Antitumor potential of an acetogenin isolated from the seed extracts of *Annona squamosa* Linn.

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Ethanolic herbal extract residue of the seeds of *Annona squamosa* Linn. were tested against Dalton’s Lymphoma Ascites (DLA) tumour cells. Then its effect on the increased lifespan of mice with DLA tumour cells was studied. At high and low dosages, it exhibited 66.67 and 56.25% increase in the lifespan. Percentage of haemoglobin (Hb) and red blood cell (RBC) counts of treated were higher than that of tumour bearing mice. The white blood cell (WBC) counts of treated were lower than that of tumour bearing mice. This is a good index of tumour recovery. Further studies were focused on mice induced with solid tumour to record the tumour volume and lifespan. It is learnt from the UV-Vis, IR, $^1$H and $^{13}$C NMR spectra analyses that annoglacin-B, an acetogenin, present in the extract residue of *A. squamosa* may be responsible for the biological activity.

Key words: Dalton’s Lymphoma Ascites (DLA), red blood cell (RBC), white blood cell (WBC), haemoglobin (Hb), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT).

INTRODUCTION

Cancer is a group of diseases in which cells are aggressive, invasive, and sometimes metastatic. Cancer may affect people at all ages. Apart from humans, forms of cancer may affect other animals and plants (Tannock and Hilp, 2007). Widespread placement of cancers eliminates surgery and radiation as options, leaving chemotherapy as the preferred medical choice. Taxol, isolated from the stem bark of *Taxus brevifolia* was ranked as “the most important new drug in cancer for 15 years”. Yet adequate drugs are not currently available to treat major solid tumours such as those of the lung, breast, prostate, and colon. There is an urgent need to find a suitable treatment for the millions of patients who are affected (Fulder, 1996). Plants still remain a traditional source of medicinal compounds; up to 40% of modern drugs may directly or indirectly be related to natural compounds. Several plant-derived compounds have been approved as anticancer drugs-vinblastine, vincristine, etoposide, teniposide, taxol, taxotere, topotecan and irinotecan, just to name a few.

Annonaceous acetogenins are a new group of compounds isolated from *Annona* plants exhibiting strong anticancer activity (Alali et al., 1999). Hence an attempt was made to isolate an acetogenin from the Indian species, *Annona squamosa* Linn. to test its antitumor potential using mice model.

EXPERIMENTAL

Herbal extract preparation

*A. squamosa* Linn. is distributed throughout India. It is a small tree / shrub approximately about 6 m in height. Its fruits are edible and the seeds are abortifacient and insecticidal and are useful in destroying lice in the hair (Warrier et al., 1996). Seeds of the *A. squamosa* Linn. premature fruits were collected, shade and air dried and ground in to powder. 150 to 200 g of the powder was packed in filter paper packs and extracted with ethanol in a soxhlet apparatus continuously for about 6 to 10 h. Then the ethanolic extract was distilled off till semisolid substances were obtained. After evaporated to dryness, these substances were stored in a -20°C deep freezer until further analysis.

Cell lines

Dalton’s Lymphoma Ascites (DLA) tumour cells were obtained through the courtesy of Amala Cancer Research Centre, Thrissur.

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Kerala, India. These tumour cells are known to grow as uniform cell suspension in the peritoneal cavity of the mice. DLA was maintained by serial transplantation from mice to mice. The ascitic fluid of the DLA was drawn out from the donor mice carrying tumour for 7 to 9 days. The freshly drawn ascitic fluid from the peritoneal cavity was washed thrice with phosphate buffer saline (PBS, pH 7.4) and diluted in PBS to a concentration of 1 x 10^6 cells / ml, and these cells were used for in vitro experiments.

**In vitro cytotoxic assay**

One million cells obtained from the above method were incubated with various concentrations of the herbal extract residue (10, 20, 50, 100 and 200 µg/ml) in a total volume of 1 ml for 3 h at 37°C. 0.1% carboxy methyl cellulose (CMC) was used as control. After incubation the viability of the cells was determined by the tryphan blue exclusion method (Talwar, 1974).

**Effect of ethanolic herbal extract residues on ascites tumour bearing animals**

BALB/c mice (20 to 25 g) were obtained from the Breeding section, Amala Cancer Research Centre, Thrissur. They were kept in groups of six in well ventilated cages in air controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water ad libitum. All animal experiments were conducted after permission from the Institutional Ethical Committee.

DLA cells were aspirated from the peritoneal cavity and washed three times with PBS. Ascites tumour cells were then inoculated to three groups (6 animals / groups) of BALB/c mice by injecting one million cells in to their peritoneal cavity. After 24 h of tumour inoculation two different dosages of herbal extract residues were given and continued for ten consecutive days.

Group I: 0.1% CMC (ip) vehicle treated control for DLA.
Group II: DLA cells + 0.1 mg/kg b.wt of A. squamosa herbal extract residue in 0.1% CMC (ip) – low dosage.
Group III: DLA cells + 0.5 mg/kg b.wt of A. squamosa herbal extract residue in 0.1% CMC (ip) – high dosage.

The death pattern of animals due to tumour burden was noted every day and the percentage of increase in life span was calculated using the formula, T-C/C x 100 where ‘T’ and ‘C’ are the number of days the treated and control animals survived respectively (Rajeshkumar and Kuttan, 2001).

**Haematological parameters**

On the 15th day post tumour inoculation, blood was collected in heparinized tubes from the respective animals by tail vein and the haematological parameters such as white blood cell count, red blood cell count and percentage of haemoglobin were determined by following the standard procedures (Rajeshkumar and Kuttan, 2001).

**Tumour cell count**

0.1 ml of the ascitic fluid was aseptically withdrawn on the 15th day post tumour inoculation using a 1 ml syringe and diluted with 0.9 ml of PBS (pH 7.4) to adjust the cell count to 1 x 10^6 cells. From the stock 0.1 ml was taken and mixed with 0.8 ml of PBS and then incubated for 3 h at 37°C. 0.1 ml of tryphan blue was then added to this. Then a drop of the resulting solution was loaded on the haemocytometer and the number of tumour cells randomly in every 100 cells was counted (Rajeshkumar and Kuttan, 2001).

**Estimation of serum glutamic pyruvic transaminase (SGPT) and serum glutamic oxaloacetic transaminase (SGOT)**

On 15th day post tumour inoculation, blood from the tail vein of treated and control mice was withdrawn individually into test tubes which were then centrifuged at low speed for about 10 to 15 min to collect the serum for enzyme analysis following the method of (Reitman and Frankel, 1957).

**Effect of herbal extract residues on the solid tumour**

Female Swiss albino mice (20 to 25 g) were obtained from the Breeding section, Amala Cancer Research Centre, Thrissur. They were kept in groups of six in well ventilated cages in air controlled room, fed with normal mice chow (Sai Feeds, Bangalore, India) and water ad libitum. All animal experiments were conducted after permission from the Institutional Ethical Committee. DLA tumour cells (1 x 10^6 cells/animal) were injected subcutaneously to the right hind limb of three groups of (6 animals / group) Swiss albino mice.

Group I: Untreated control for DLA
Group II: DLA cells + 0.1 mg/kg b.wt of A. squamosa extract residue in 0.1%
CMC (ip) - low dosage
Group III: DLA cells + 0.5 mg/kg b.wt of A. squamosa extract residue in 0.1%
CMC (ip) - high dosage

After 24 h of tumour inoculation different dosages of herbal extract residue were given and continued for 10 consecutive days. Initial diameter of the hind limb was noted using vernier calipers. From the 7th day onwards the tumour diameter was measured every 3rd day and recorded up to 40 days. The tumour volume was calculated using the formula, \( V = \frac{4}{3} \pi r^2 \times H \). The survival of the animal was also recorded up to 60 days.

**Spectral analysis**

Semisolid substances recovered from ethanolic herbal extract of A. squamosa were subjected to UV – VIS, IR and NMR analyses.

**Statistical analysis**

Results were expressed as mean ± standard deviation and student’s ‘t’ test was used.

**RESULTS**

Results of the in vitro cytotoxicity test were presented in Table 1. A. squamosa extract residue showed 99% cytotoxicity at 200 µg concentration and at 100 µg concentration also, it registered the toxicity of 98%. With regard to tumour cell count, A. squamosa extract residue both at low and high dosage levels led to the decreased formation of 7.13 ± 0.25 x 10^6 tumour cells and 4.23 ± 0.06 x 10^6 tumour cells in the peritoneal activity of the mice (Table 2) respectively.

Normal animals had the Hb content of 19.77 ± 0.70%
### Table 1. Cytotoxic effect of ethanolic herbal extract residue on DLA tumour cells.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Concentration of herbal extract residue (µg)</th>
<th>% of cytotoxicity Control (CMC 0.1%)</th>
<th>A. squamosa</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0</td>
<td>99</td>
</tr>
</tbody>
</table>

One million cells were incubated with various concentrations of the herbal extract residue (10, 20, 50, 100 and 200 µg/ml) in a total volume of 1 ml for 3 h at 37°C. After incubation the % viability of the cells was determined by the trypan blue exclusion method. Duplicates were carried out.

### Table 2. Effect of ethanolic herbal extract residue on DLA tumour cell counts.

<table>
<thead>
<tr>
<th>Control/Herbal extract residue treated animal groups</th>
<th>Dosage (mg.b.wt.kg⁻¹)</th>
<th>Cells (10⁶/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>A. squamosa</td>
<td>0.1</td>
<td>7.13±0.25*</td>
</tr>
<tr>
<td>Treated</td>
<td>0.5</td>
<td>4.23 ± 0.06*</td>
</tr>
</tbody>
</table>

BALB/c mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting 1 × 10⁶ cells into the peritoneal cavity of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given and continued for ten consecutive days. Tumour cell count was done on the 15th day post tumour inoculation by drawing 0.1 ml of the ascitic fluid from the animals treated with herbal extract residues and from the control. Tryphan blue exclusion method was followed. * P<0.05.

### Table 3. Percentage of Hb, RBC and WBC counts of the test animals treated with low and high dosage of ethanolic herbal extract residue after tumour induction.

<table>
<thead>
<tr>
<th>Control/Herbal extract residue treated animal groups</th>
<th>Dosage (mg.b.wt.kg⁻¹)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour bearing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. squamosa</td>
<td>0.1</td>
<td>11.58 ± 1.08 NS</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>14.71 ± 1.08 NS</td>
</tr>
</tbody>
</table>

A. squamosa treated animals showed only about 12.45 ± 1.43% of Hb both at high and low dosages. Red blood cell (RBC) counts of normal and tumour bearing animals were 12.64 ± 1.25 cells/mm³, 6.8 ± 0.17 cells/mm³. A. squamosa extract residue treated animals showed the values of 8.99 ± 1.4 cells/mm³ and 7.41 ± 0.24 cells/mm³ both at high and low dosages respectively. Normal and tumour bearing animals had the white blood cell (WBC) counts of 13300 ± 1389 cells/mm³ and 68666 ± 3762 cells/mm³ respectively whereas A. squamosa treated ones registered the WBC counts of 43483 ± 2472 cells/mm³ and 46233 ± 2599 cells/mm³ both at high and low dosages respectively (Table 3). SGPT and SGOT enzyme activities of test animals were found to be decreased than that of tumour bearing animals (Table 4). A. squamosa extract residue exhibited 66.67 and 56.25% increase in the life span of animals induced with DLA at 0.5 and 0.1 mg/Kg b. wt dosages respectively (Table 5).

Both at high and low dosage levels, A. squamosa extract residue was able to keep the tumour volume between the ranges 0.31 ± 0.02 mm³ and 0.33 ± 0.03 mm³, when compared to the controls' tumour volume of whereas the tumour bearing animals had that of 14.71 ± 0.89% and 11.58 ± 1.08% of Hb both at high and low dosages. Red blood cell (RBC) counts of normal and tumour bearing animals were 12.64 ± 1.25 cells/mm³, 6.8 ± 0.17 cells/mm³. A. squamosa extract residue treated animals showed the values of 8.99 ± 1.4 cells/mm³ and 7.41 ± 0.24 cells/mm³ both at high and low dosages respectively. Normal and tumour bearing animals had the white blood cell (WBC) counts of 13300 ± 1389 cells/mm³ and 68666 ± 3762 cells/mm³ respectively whereas A. squamosa treated ones registered the WBC counts of 43483 ± 2472 cells/mm³ and 46233 ± 2599 cells/mm³ both at high and low dosages respectively (Table 3). SGPT and SGOT enzyme activities of test animals were found to be decreased than that of tumour bearing animals (Table 4). A. squamosa extract residue exhibited 66.67 and 56.25% increase in the life span of animals induced with DLA at 0.5 and 0.1 mg/Kg b. wt dosages respectively (Table 5).

Both at high and low dosage levels, A. squamosa extract residue was able to keep the tumour volume between the ranges 0.31 ± 0.02 mm³ and 0.33 ± 0.03 mm³, when compared to the controls' tumour volume of
0.75 ± 0.06 mm³ (Figure 1). There was no much difference between these two dosage effects. Concurrently percentage of increase in the life span of animals induced with solid tumour was studied up to 60 days. A. squamosa extract residue exhibited 35.13 and 30.63% of increase in the life span of animals induced with solid tumour at high and low dosages respectively (Table 6).

The crude ethanol extract residue of A. squamosa extract residue was investigated for its composition by recording the UV-VIS, IR and ¹H and ¹³C NMR spectra. ¹H NMR spectrum indicated the presence of annoglacin-B in the crude mixture (Figures 2 to 6) by the signals at 3.6, 3.8 and 3.4 ppm. The presence of olefinic hydrogens was also noticed in the downfield region. In the ¹³C NMR spectrum signals at 71.83, 74.0, 82.57 and 82.64 ppm were all indicative of the presence of annoglacin-B in the extracts residue. The IR spectrum was also matching with the reported pattern for most of the bands. The λ max noticed in the UV region of the UV-VIS spectrum of the crude extract residue appeared around the region 220 to 240 nm due to the π → π* absorption.

DISCUSSION

At 200 μg concentration, A. squamosa herbal extract residue showed 99% of cytotoxicity. Hence it was decided to carryout further studies on mice after tumour inoculation. Preethi et al. (2006) reported that Ruta graveolens was cytotoxic towards DLA and Ehrlich ascites carcinoma (EAC) only at higher concentration. The 100% cytotoxicity was attained only at 16 mg/ml. Jelly et al. (2005) investigated that the 70% methanolic extract of Holostemma adakodien shows 50% cytotoxicity at a concentration of 750 µg/ml against DLA cells. The alcoholic Boerhavia diffusa extract was 100% cytotoxic towards DLA at a concentration of 500 µg (Lini and Kuttan, 2000).

Methanolic extract of Piper longum was toxic at 250 µg/ml to DLA cells and 100 µg/ml to EAC cells. The aqueous extract was non toxic to both DLA and EAC cells even at a concentration of 500 µg/ml. Babu et al. (2001) published that 70% ethanolic extract of Cuscuta reflexa was toxic to DLA and EAC cells at a concentration of 1.2 mg/ml and 800 µg/ml respectively for causing 50% tumour cell death.

In the present investigation, in vitro cytotoxic studies against the DLA cells with the extract residue of A. squamosa, are found to coincide with the results of already reported studies (Colom et al., 2008) and we also observed that the concentrations required to cause >90% toxicity are lesser than that required in the earlier reports. Effect of herbal extract residue on the increased life span of animals with DLA was studied upto 45 days. At high and low dosage levels, A. squamosa herbal extract residue registered 66.67 and 56.25% increase in the life span of the animals induced with DLA respectively. As far
Figure 1. Measurement of tumour volume of animals treated with ethanolic herbal extract residue. Swiss albino mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1 \times 10^6$ cells into the right hind limb of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given by keeping one group each for low dosage and high dosage and continued for ten consecutive days. Initial diameter of the hind limb was noted using vernier calipers. From the 7th day onwards the tumour diameter was measured every 3rd day and recorded up to 40 days. The tumour volume was calculated using the formula, $V = \frac{4}{3} \pi r_1^2 \times r_2$. The treated cells were significantly different over control at $P<0.01$. Filled circle Control (0.1% CMC); unfilled circle A. squamosa high dosage and filled triangle A. squamosa low dosage, the error bars indicates±2 SD.

Table 6. Effect of ethanolic herbal extract residue on the percentage of increased lifespan of animals with solid tumour.

<table>
<thead>
<tr>
<th>Control/Herbal extract residue treated animal groups</th>
<th>Dosage (mg.b.wt.kg$^{-1}$)</th>
<th>Mean survival time (days) Mean ± SD</th>
<th>% of increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>44.4±0.89</td>
<td></td>
</tr>
<tr>
<td>A. squamosa</td>
<td>0.1</td>
<td>58±2.9*</td>
<td>30.63</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>60*</td>
<td>35.13</td>
</tr>
</tbody>
</table>

Swiss albino mice were segregated into three groups (6 animals/group) and induced with ascites tumour by injecting $1 \times 10^6$ cells into the right hind limb of the test animals. After 24 h of tumour inoculation, different dosages of drugs were given by keeping one group each for low dosage and high dosage and continued for ten consecutive days. The survival of the animal was also recorded up to 60 days. *$P<0.001$.

as Hb estimation and RBC counts are concerned, all the values of A. squamosa herbal extract residue treated mice were generally higher than that of tumour bearing animals. This clearly shows that the extract residue has no side effects as far as blood parameters are concerned. Regarding WBC counts, all the values were lower than that of tumour bearing animals. Low WBC counts are good index of tumour recovery (Clarkson and Burchenal, 1965; Obiling and Guerin, 1954). SGPT and SGOT enzyme activities of test animals were found to be decreased than that of tumour bearing animals and also found to be dosage dependent.

Life span of DLA bearing animals was increased by methanolic extract of R. graveolens. At 400 and 200 mg/Kg b. wt dosages, the increase in life span was 21.6 and 43.2% respectively (Preethi et al., 2006). Likely the life span of DLA bearing mice treated with alcoholic extract of B. diffusa was significantly increased with an increase in life span of 64% (Lini and Kuttan, 2000). Serum and tissue levels of LPO, ALP and GPT which were elevated by CTX was reduced by Emblica officinalis (EO) and Chayavana prash (CHY) treatment (Joy and Kuttan, 1998).

Earlier reports clearly state that herbal extracts which
have potent antioxidant activities enhance the percentage of increase in the life span of animals induced with DLA tumour cells and in some cases induced with carcinogens. In the present investigation too, mice bearing DLA and treated with herbal extract residue showed the percentage of increase in the life span. This might be due to its good antioxidant activities. In this way, results of the present study stand in good agreement with the already reported studies in general. Variations in the percentage of increase in the life span of animals bearing tumour might be due to variations in the type of extracts used and the degree of antioxidant activities elicited.

Effect of herbal extract residues on solid tumour development was studied for 40 days. Based on the results, it was found that A. squamosa extract residue was able to keep the tumour volume under check. No much difference was noticed between the low and high dosages. This might be due to slight toxicity of A. squamosa at high dosage. In comparison with earlier reports, A. squamosa seemed to be better as its dosage was comparatively low. Percentage of increase in the life span of animals induced with solid tumour by DLA cells was also studied concurrently up to 60 days. High and low dosages of A. squamosa exhibited the 35.13 and 30.63% increase in the life span of animals induced with solid tumour by DLA cells respectively. Jelly et al. (2005) reported that the tumour volume in untreated control mice was 4.84 ± 1.23 mm³ on 30th day and this was reduced to 0.68 ± 0.39 mm³ by the administration of methanolic extract of Holostemma adakodien root at concentration of 25 mg/Kg b. wt. The Ethanolic extract of Lentinus dicholamellatus showed profound antitumour activity in a dosage dependent manner. The extract decreased the tumour volume by 56.62 and 67.94% respectively, when administered at concentrations of 250 and 500 mg/Kg body weights to animals after implantation of solid tumour by DLA cell line (Nitha et al., 2005).

Oral administration of the berberine hydrochloride (5, 2.5 and 0.5 mg/Kg b.wt) was found to inhibit the sarcoma development by 60, 53 and 33% respectively in female wistar rats of 120 to 150 g b. wt (Anis et al., 2000). Oral administration of methanolic extract of Emilia sonchifolia could reduce significantly the solid tumour induced with DLA in Swiss albino mice. On the 30th day the tumour volume of the control animal was 4.25 mm³ and that of drug treated (100 mg/Kg b.wt) was 1.25 mm³ (Shylesh and Padikkala, 1998). On the 40th day oral administration of 75% methanolic extract of Picrorhiza kurroa (750 mg/Kg b.wt) retarded the development of solid tumours induced with DLA by 44% in male Swiss albino mice (Joy and Kuttan, 1998).

In line with already studied reports, A. squamosa was able to keep the tumour volume between the ranges 56.06 to 58.72%. A. squamosa herbal extract residue showed considerable tumoricidal activity that is, > 50% thereby reducing the tumour volume to significant level. Encouraging results were therefore noticed in the percentage of increase in the lifespans of solid tumour bearing animals by 35.13% altogether when compared to that of control animals.

It is learnt from the spectral analysis that Annoglacin-B, present in the extract residue of A. squamosa may be responsible for the biological activity. Alali et al. (1999) reported that acetogenins are present in the seeds, roots, bark, stems and fruits of most annona trees, and appear to have great potential in anti-cancer treatments. In the present study spectral results indicate the presence of annoglacin-B in the crude mixture and its presence in the related species was already reported. Present investigation strongly recommends scientific validation for all that supposed to be anticancer herbs by carrying out in vitro as well as in vivo studies on animal models.
Figure 3. IR spectrum of ethanolic extract residue of *A. squamosa*. 
Figure 4. $^1$H NMR spectrum of ethanolic extract residue of *A. squamosa*.
Figure 5. $^{13}$C NMR spectrum of ethanolic extract residue of *A. squamosa*. 
because of the different behaviour of the cancer cells against the same testing drug in these two different systems and also the very fact that animals as well as human bodies are not merely pool of cells. As annoglacin-B, an annonaceous acetogenin exhibits considerable antitumour potential it may be taken into account for further clinical evaluations in future.

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