Antitumor activity of *Sida Veronicaefolia* against Ehrlich Ascites Carcinoma in mice

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**ABSTRACT**

The acetone and ethanol extracts from the leaves of *Sida Veronicaefolia* i.e. AESV and EESV respectively were evaluated for antitumor activity against Ehrlich Ascites Carcinoma (EAC) bearing Swiss albino mice. The extracts were administered at the doses of 500 mg/kg body weight per day for 14 days after 24 h of tumor inoculation, 24 h after the last dose, with fasting, the mice were sacrificed. The present study deals with the effect of AESV and EESV on mean survival time, tumor volume, tumor weight, tumor cell count, body weight, peritoneal cell count, haematological studies and *In vitro* cytotoxicity. AESV and EESV caused significant decrease in tumor volume, tumor weight, tumor cell count, body weight and it prolonged the life span i.e. mean survival time of EAC-tumor bearing mice and normal peritoneal cell count in normal mice. Haematological profile converted to more or less normal levels in AESV and EESV treated mice. AESV and EESV also exhibited significant cytotoxic activity at 200 µg/ml, but higher cytotoxic activity was found in AESV. The results indicate that AESV and EESV exhibited significant antitumor activity in EAC-bearing mice.

**Keywords:** Antitumor Activity, *Sida Veronicaefolia*, Ehrlich Ascites Carcinoma (EAC).

**INTRODUCTION**

The use of medicinal plants to treat diseases is as old as human civilization. Human beings of all ages in both developing and undeveloped countries use plants in seek to treat numerous diseases and to get relief from physical ailments. Cancer is a class of diseases in which a cell or a group of cells display uncontrolled growth, invasion and sometimes metastasis. It is the largest non-communicable disease and it has a sizable contribution in the total number of deaths. The World cancer report documents that cancer rates are set to increase at an alarming rate globally. Cancer rates could increase by 50% new cases for the year 2020¹.

India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani, and Siddha. Only a few of them have been scientifically explored. There were good number of plant products such as flavonoids, terpenes, alkaloids²,³,⁴ and these can are used as remedies to treat various diseases and disorders. Because of their distinct pharmacological qualities including cytotoxic and cancer chemopreventive effects, it is inspired many scientists to take up independent investigations on a number of medicinal plants.

*Sida Veronicaefolia*, family Malvaceae, is a straggling way side herb found very often growing in shady places. It grows mainly in clearing in the forest and as weeds in the over grown grass of public parks and gardens.⁵ It is also known as Rajbala, Bhumibala, Farid buti, Shakibala, etc. It has a capability to remove the three *doshas* from the body, and to provide strength and glow to the body.⁶

*Sida Veronicaefolia* is very popular with rural womenfolk, especially in the areas where it grows in its natural habitat, and is used extensively in traditional medicine for shortening and reducing the pain of childbirth. It is believed to render parturition almost painless and leads to shorter period of postpartum bleeding. Soup of this plant is taken in the last days of pregnancy.³ Lutterodt reported that alcoholic extract of *Sida veronicaefolia* has abortifacient effect in pregnant rats. An oral dose produces abortifacient effect when administered from 15⁶-17⁶/7/day of pregnancy.³ It is also reported that water soluble fraction from an alcoholic extract of *Sida veronicaefolia* has muscarine like active principle.⁸ Literature survey revealed that no detailed phytochemical work has been done on this plant and yet not being screened for its cytotoxic activity. The aim of present study was to investigate cytotoxic activity by using *In vivo* and *In vitro* models.

**METHOD AND MATERIALS**

**Collection and Authentication of the leaves**

The leaves of *Sida veronicaefolia* was collected from Sanjivini botanical garden, Bhopal, India in month of July 2009. The leaves were authenticated by Dr. Sayeeda Khatoon, chemotaxonomist and the voucher specimens were deposited in the departmental herbarium for future reference.

**Preparation of Crude Drug for Extract**

The authenticated leaves were used for the preparation of the extract. The leaves were collected and dried under shade and then coarsely powdered with the help of mechanical grinder. The powdered was passed through sieve no. 40 and stored in an airtight container for extraction.

**Preparation of extracts of *Sida veronicaefolia***

The powdered leaves (500 g) were sequentially extracted using petroleum ether, chloroform, ethanal and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation.

**Preliminary phytochemical screening**

Extracts of *Sida veronicaefolia* was subjected to qualitative tests for the identification of various active constituents viz. carbohydrate, glycoside, alkaloid, amino acids, flavonoids, fixed oil, tannins, gum and mucilage, phyto-sterols etc. The phytoconstituents were identified by chemical tests, which showed the presence of various constituents in the different extracts.

**Pharmacological Evaluation**

**Animals**

Swiss Albino mice (20-25 gm) of either sex and of approximately the same age, procured from Institute of Animal Health and Veterinary Biological,
Acute Toxic Study

This study was carried out on the basis of OECD 423 guidelines, both extracts were non toxic up to 5000mg/kg hence the LD50 was 5000mg/kg and 1/10 LD50 was selected as dose for the study.

Anticancer activity

Cells

EAC cells were obtained through the courtesy of Amala Cancer Research Center, Thrissur. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse.11

Experimental Design

The animals (Swiss albino mice weighing 20-25 g) were divided into 5 groups consisting of 12 animals. Animals were fed with basal diet and water throughout the experimental period. All the groups were injected with EAC cells except the group I. This was taken as day zero. From day 1st, normal saline (5 ml/kg) was given in group I, 5-fluorouracil (20mg/kg), AESV (500 mg/kg) and EESV (500 mg/kg) were given to group III, group IV and group V respectively, for 14 consecutive days, whereas group II was serve as a tumor control group and normal saline (5 ml/kg) was given to this group also, on day 15th half of the mice from each group were sacrificed, 24h after last dose, for the determination of tumor volume, tumor weight, haematological parameters etc, and rest were kept with food and water ad libitum to check the increase in the life span of the tumor hosts.12

Effect on Mean survival time

Animals were inoculated with 1x10^6 cells/mouse on day ‘0’ and treatment with AESV and EESV was started 24 h after inoculation, at a dose of 500 mg/kg/day, p.o. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for 14 days. The median survival time (MST) of each group was noted. The antitumor efficacy of AESV and EESV were compared with that of 5-fluorouracil (Dabur Pharmaceutical Ltd, India; 5-FU, 20 mg/kg/day, i.p. for 14 days). The MST of the treated groups was compared with that of the control group using the following calculation:12,13

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

Mean survival time = [1st Death + Last Death] / 2

The result was shown in Table no 1.

Table 1. Effect on the survival of tumor bearing mice

<table>
<thead>
<tr>
<th>S No</th>
<th>Treatment</th>
<th>Mean Survival Time (Days)</th>
<th>Increase in life span (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tumor Control</td>
<td>21.50 ± 2.73</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>5-FU (20mg/kg, i.p)</td>
<td>40.16 ± 2.13*</td>
<td>86.79%</td>
</tr>
<tr>
<td>3</td>
<td>AESV (500 mg/kg, p.o)</td>
<td>35.16 ± 2.8*</td>
<td>63.50%</td>
</tr>
<tr>
<td>4</td>
<td>EESV (500 mg/kg, p.o)</td>
<td>34.16±4.5*</td>
<td>58.88%</td>
</tr>
</tbody>
</table>

n=6 animals in each group, *P<0.01 Vs control. Days of treatment = 14, Values are expressed as mean ± SEM

Effect on tumor volume and tumor weight

On 15th day, after 24h of dose, 6 mice from each group were dissected and the ascitic fluid was collected from peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. The tumor weight was measured by taking the weight of mice before and after collection of ascitic fluid from peritoneal cavity.12 13 The result was shown in table no 2.

Effect on tumor cell count

The ascites fluid withdrawn from the peritoneal cavity of the mice was taken in WBC pipette and diluted 100 times with normal saline. A drop of a diluted cell suspension was placed on the neubauers chamber and the number of cells in the 64 square was counted. The viability and non viability of cells was checked by tryphan blue method. On staining viable cells did not take the dye whereas the non viable cells were stained blue.12 13 The result was shown in table no 2.

Table 2. Effect on Tumor volume, Tumor weight and Tumor cell count

<table>
<thead>
<tr>
<th>Treatment/dose</th>
<th>Tumor Volume (ml)</th>
<th>Tumor weight (gm)</th>
<th>Tumor cell count X10^6/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AESV (500 mg/kg, p.o)</td>
<td>24.33±0.33</td>
<td>28.5±0.76*</td>
<td>31±0.25*</td>
</tr>
<tr>
<td>EESV (500 mg/kg, p.o)</td>
<td>25.43±0.33</td>
<td>30.5±0.88*</td>
<td>35±0.30*</td>
</tr>
</tbody>
</table>

n=6 animals in each group, *P<0.01 Vs control. Days of treatment = 14, Values are expressed as mean ± SEM

Effect on body weight

Treatments were continued for 14 days. Body weights were recorded every 7th day till 40 days of treatment or till the death of the animal.12 13 The result was shown in Table no 3.

Table 3. Effect on body weight

<table>
<thead>
<tr>
<th>Parameter Normal</th>
<th>Tumor Control</th>
<th>Tumor</th>
<th>5 FU</th>
<th>AESV</th>
<th>EESV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb(g/dl)</td>
<td>14.3±0.10</td>
<td>14.0±0.05*</td>
<td>13.2±0.10*</td>
<td>12.9±0.10*</td>
<td></td>
</tr>
<tr>
<td>RBC(million/mm^3)</td>
<td>4.68±0.06</td>
<td>4.11±0.04*</td>
<td>3.13±0.06*</td>
<td>3.88±0.04*</td>
<td></td>
</tr>
<tr>
<td>WBC(million/mm^3)</td>
<td>7.48±0.03</td>
<td>7.19±0.07*</td>
<td>8.23±0.02*</td>
<td>9.58±0.02*</td>
<td></td>
</tr>
<tr>
<td>Platelets (%)</td>
<td>8.21±0.06</td>
<td>13±2±0.2*</td>
<td>8.65±0.04*</td>
<td>9.6±0.05*</td>
<td></td>
</tr>
<tr>
<td>PCV(%)</td>
<td>16.5±0.42</td>
<td>31.5±42*</td>
<td>19.5±42*</td>
<td>21.3±33*</td>
<td></td>
</tr>
<tr>
<td>Neutrophils%</td>
<td>30.83±0.60</td>
<td>31.83±0.7*</td>
<td>42.16±0.60*</td>
<td>38±1.78*</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes%</td>
<td>68.5±4.22</td>
<td>64.66±4.22*</td>
<td>54.0±6.8*</td>
<td>59.5±4.2*</td>
<td></td>
</tr>
<tr>
<td>Monocytes%</td>
<td>1.16±0.16</td>
<td>2.16±0.16*</td>
<td>1.33±0.21*</td>
<td>1.50±0.22*</td>
<td></td>
</tr>
</tbody>
</table>

Values were expressed as mean±SEM, n=6 in each group. *P< 0.001 Vs Normal control, $P<0.001 Vs Tumor control, #P<0.005 Vs Normal control, ns = not significant

Effect on normal peritoneal cells

Five groups of normal mice (n=6) were used for the study. First two groups were treated with 500 mg/kg, p.o. of acetone and ethanol extracts only once for a single day and other two groups received the same treatment for two consecutive days. The untreated group was used as control. Peritoneal exudate cells were collected after 24 h treatment by repeated intraperitoneal wash with normal saline and counted in each of the treated groups and compared with those of the untreated group.14

Effect on haematological parameters

Both treatments were given for 14 days to each group (except group III), on the 15th day, blood was drawn by retro orbital plexus method. WBC count, RBC count, haemoglobin, protein and packed cell volume were determined using Liesman stain solution.15 16 17

Red blood cells (RBC), White blood cells (WBC) and Haemoglobin (Hb) were estimated with the help of haematology analyzer (Medonic CA620, Boule, Sweden). The result was shown in Table no 4.

Table 4.Effect of AESV and EESV on Hematological Parameters

<table>
<thead>
<tr>
<th>Parameter Normal</th>
<th>Tumor Control</th>
<th>Tumor</th>
<th>5 FU</th>
<th>AESV</th>
<th>EESV</th>
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<tbody>
<tr>
<td>Hb(g/dl)</td>
<td>14.3±0.10</td>
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<td></td>
</tr>
</tbody>
</table>

Values were expressed as Mean ± SEM, n=6 in each group. *P< 0.001 Vs Normal control, $P<0.001 Vs Tumor control, #P<0.005 Vs Normal control, ns = not significant

References

2. Mhow, Indore, Madhya Pradesh were used for the study. They were housed in polypropylene cages and fed with standard rodent pellet diet (Hindustan Lever Limited, Bangalore) and water ad libitum. The animals are exposed to alternate cycle of 12 hrs of darkness and light each. Before each test, the animals are fasted for at least 12 hrs; the experimental protocols were subjected to the scrutinization of the Institutional Animals Ethical Committee and were cleared by the same. All experiments were performed during morning according to CPCSEA guidelines for care of laboratory animals and the ethical guideline for investigations of experimental pain in conscious animals.
Invitro Cytotoxicity

Cell Cultures

The EAC cell line was procured through the courtesy of Amala Cancer Research Center, Thrissur and maintained at Pharmacology Department, TIT-Pharmacy, Bhopal in Dulbecco’s modified eagle medium (DMEM) at 37°C and 5% CO₂ using standard cell culture methods. At confluence, cells were trypsinised and equally distributed in two standard cell culture flasks and were allowed to adhere for 24hr. In order to evaluate the effect of AESV and EESV on cancer cells, cells were transferred in 96 well cell culture plate and incubated for 24hr. After confluence, MTT assay and Neutral red uptake cytotoxic assay have been conducted to evaluate the cell death caused by the extracts.

In Vitro Cytotoxic Assay

In Vitro cytotoxic activity was found using MTT assay and Neutral red uptake cytotoxic assay.

MTT Assay

25mg of MTT powder was dissolved in 5ml PBS then filtered it with the help of 10ml syringe and syringe filter. Incubated cell plates were taken out from the incubator, and discard the culture media from the plates. Culture media was replaced by the extract containing culture media. Then the plates were incubated in CO₂ incubator for 24 hrs for the action of extracts. 5 hours before the end of the incubation, add 20µl of MTT solution to each well containing cells. Incubate the plate at 37°C for 5 hours. Remove media and add 200µl of DMSO to each well and pipette up and down to dissolve crystals. Transfer to plate ELISA reader and measure absorbance at 550nm to get optical density. Then calculate the % inhibition using the formula

\[
\% \text{ inhibition} = \left(\frac{OD \text{ of untreated} - OD \text{ of drug Treated}}{OD \text{ of untreated}}\right) \times 100
\]

The result was shown in Table no 5.

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Concentration</th>
<th>Optical density</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>No treatment</td>
<td>0.3660</td>
<td>0.00 ± 1.31%</td>
</tr>
<tr>
<td>2</td>
<td>AESV</td>
<td>200 µg/ml</td>
<td>0.0226</td>
<td>93.83 ± 3.48%</td>
</tr>
<tr>
<td>3</td>
<td>EESV</td>
<td>200 µg/ml</td>
<td>0.0157</td>
<td>95.71 ± 3.45%</td>
</tr>
</tbody>
</table>

8 wells /group OD at 550 nm, Values are expressed as mean ± SEM.

Neutral Red Uptake Cytotoxic assay

NR dye (3.3gm) was dissolved in 100 ml of double distilled water and then this stock solution was filtered by using syringe filter. It was stored at room temperature and used within 6 months. 1 ml of NR stock solution was dissolved in the 99 ml of culture media to get the final concentration 0.33%. Incubated cell plates were taken out from the incubator, and discard the culture media from the plates. Culture media was replaced by the extract containing culture media. Then the plates were incubated in CO₂ incubator for 24 hrs for the action of extracts. The extract containing culture media was then replaced with NR-containing medium. Plates were again placed to incubator for 4-8 hours depending on cell type and maximum cell density. At the end of the incubation period, the medium was carefully removed and the cells were quickly washed with PBS. The washed solution was removed and the incorporated dye was then solubilized in a volume of Neutral Red Assay Solubilization Solution (ethanol:acetic acid) equal to the original volume of culture medium. The plates were allowed to stand for 10 minutes at room temperature. Gentle stirring in a gyratory shaker or pipetting up and down (trituration) enhanced mixing of the solubilized dye. The background absorbance was measured at 540 nm using ELISA reader to get optical density and pictures were captured using microscope. Then calculate the % inhibition using the formula

\[
\% \text{ inhibition} = \left(\frac{OD \text{ of untreated} - OD \text{ of drug Treated}}{OD \text{ of untreated}}\right) \times 100
\]

The result was shown in Table no 6 and in Fig no 1.
experimental groups was assessed by One-way ANOVA followed by Newman-Keuls Multiple Comparison Test. The value of probability less than 5% (P < 0.05) was considered statistically significant.

RESULT
The phytoconstituents were identified by chemical tests. The extracts showed the presence of flavonoids, phenolic compounds, tannins, alkaloids, phytosterols, saponins, proteins and amino acids.

Effect on Mean survival time
The MST for the Tumor control was 21.50 ± 2.73 days, whereas it was 35.16 ± 2.8 days, 34.16 ± 4.5 days, and 40.16 ± 2.13 days for the groups treated with AESV and EESV (500 mg/kg/day, p.o.) and 5-FU (20 mg/kg/day, i.p.) respectively. The % increase in the lifespan of tumor-bearing mice treated with AESV, EESV and 5-FU was found to be 63.50 %, 58.88 % and 86.79% respectively (P< 0.01) as compared to the control group. The effect of AESV and EESV on the survival of tumor-bearing mice was shown in Table 1.

Effect on tumor volume, tumor weight and tumor cell count
There was reduction in the tumor volume, tumor weight and tumor cell count of mice treated with AESV and EESV (P<0.001) as showed in table no 2. Tumor volume of tumor control animals was 6.70 ± 0.16ml, whereas for the extract-treated group it was 2.15 ±0.09* ml and 2.56 ±0.10* ml for AESV and EESV respectively. Tumor weight of tumor control animals was 6.87 ±0.21 g, whereas for the extract-treated group it was 2.25 ±0.25* g and 2.71 ±0.31* g for AESV and EESV respectively.

Effect on body weight
There was a significant decrease in the weight gain by the AESV and EESV treated mice when compare with tumor control as showed in table no 3.

Effect on normal peritoneal cells
The average number of peritoneal exudate cells per normal mouse was found to be 5.8±0.1×10^6. Single treatment with AESV and EESV (500 mg/kg) enhanced peritoneal cells to 9.7±0.37 ×10^6 and 9.1±0.1 ×10^6, while two consecutive treatments also enhanced the number to 14.3±0.27×10^6 and 13.2±0.2×10^6 respectively (P<0.001). At the same time interval, AESV and EESV (500 mg/kg/day, p.o.) treatment could change these altered parameters to near normal.

In Vitro Cytotoxicity
The results of In Vitro cytotoxic test were shown in Table 5 and 6. Both the extracts shows remarkable cytotoxic activity against the tested cells but higher cytotoxic activity was found in AESV.

CONCLUSION
The present study was carried out to evaluate the antitumor effect of AESV and EESV in EAC-bearing mice. The AESV and EESV treated animals at the doses 500 mg/ kg significantly decrease the tumor volume, tumor weight, tumor cell count, body weight, and brought back the haematological parameters to more or less normal levels. In EAC-bearing mice, a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid increase in ascites fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals. The AESV and EESV decreased the ascites fluid volume, viable cell count, and increased the percentage of life span. It may be concluded that AESV and EESV by decreasing the nutritional fluid volume and arresting the tumor growth, increases the life span of EAC-bearing mice.

A significant enhancement of peritoneal cell count was observed. The effect of AESV and EESV treatment on the peritoneal exudate cells of normal mice is an indirect method of evaluating its inhibitory effect on tumor cell growth. Normally, a mouse contains about 5 x 10^6 peritoneal cells, 50% of which are macrophages. AESV and EESV treatment was found to enhance peritoneal cells count. These results demonstrate the indirect inhibitory effect of AESV and EESV on EAC cells, which is probably mediated by the enhancement and activation of either macrophage or cytokine production.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or haemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. In EAC control group, a differential count the presence of neutrophils increased, while the lymphocyte count decreased, the observed leucocytopenia indicates a common symptom of immunosuppression in many types of cancers and one of the causes of neutropenia is myeloid growth factors which are produced in malignant process as part of a paraneoplastic syndrome. In addition to this another factor granulocyte colony stimulating factor produced by the malignant cells has also been attributed to be the cause of neutropenia because of its action on bone marrow granulocytic cells in cancer. After the repeated treatment, AESV and EESV able to reverse the changes in altered neutrophils and lymphocytes count.

Treatment with AESV and EESV brought back the haemoglobin content, RBC, and WBC count more or less to normal levels and this indicates that AESV and EESV posses protective action on the haematopoietic system.Preliminary phytochemical screening indicated the presence of flavonoids, phenolic compound, tannins, alkaloids, phytosterols, saponins, proteins and amino acids. Flavonoids have been shown to possess antituagenic and antimalignant effects. Moreover, flavonoids have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis. Tannins and phenolic compounds are considered to have cancer-preventive properties. These induced cell death in cancer cells by concentration-dependent decrease of ATP and a deteriora-

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