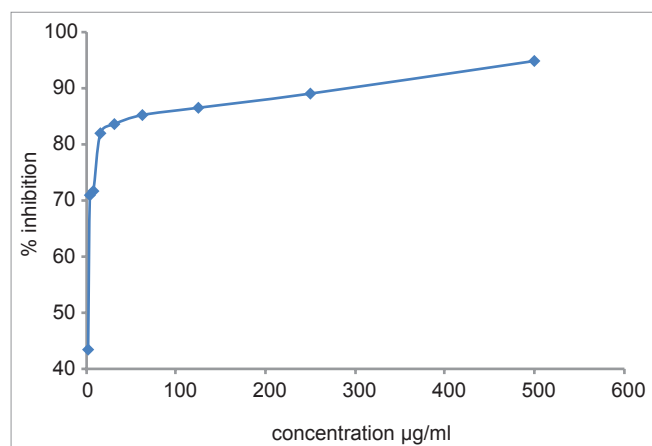


Figure 1: Cytotoxicity of chloroform extract against Ehrlich's ascites carcinoma cell line

In the tumor control mice, the hematological parameters were found to be significantly altered on day 15 when compared to the normal group. There was an increase in total WBC count with a reduction in RBC count and hemoglobin content. The extract treated group showed a significant effect on increasing life span and retrieved all the parameters to the normal level.

In vivo anticancer activity

Weight variation

Significant weight gain of about 62% was observed in tumor inoculated mice by day 15. Administration of 5-fluorouracil significantly reduced weight gain as compared with that of EAC control. The group treated with the extract of 400 mg/kg concentration also shown a reduction in weight gain.

Survival time parameter

Reliable criteria for judging the value of any anti-cancer agent is the prolongation of life span of animals.^[13] The effect of the extract on survival of tumor-bearing mice is shown in Table 1. The mean survival time was prolonged by the administration of the standard drug, 5-fluorouracil. Even the extract significantly prolonged the mean survival time ($P < 0.001$) at both the concentration when compared to control. The increase in lifespan of extract treated animals was found to be more than 50%. By convention, a 25% increase in lifespan is considered as possible anticancer activity of a test compound.

Treatment with extract reduced the tumor-viable count, the ascitic cell volume and packed cell volume in a significant manner ($P < 0.001$) as compared to that of EAC control group. Further, nonviable tumor cell count was increased significantly when compared with the EAC control [Table 2].

Hematological parameters

Table 3 shows the effect of the extract on hematological parameters. Most chemotherapeutic agents induce collateral effects. Direct myelotoxicity is often observed in patients

Table 1: Antitumor activity of *Sphaeranthus amaranthoides* extract with regard to mean survival time and increase in life span

Group	Mean survival time	Percentage increase in life span
EAC control	15	-
Treated with chloroform extract 200 mg/kg	23.6*	57
Treated with chloroform extract 400 mg/kg	27.2*	81
Treated with 5-fluorouracil	29.6*	97

Mean±SEM. $P < 0.001$ compared with EAC control. SEM=Standard error of mean; EAC=Ehrlich's ascites carcinoma. *Represents significant difference at $P < 0.001$ level when compared with EAC control

Table 2: Antitumor activity of *Sphaeranthus amaranthoides* extract with regard to tumor volume, viable tumor cells count and nonviable tumor cells count (mean±SEM, n=5)

Group	Ascitic cell volume in ml	Packed cell volume in ml	Viable cell count/ml	Nonviable cell count/ml
EAC control	5.02±0.14	2.4±0.09	7.52±0.82	0.32±0.02
Treated with chloroform extract 200 mg/kg	1.8±0.05	0.64±0.02	3.42±0.17	1.48±0.15
Treated with chloroform extract 400 mg/kg	1.03±0.01	0.4±0.05	1.52±0.15	2.88±0.16
Treated with 5-fluorouracil	0.86±0.02	0.43±0.01	0.85±0.03	3.46±0.05

Mean±SEM. $P < 0.001$ while treated group compared to EAC control. SEM=Standard error of mean; EAC=Ehrlich's ascites carcinoma

Table 3: Effect of *Sphaeranthus amaranthoides* extract on hematological parameters of Swiss albino mice

Treatment	Total WBC (cells/ml×10 ⁶)	Total RBC (cells/ml×10 ⁶)	Hb content
Normal control	8.28±0.29	4.00±0.16	14.82±0.35
EAC control	17.98±0.13*	2.83±0.09*	10.02±0.18*
Treated with chloroform extract 200 mg/kg	15.36±0.42*	3.22±0.09**	12.44±0.18*
Treated with chloroform extract 400 mg/kg	11.56±0.52*	3.79±0.15	14.02±0.18
Treated with 5-fluorouracil	7.82±0.46	3.94±0.25	16.12±0.35**

Mean±SEM; n=5. $P < 0.001$ when compared to eac control; * $P < 0.05$; **Not significant. SEM=Standard error of mean; EAC=Ehrlich's ascites carcinoma; RBC=Red blood cell; WBC=White blood cell

undergoing anticancer treatments. Hence, the evaluation of toxicity can be accomplished by blood count evaluation.^[14] Induction of tumor increased significantly ($P < 0.05$) the total number of WBC by almost two times. However, the effect was reversed significantly by 5-fluorouracil administration. The extract at both the concentration significantly ($P < 0.05$) reversed the tumor-induced rise in total counts of WBC. However, the effect was less when compared with the standard. There was a significant decrease in RBC and Hemoglobin due to tumor induction. This was significantly ($P < 0.05$) reversed towards normal by standard drug and the tested extract.

CONCLUSION

It was found that the treatment with the drug enhanced the nonviable cell count and decreased the viable cell count. It may be due to the absorption of extract by viable cells that leads the lysis of the cells through the activation of macrophages or some cytokinin production in the peritoneal cavity. These effects were almost comparable to 5-fluorouracil – the standard drug used in the study.

The prolongation of life span (ILS) by treatment with the extract was found to be comparable to that of the standard drug 5-fluorouracil. These findings imply that the extract might be having some anticancer principles.

Further, possible mechanism of the chloroform extract for anticancer effect should be explored.

Inoculation of EAC in the mice caused an increase in body weight, ascites, and the mice became inactive and slow. From the *in vivo* cancer model (Ehrlich ascitic carcinoma model), it was inferred that the extract significantly ($P < 0.05$) reversed the tumor-induced changes in the parameters examined namely % increase in body weight, % increase in lifespan, viable tumor cell count, and hematological parameters (total WBC, RBC, and hemoglobin count).

Anemia and myelosuppression are the major problem encountered in cancer chemotherapy.^[15] The reduction in RBC and hemoglobin percentage in tumor-bearing mice may be due to iron deficiency or due to hemolytic or myelopathic conditions.^[16] Treatment with the extract reversed the blood count and hemoglobin content near to normal values. This indicates that the extract possess a significant anti-cancer activity. The antioxidants present in the plant may be responsible for this effect.^[17]

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