Preclinical Evaluation of the Anticancer Activity of Hydroalcoholic Stem Bark Extract of *Alstonia scholaris* in Ehrlich Ascites Carcinoma Transplanted in the Swiss Albino Mice

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**Abstract**

**Background:** Cancer has emerged as the second largest killer disease in the world and mortality rates for solid tumors remain unchanged. Therefore there is need to screen, new remedies to treat cancer.

**Materials and methods:** The antineoplastic activity of different doses of hydroalcoholic extract of *Alstonia scholaris* (L.) R. Br. (ASE) was studied in Swiss albino mice, transplanted with Ehrlich Ascites Carcinoma cells (EAC) in its peritoneal cavity. The effect of ASE injected at different stages of tumor development was evaluated by studying tumor free survivors. In another study EAC cells were treated with Echitamine Chloride (ECL) in vitro and transplanted back into mice and tumor free survivors determined. The glutathione and lipid peroxidation were also measured after ASE treatment.

**Results:** The daily administration of ASE in tumor bearing mice caused a dose-dependent remission of tumor, and highest regression was observed at 480 mg/kg b. wt. this dose showed some toxic effects and next lower dose i.e. 420 mg/kg was considered as the suitable dose, where 33.33% of the animals survived up to 120 days post-tumor cell inoculation, as against no survivors in the saline treated control and positive cyclophosphamide treated groups. The ASE caused a dose-dependent elevation in the median survival time (MST) and average survival time (AST), causing a corresponding increase in the median life span (MILS) and average life span (IALS) of experimental animals. Treatment of EAC mice with 420 mg/kg ASE or ECL also retarded tumor development even during the late stages of tumor development, while cyclophosphamide was completely ineffective. The in vitro treatment of EAC cells with ASE or ECL and their transplantation in mice showed that ASE treatment was more potent than ECL treatment as evident by increased MST and AST in the former when compared with the latter. Assay of glutathione and lipid peroxidation after 240, 420 mg/kg ASE or 25 mg/kg cyclophosphamide led to a decline in glutathione contents and increase in lipid peroxidation.

**Conclusions:** Our study shows that ASE treatment kills the tumor cells effectively increasing long-term disease free survival of experimental mice, which may be due to reduced glutathione and increased lipid peroxidation.

**Keywords:** *Alstonia scholaris*; Mice; Ehrlich Ascites Carcinoma; Echitamine; Cyclophosphamide; Median Survival Time; Average Survival Time; Glutathione; Lipid Peroxidation

Introduction

The plants have provided many useful products including medicines to the mankind since time immemorial and had significantly contributed in human healthcare [1]. The infectious diseases took toll of human population during ancient times. However due to the scientific development in modern medicine, it has been possible to eradicate most of the infectious diseases or reduce their occurrence to a minimum in the modern era. The technological advancement and lifestyle changes have resulted in the increased frequency of cardiopulmonary diseases and cancer. The cancer has emerged as the second largest killer disease in the world and the analysis of 2012 cancer incidence data reveals that cancer incidence is twice higher in the developed countries than the less developed countries [2]. It is speculated that by the year 2016 approximately 1,685,210 new cancer cases will be diagnosed with a mortality of 595,690 in the United States alone [3]. Approximately, 700,000 new cases were diagnosed in India and the incidence is expected to increase five folds by 2025 according to a WHO report [4]. There has been a rapid advancement in the cancer treatment strategies and despite of the availability of large number of cancer treatment modalities, complete cure of cancer remains tricky, except for the hematological malignancies, which have highest cure rates after treatment [3]. Further, the modern synthetic cytotoxic drugs cause severe systemic toxicity in the patients receiving them and treatment of cancer puts economic pressure on the family of a patient and are unaffordable by a large number of poor patients, who suffer from cancer. The other drawback of cytotoxic drugs is that their use is associated with the development of second malignancies in the patients who survive after the treatment of cancer [5]. This clearly indicates that there is a need to search new remedies, which are cheaper economically and do not have severe side effects of the modern drugs. Humans have used plants for healthcare and the botanicals may provide a cheaper alternative to the exotic synthetic formulations with much lesser toxic implications and their screening provides an interesting approach for new drug discovery.

The plants have traditionally provided several remedies for the treatment of various ailments in man, since time immemorial. Several plant products have proved beneficial in the treatment of various malignancies in the modern medicine. Many potent antineoplastic agents, like vinca alkaloids (vinblastine, vincristine), epipodophyllotoxins (etoposide and teniposide), taxol, etc. which are in frequent clinical use have been derived from plants and are used to treat several neoplastic diseases in man [6,7]. Vinca alkaloids are active against many solid tumors, and hematological malignancies can be effectively treated using them [8-10]. Epipodophyllotoxins have been reported to be active in several rodent and human neoplasms, including Hodgkin's disease, small cell lung carcinoma, testicular tumors, diffuse histiocytic lymphoma, testicular cancer, and certain lymphomas [11-13]. Taxol is an antimitotic agent and cells resistant to vinca alkaloids because of mutations in tubulin remain sensitive to taxol. It is clinically found to be effective against broad spectrum of malignant tumors including, breast cancer, malignant melanomas and carcinomas of the ovary [14,15].

*Alstonia scholaris* (L.) R. Br, a tree belonging to the family Apocynaceae, has been used since time immemorial in the
folklore and traditional systems of medicine in India, to treat several diseases. The ripe fruits of the plant are used in syphilis, insanity and epilepsy. It is also used as a tonic, antiperiodic and anthelmintic. The milky juice of A. scholaris is applied to treat ulcers. Its bark is extensively used in many compound herbal formulations [16]. It is a bitter tonic, alternative and febrifuge and is reported to be useful in the treatment of malaria, diarrhoea and dysentery [16-20]. The alkaloid corallstone and coraltostonide present in the plant have been found to be active against P. falciparum and pentacyclic triterpenoids isolated from A. scholaris have been found to exert antibacterial activity [21,22]. The leaf extracts have been reported to possess antimicrobial properties [23]. A. scholaris has also been reported to inhibit liver injuries induced by carbon tetrachloride, beta-D-galactosamine, acetaminophen and ethanol as observed by the reduced elevation of serum transaminases level and histopathological changes such as cell necrosis, inflammatory cell infiltration [24]. The alcoholic extract of the stem bark of Alstonia has also been reported to possess anticancer activity in HS1 human sarcoma in the embryonated egg [18]. Our earlier studies have shown that Alstonia possesses anticancer, chemopreventive and radiosensitizing activities [25-27]. In addition, it has also been found to be non-teratogenic up to a dose of 240 mg/kg [25]. Therefore, the present study was undertaken to evaluate the anticancer activity of the alcoholic extract of the stem bark of A. scholaris in mice bearing Ehrlich ascites carcinoma.

Materials and Methods

Preparation of the Extract

The identification of the plant Alstonia scholaris (L.) R. Br. (family Apocynaceae) was done by Dr. G. K. Bhat (a well-known taxonomist) Department of Botany, Poornma Pratna College, Udupi, India and the herbarium specimen (Voucher No. ASU 034) has been stored with us. The non-infected stem bark of the tree was carefully peeled off, shade dried, and coarsely powdered with the help of a hand club. The stem bark powder was extracted with 85% ethyl alcohol in a Soxhlet apparatus extensively for 3 days. The cooled liquid extract was concentrated by evaporating its liquid contents, with an approximate yield of 28%. Henceforth, the extract of Alstonia scholaris will be abbreviated as ASE.

Animal Care and Handling

The guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India) were strictly followed in animal care and their handling. The study was approved by the Institutional Animal Ethical Committee of Kasturba Medical College, Manipal, India, where the study was carried out. Usually female Swiss albino mice of 10 to 12 weeks age with 30 to 36 g body weight (b. wt.) were procured from an inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and light (14 and 10 h of light and dark, respectively). The animals were given sterile food and water ad libitum. Four animals were kept in a sterile polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment.

Tumor model

Ehrlich ascites carcinoma (EAC) was supplied by the Cancer Research Institute (ACTREC), Mumbai, India. The EAC was propagated and maintained by serial intraperitoneal transplantation of 10^4 viable EAC cells in female mice in an aseptic environment. The day of tumor inoculation was designated as day zero.

Preparation of Drug and Mode of Administration

A known quantity of ASE was dissolved in 100 µl of ethanol and further diluted with the 0.5% carboxy methyl cellulose dissolved in sterile physiological saline (SPS). Cyclophosphamide (CPA) was dissolved in SPS containing 0.5% carboxymethyl cellulose (CMC). The SPS, ASE or CPA was intraperitoneally administered in the female tumor bearing mice.

Acute Toxicity Studies

The acute toxicity of the ASE was determined according to Prieur et al. [28] and Ghosh [29]. Briefly, the animals (non-tumor bearing) were allowed to fast by withdrawing the food and water for 18 h. Thefasted animals were divided into several groups of 10 each. Each group of animals was injected with 600, 800, 900, 1000, 1100, 1200, 1300 and 1400 mg/kg body weight (b. wt.) of ASE intraperitoneally. The animals were provided with food and water immediately after the drug administration. Mortality of the animals was observed up to 14 days post drug treatment.

Selection of Optimum Dose

The dose of ASE was selected following the standard protocol recommended by the National Service Center of Cancer Chemotherapy, CCNSC, USA [30]. Twenty-four hours after the tumor inoculation, the animals were divided into the following groups:

- **SPS control:** The animals of this group received 0.3 to 0.36 ml of sterile physiological saline (SPS) containing 0.5 % carboxymethyl cellulose once daily, consecutively for 9 days.
- **CPA control:** This group of animals was injected once daily with 25 mg/kg b. wt. of the freshly prepared CPA dissolved in sterile saline containing 0.5% carboxymethyl cellulose consecutively for nine days and served as the positive control.
- **ASE group:** The animals of this group were injected with 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 and 600 mg/kg b. wt. ASE once daily, consecutively for nine days.

Stage Specific Evaluation

The tumors were inoculated and allowed to grow for 1, 3, 6, 9, 12 and 15 days and for reasons of clarity these days have been designated as stage I, II, III, IV, V and VI, respectively. The tumor bearing animals of these stages were divided into the following groups:

- **ASE group:** The animals of this group were administered once daily with 420 mg/kg b. wt. ASE (the best dose) for nine consecutive days at I, II, III, IV, V or VI stage of tumor development.
- **CPA group:** This group of animals received 25 mg/kg b. wt. of cyclophosphamide in a similar fashion and served as concurrent positive control.

In Vitro to In Vivo Studies

A separate experiment was carried out to investigate the early and delayed cell death after EAC treatment, where the EAC cells were collected aseptically from the peritoneum of a seven day old tumor, washed in sterile PBS followed by serum free RPMI medium. The cells were resuspended in the serum free RPMI medium at a density of 10⁵ cells/ml in several culture flasks. The cells were treated with 30, 36, 42, 48, 54 or 60 µg/ml of ASE or 100 µg/ml echitamine chloride (ECL) with occasional shaking. The cultures were incubated at 37°C for 6 h in an atmosphere of 5% CO₂ and 95% air in a CO₂ incubator.

The immediate cytotoxic effect of ASE and ECL was evaluated by using the trypan blue dye exclusion test. Briefly, four hours after the
drug/s exposure the cells were washed in sterile PBS and the cell viability was determined using trypan blue dye exclusion test using a hemocytometer under an inverted transmitted light microscope (Leitz, Wetzlar, Germany). The number of viable and dead cells were scored and expressed as percent live cells. The delayed cell death or the reproductive cell death was calculated by injecting 10⁶ viable EAC cells exposed to ASE or ECL in normal healthy mice as described earlier.

The animals of all the experiments were monitored regularly for alteration in body weight, signs of toxicity and mortality. The weight of animals was recorded every third day up to 30 days after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of five grams per mouse was considered as an index of toxicity [30]. The animal survival was monitored daily up to 120 days, since the survival of animals up to 120 days is roughly equivalent to 5 years survival in man [31]. The tumor response was assessed on the basis of median survival time and tumor free survival. The median survival time (MST), and the average survival time (AST) were calculated from the animals dying within 120 days and those surviving 120 days were excluded from it. The MST and AST were calculated as follows:

\[
\text{MST} = \frac{\text{First death} + \text{last death in the group}}{2}
\]

\[
\text{AST} = \frac{\text{Sum of animal death on different days}}{\text{Number of animals}}
\]

The increase in median life span (% IMLS) and increase in average life span (% IALS) were also calculated using the following formulae:

\[
\text{IMLS} = \frac{\text{MST of treated mice} - \text{MST of control}}{\text{MST of control}} \times 100
\]

\[
\text{IALS} = \frac{\text{AST of treated mice} - \text{AST of control}}{\text{AST of control}} \times 100
\]

Biochemical Estimations

A separate experiment was performed to estimate glutathione and lipid peroxidation in the tumor cells. The animals were inoculated with tumor cells as described above and the tumor was allowed to grow for six days. On the seventh day, the tumor bearing animals were divided into the following groups:

- **SPS control**: The animals of this group received 0.3 to 0.36 ml of sterile physiological saline (SPS) containing 0.5% carboxymethyl cellulose.

- **CPA control**: This group of animals was injected once with 25 mg/kg b. wt. of the freshly prepared CPA dissolved in sterile saline containing 0.5% CMC.

- **ASE group**: The animals of this group were administered with a single injection of 240 or 420 mg/kg b. wt. of the ASE dissolved in sterile saline containing 0.5% CMC.

Four animals from each group were sacrificed at 1.5, 3, 6, 9, 12, 18 or 24 h after the drug administration. The tumor cells were aspirated in aseptic condition and were washed with SPS thrice. The cells were counted under the inverted microscope and 1 × 10⁶ cells were sonicated (Virsonic, USA) and processed for the estimation of glutathione and lipid peroxidation assays as described below.

Glutathione (GSH)

GSH contents were measured by the method of Moron, et al. [32]. Briefly, proteins were precipitated by 25% TCA, centrifuged and the supernatant was collected. The supernatant was mixed with 0.2 M sodium phosphate buffer pH 8.0 and 0.06 mM DTNB and incubated for 10 minutes at room temperature. The absorbance of the sample/s was read against the blank at 412 nm in a UV-Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Japan) and the GSH concentration was calculated from the standard curve.

Lipid Peroxidation (LOO)

LOO was measured by the method of Beuge and Aust [33]. Briefly, the homogenate was mixed with TCA-TBA-HCl and heated for 15 min in a boiling water bath. After centrifugation the absorbance was recorded at 535 nm using a UV-Visible double beam spectrophotometer (Shimadzu UV-260, Shimadzu Corp, Japan). The lipid peroxidation has been expressed as MDA in nmol per 10⁶ cells. The concentration of LOO in the sample was determined against the standard curve of TMP (1,1,3,3-tetramethoxy propane).

Statistical Analysis

The statistical significance between the treatments was determined using the “Z” test for the survival studies, while the student’s t test was used for the cytotoxicity and biochemical studies. All the data are expressed as mean ± SEM (standard error of the mean).

Results

Acute Toxicity Studies

The administration of different doses viz. 600, 800 and 900 mg/kg b. wt. of ASE did not induce any mortality during the whole observation period. However, a further increase in the ASE dose to 1000 mg/kg b. wt. caused a 20% reduction in the survival of mice. An increase in the dose of ASE up to 1200 mg/kg b. wt. resulted in a 50% reduction in the survival of mice and 80% of the animals died when the drug dose was raised up to 1300 mg/kg. 100% mortality was observed at 1400 mg/kg and thereafter up to a dose of 1500 mg/kg b. wt. of ASE, the last dose evaluated.

Selection of Optimum Dose

There was no spontaneous regression of tumors in the mouse transplanted with the EAC cells and the animals exhibited a constant weight gain and increase in the volume due to tumor cell multiplication and growth (figure 1). The MST was found to be approximately 19 days in the SPS group, while the AST was 18 days (table 1).

The treatment of 24 h old tumors with 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540 or 600 mg/kg ASE or 25 mg/kg CPA inhibited the weight increase in all the treatment group indicating arrest of tumor cell proliferation and growth (figure 1). The administration of 480, 540 and 600 mg/kg, was accompanied with toxic side effects like ruffling of hair, sluggishness and lacrimation administration of 480, 540 and 600 mg/kg, was accompanied with toxic side effects like ruffling of hair, sluggishness and lacrimation of the animals. A separate experiment was performed to estimate glutathione and lipid peroxidation in the tumor cells. The animals were inoculated with tumor cells as described above and the animals was allowed to grow for six days. On the seventh day, the tumor bearing animals were divided into the following groups:

- **SPS control**: The animals of this group received 0.3 to 0.36 ml of sterile physiological saline (SPS) containing 0.5% carboxymethyl cellulose.

- **CPA control**: This group of animals was injected once with 25 mg/kg b. wt. of the freshly prepared CPA dissolved in sterile saline containing 0.5% CMC.

- **ASE group**: The animals of this group were administered with a single injection of 240 or 420 mg/kg b. wt. of the ASE dissolved in sterile saline containing 0.5% CMC.

Four animals from each group were sacrificed at 1.5, 3, 6, 9, 12, 18 or 24 h after the drug administration. The tumor cells were aspirated in aseptic condition and were washed with SPS thrice. The cells were counted under the inverted microscope and 1 × 10⁶ cells were sonicated (Virsonic, USA) and processed for the estimation of glutathione and lipid peroxidation assays as described below.

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Statistical Analysis

The statistical significance between the treatments was determined using the “Z” test for the survival studies, while the student’s t test was used for the cytotoxicity and biochemical studies. All the data are expressed as mean ± SEM (standard error of the mean).
35.5, 48.5, 53.5 and 65.5 days, respectively when compared with the SPS treated group. The AST was also elevated up to 30, 35, 49, 55 and 64 days for 240, 300, 360, 420 and 480 mg/kg ASE, respectively. The ASE treatment increased the MST by 1.23 (240 mg), 1.5 (300 mg), 2.7 (360 mg), 3.1 (420 mg) and 4.2 (480 mg) folds when compared with the CPA group. The IMLS also showed an elevation up to 71, 87, 155, 181 and 245% for 240, 300, 360, 420 and 480 mg/kg ASE treatment, respectively. The treatment of mice with 240, 300, 360, 420 and 480 mg/kg ASE also caused an elevation in the IALS up to 65, 94, 175, 208 and 258% respectively (table 1).

Of all the ASE doses tested, the highest anticancer activity was observed for 360, 420 and 480 mg/kg, where 16.7, 33.3 and 41.7% healthy survivors were observed at the end of 120 days (figure 2). However, out of all the above three doses 420 mg/kg was considered as the best dose as it did not induce any toxic effects in the form

**Figure 1:** Alterations in the weight changes (g) in the Ehrlich's ascites carcinoma bearing mice treated with sterile physiological saline (closed squares), 25 mg/kg cyclophosphamide (closed circles) and *Alstonia scholaris* extract (triangles). a: 30, b: 60, c: 90, d: 120, e: 180, f: 240, g: 300, h: 360, i: 420, j: 480, k: 540 and l: 600 mg/kg *Alstonia scholaris* extract.
Stage Specific Evaluation

The stage specific evaluation of the anticancer activity of ASE was carried out in tumor bearing animals on 1, 3, 6, 9, 12 or 15 days (stage I, II, III, IV, V and VI, respectively) by administering 420 mg/kg ASE or 25 mg/kg CPA for nine days consecutively at stage I, II, III, IV, V or VI. Both the drugs were effective in reducing the weight gain in the animals due to tumor development, especially during the early stages, however, ASE was found to be more efficient than CPA even in the mid stages of tumor progression. The use of ASE even during the late stages of tumor development proved effective in reducing the tumor burden and weight gain (figure 3).

The administration of 25 mg/kg CPA exerted a significant anticancer activity only when administered in the early stages of tumor development, which is corroborated by the body weight loss of debility, loss of body weight and death and further studies were carried out using this dose of ASE.

Stage Specific Evaluation

The stage specific evaluation of the anticancer activity of ASE was carried out in tumor bearing animals on 1, 3, 6, 9, 12 or 15 days (stage I, II, III, IV, V and VI, respectively) by administering 420 mg/kg ASE or 25 mg/kg CPA for nine days consecutively at stage I, II, III, IV, V or VI. Both the drugs were effective in reducing the weight gain in the animals due to tumor development, especially during the early stages, however, ASE was found to be more efficient than CPA even in the mid stages of tumor progression. The use of ASE even during the late stages of tumor development proved effective in reducing the tumor burden and weight gain (figure 3).

The administration of 25 mg/kg CPA exerted a significant anticancer activity only when administered in the early stages of tumor development, which is corroborated by the body weight

### Table 1: Effect of treatment schedule on the median survival time (MST) and average survival time (AST) of mice transplanted with Ehrlich’s ascites carcinoma cells. a = p < 0.05; b = p < 0.001; c = 0.002; d = 0.0001 (compared with SPS).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MST</th>
<th>IMLS</th>
<th>AST</th>
<th>IALS</th>
<th>Percent survival (day)</th>
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<td>30</td>
<td>60</td>
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**Dose selection**

**Tumor stages**

**In vitro to in vivo studies**

### Figure 2: The median survival time (MST) and average survival time (AST) in the Ehrlich’s ascites carcinoma bearing mice treated with different doses of *Alstonia scholaris* extract and 25 mg/kg cyclophosphamide once daily for nine consecutive days. Solid bars: MST, mottled bars: AST.
changes (figure 3). None of the animals treated with CPA at various stages of tumor development survived up to 120 days post-tumor cell inoculation (table 1). The MST for the tumor bearing animal that received CPA at stage I, II, III, IV, V and VI was 29, 26.5, 24, 21.5, 19 and 18.5 days, respectively (figure 4a). An IMLS of 57.9, 39.5, 26.3 and 13.15% was observed for stage I, II, III, and IV respectively. The administration of CPA at the end stages (i.e. V and VI) proved ineffective in increasing the IMLS. The IALS also decreased with the increase in the tumor stages in the CPA group and it was 55.8, 46.6, 28.5, 17.4, 5.3 and 1.5% for stage I, II, III, IV, V and VI respectively (table 1).

The treatment of mice with 420 mg/kg ASE at various stages of tumor development resulted in an increase in the MST up to 53.5, 47, 42, 33, 23 and 20 days and AST to 55.4, 40.4, 37, 29.7, 23.4 and 19.7 at stage I, II, III, IV, V or VI, respectively, when compared with the CPA treatment (figure 4a, b). The IMLS and IALS also increased after ASE treatment depending on the stage of the tumor. The IMLS and IALS declined depending on the treatment stage and the lowest values were observed for stage VI. The IMLS of 181.6, 147.4, 121, 73.7, 21 and 5.3% while the IALS of 208, 124.8, 105.9, 65.1, 30.3 and 9.9% was found for stage I, II, III, IV, V and VI respectively (table 1).

The administration of ASE was better than CPA as 41.7, and 16.7% survivors were observed at the end of 120 days for stage I and III respectively, while no survivors were reported for the CPA group. The IMLS was 3.1, 3.7, 4.6 and 5.6 folds greater for stage I,
Studies

In Vivo

In Vitro to In Vivo Studies

Exposure of cells to PBS in vitro for 4 h did not induce any adverse effect on the cell viability and a maximum number of viable cells were observed in the PBS treated group (99.5%). However, the exposure of EAC cells to 100 µg/ml of ECL, the positive control resulted in approximately 21% reduction in the viable cells. Treatment of EAC cells to different concentrations of ASE resulted in a concentration dependent reduction in the cell viability (figure 5). The exposure of EAC cells to 24, 30 and 36 µg/ml caused a gradual decrease in the cell viability, where 98, 95 and 87% viability was observed. A further increase in the drug dose up to 42 µg/ml caused an abrupt decline in the viable cell population, where only 75% viable cells were observed (table 2). A 39% reduction in the viable cell population was observed after treatment of EAC cells with 60 µg/ml ASE, where the viability reached a nadir of 61% (table 1).

The inoculation of the SPS treated EAC cells into the healthy mice (non-tumor bearing) exhibited a constant weight gain due to the tumor cell multiplication and growth as was observed for the other experiments (figure 6). The MST was found to be 19.5 for this group, while the AST was 18.4 days (figure 7). The exposure of EAC cells to 100 µg/ml of ECL resulted in a MST and AST of 25 and 24.4 days, and the IMLS and IALS increased up to 28.2 and 32.6%, respectively (table 1).

Table 2: Cytotoxic effect of various doses of ASE and 100 µg/ml ECL on Ehrlich's ascites carcinoma cells. a = p < 0.05; b = p < 0.01; c = p < 0.001 (compared with SPS). ASE = Alstonia scholaris extract; SPS = Sterile physiological saline; CPA = Cyclophosphamide; ECL = Echitamine chloride

Biochemical Estimations

Glutathione (GSH): The treatment of mice with 240 and 420 mg/kg ASE resulted in a time dependent decline in the GSH contents in the

**Figure 6**

EAC cells and a nadir was observed at 6 h after treatment. Thereafter, GSH contents elevated steadily and reached almost normal level for 240 mg/kg ASE or CPA (figure 8a). The CPA treatment also caused a decline in the GSH contents and a maximum decline was observed at 9 h post-treatment (table 3).

**Lipid peroxidation (LOO):** Treatment of mice with 240, 420 ASE and 25 mg/kg of CPA resulted in the enhancement of lipid peroxidation with time (table 3). The peak lipid peroxidation was observed for 420 mg/kg ASE followed by 25 mg/kg CPA or 240 mg/kg ASE. The greatest elevation was observed at 9 h post treatment for ASE, while it was 12 h for CPA treatment (figure 8b).

**Discussion**

Chemotherapy has been the mainstay of cancer treatment for the past 60 years and it continues to play a major role in the control of advanced stages of malignancies and also as a prophylactic against possible metastases in combination with radiotherapy. The goal of cancer therapy is to completely eradicate the neoplastic...
cells without causing any appreciable damage to the normal tissues of the host. However, most of the modern clinically used chemotherapeutic drugs exhibit severe normal toxicity, resulting in undesirable side effects. Moreover, many of the potent drugs are mutagenic, carcinogenic, teratogenic and produce second malignancies which prove fatal to the patients [5]. Therefore, a need is felt to find alternative drugs, which are highly effective at non-toxic doses, inexpensive and accessible to one and all. Plants have formed the basis of sophisticated traditional systems of medicines that have been in existence for thousands of years in India [34]. These plant-based medicinal systems continue to play an important role in healthcare, and it has been estimated by the World Health Organization that approximately 80% of the world’s inhabitants rely mainly on traditional medicines for their primary healthcare [35]. Plant products also play an important role in healthcare systems of the remaining 20% of the population mainly residing in the developed countries. The use of some herbs has attracted a great deal of attention as one of the alternative cancer therapies from the point of less toxicity and cost benefit. Several clinical trials using herbs or natural products are being undertaken even in USA [36]. Furthermore, some of the herbal medicines and their constituents have been reported to inhibit the proliferation of cancer cells directly and also have been found to be clinically useful [37]. Therefore, an attempt has been made to evaluate the anticancer activity of the stem bark extract of *Alstonia scholaris*, which is commonly used in Ayurvedic system of medicine for various purposes.

The treatment of animals with ASE up to a dose of 900 mg/kg did not induce toxicity and no drug-induced mortality was observed. The LD<sub>50</sub> for the drug induced acute mortality was found to be 1,200 mg/kg b. wt. Similarly in our early study 2000 mg/kg oral administration of ASE was found to be non toxic [38]. The present finding that the crude extract of the stem bark has a strong tumoricidal and tumor growth inhibitory activity is very encouraging. Even though, the effect increases with the drug dose, the highest drug doses screened in this study (480, 540 or 600) have shown toxic manifestations like diarrhea, body weight loss and mortality. However no such symptoms were associated up to 420 mg/kg. Therefore, 420 mg/kg ASE the dose which produced highest anticancer effect after 480 mg/kg was considered as an optimum dose. The finding that the animals are able to tolerate cumulative dose of 3,780 mg (420 mg/kg, which is more than 1/3 of the LD<sub>50</sub> daily for nine days) without serious side effects is significant from the clinical point. The echitamine, one of the alkaloids isolated from ASE has been found to be toxic at a low dose [39]. The lower toxicity of ASE may lie in its composite nature, where the presence of several chemicals in it could counteract the toxic implications of the other components without serious compromise on the anticancer effect, which is in conformation with the Ayurvedic philosophy.

The SPS treated group of animals showed continuous tumor development in the SPS group as evidenced by a constant increase in the animal weight and finally death due to tumor burden. The first death in SPS group was recorded on day 16 and none of the thirty animals used in the study survived beyond day 22 post-tumor cell inoculation. In contrast, the administration of different doses of ASE (viz. 30, 60, 90, 120, 180, 240, 300, 360, 420, 480, 540, and 600 mg/kg) caused inhibition of tumor development, which is evident by the reduced speed in weight gain due to restriction of cell multiplication. However, the best activity was seen in the group of animals receiving 240, 300, 360, 420 and 480 mg/kg ASE, which is
reflected by an increase in the MST by 1.23, 1.5, 2.68, 3.13 and 4.22 and the AST by 1.15, 1.68, 3.13, 3.72 and 4.61 folds when compared with the CPA group, which has been used as positive control. The ASE treatment at a dose of 360, 420 and 480 mg/kg increased the 120 day survival by 16.66, 33.33 and 41.66 % respectively, which when extrapolated corresponds to 5 year survival in human beings [31], however, no such effect was observed for the CPA treatment.

The in vitro to in vivo studies have also confirmed the efficacy of ASE as an anticancer agent, where four hours treatment with ASE has increased the survival of the tumor-bearing mice significantly. As far as the authors are aware, this is the first systematic report of the anticancer activity of ASE in vivo. The other Indian medicinal plants like Euvartania heynacana, Rubia cordifolia, Tylophora indica, Withania somnifera, Hygrophila spinosa, Podophyllum hexandrum and Tinospora cordifolia have been reported to possess antineoplastic activity in different tumor models in vivo and in vitro [40-51].

Out of all the ASE doses screened, 420 mg/kg was considered to be the best dose as it resulted in 33.33% survivors, at the end of 120 days and did not induce any toxic effects in the form of debility, loss of body weight and death. However, no survivors were reported for the SPS and CPA groups up to 120 days post-treatment. The alkaloid fraction of Alstonia scholaris has been reported to increase the survival of EAC mouse earlier [27]. The other plant extracts including Tylophora indica, and Aphananthis polysychna, have also shown an optimal activity only at a particular drug dose beyond which, it was either ineffective or toxic to the animals [41,52]. Alstonine, one of the alkaloids present in ASE has been reported to be a cytotoxic in VCB lymphoma and Ehrlich ascites carcinoma cells [53]. The methanolic extracts of the root barks of A. macrophylla, A. glaucescens, and A. scholaris have been reported to be cytotoxic against two human lung adenocarcinoma (MOR-P) and large cell carcinoma (COR-L23) cell lines [54]. Activity-directional fractionation of A. scholaris led to the isolation of the alkaloids, talcarpine, villalstonine, pleiocarpamine, and macralstonine. The bisindole and villalstonine were found to possess pronounced activity in both cell lines, while pleiocarpamine, O-methylmacralstonine and macralstonine were all considerably less active than villalstonine. Out of the thirteen indole alkaloids isolated from the root bark of sister species A. macrophylla, the pronounced cytotoxic activity has been found for bisindoles O-acetylmacralstonine, villalstonine and macrocarpamine against the human cancer cell lines, MOR-P (adenocarcinoma), COR-L23 (large cell carcinoma), StM11t 1a (melanoma), Caki-2 (renal cell carcinoma), MCF7 (breast adenocarcinoma) and LS174T (colon adenocarcinoma) [55]. The alkaloid echitamine, present in the stem bark of A. scholaris has been reported to be cytotoxic in vitro and in vivo [56].

The clinical efficacy of an anticancer agent lies in its ability to inhibit the proliferation of tumors not only in early stages but also in the late stages of its development. Therefore, the stage specific antineoplastic activity of 420 mg/kg of ASE was evaluated at different stage of the tumor development with respect to the efficacy of 25 mg/kg of CPA. The results from the stages specific evaluation show that both ASE and CPA inhibited the increase in the body weight gain in animals due to tumor development during the early stages effectively, which may be due to an efficient tumor cell kill by ASE. The ASE was also found to be more efficient than CPA in mid as well as late stages of tumor development as evident by the inhibition in tumor gain and increase in the life span. The administration of ASE was able to enhance the 1MLS by 3.13, 3.73, 4.6 and 5.6 folds for I, II, III, IV and V stages, respectively. Similarly, the IALS was also elevated by 3.72, 2.67, 3.73, 5.73 and 6.35 folds for stage I to VI, respectively, when compared with concurrent CPA group. Treatment of tumor bearing mice with ASE at stage I and III increased the survival by 41.66 and 16.66 % respectively at the end of 120 days post tumor inoculation, for stages I and III, no survivors has been reported for the CPA group. The studies of the anticancer activity of plants at different stages of tumor development are scanty, however, a similar effect has been observed earlier in the EAC tumor receiving Aphamamixis polyestichca [52]. The information on the treatment in advanced stages of tumor development is lacking. The stage specific evaluation of ASE has shown that ASE is capable of inhibiting the tumor development even at late stages. This is significant with respect to clinical situation, where the patients turn up late for cancer treatment.

The mechanism of action of herbal drugs and their extract preparations differ in many respects from that of the synthetic drugs or single substances [57]. It can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating. As it is observed that the desired activity of an active compound is rarely present in adequate quantity and when present may contain unwanted activities. Further, it has also been observed that in plants, certain other compounds may be of help in enhancing the potency of the active compounds resulting in an additive or synergetic positive effect and at the same time reducing the toxic implications for the treatment, which in its final total gives immense benefit [58].

The exact mechanism of cell killing effect of ASE is not known. The tumoricidal effect of ASE in the present study may be due to several putative mechanisms. The ASE treatment caused a significant elevation in the LOO and subsequent decline in GSH levels in the EAC cells, which may be responsible for inflicting the damage to tumor cells and arresting the further growth of the tumor. The rise in LOO by ASE may inflict damage to the cell DNA, which may inhibit further division of tumor cells and also kill tumor cells leading to shrinkage of the tumor. This is supported by increased lipid peroxidation, which has been reported to cause damage to the DNA [59]. The ASE may have stimulated apoptosis in the tumor cells resulting in the continuous remission of the tumor, thereby eradicating the disease. The ASE treatment may have inhibited the DNA synthesis or mitosis of cells leading to arrest of cell proliferation and further tumor development. Echitamine chloride, one of the constituent of ASE has been reported to inhibit DNA synthesis [60-61]. Our earlier study has indicated that presence of echitamine chloride in ASE and its cytotoxic effects in vitro and in vivo [56]. It may also have acted by inhibiting serine proteases. The protease inhibitors have also been reported to inhibit tumor invasion, metastasis and angiogenesis [62] and the presence of triterpenoids in the ASE may also have contributed to anticancer activity. The triterpenoids (Lupeol linoleate, lupeol palmitate and alpha-amyrin linoleate), present in Alstonia have been reported to inhibit serine proteases and PKA [63]. ASE may have caused immune modulation resulting in the tumor cell kill and shrinkage. A. scholaris has been reported to possess immunostimulatory effect in BALB/c mouse and also to enhance the phagocytic activity in normal as well as immunosuppressed mice. It has also prevented the prednisone-induced decline in immune modulation and increased the lytic activity of peritoneal exudate cells against Escherichia coli [64]. The presence of ASE may have inhibited the transcriptional activation of NF-kB and COX-II, which provide survival advantage to tumor cells.

Conclusions

From our study it is clear that ASE, inhibited the growth of EAC cells and the optimum non-toxic dose of 420 mg/kg was nearly 1/3rd of the LD50 (1200 mg/kg) dose. The non-toxic nature of this drug may lie in its composite status. The exact mechanism of action of ASE is not known, however, the anticancer effect of ASE may be
due to multiple actions on the tumor cells that would have helped to kill the tumor cells effectively resulting in the complete regression of tumors in the survivors. Alternatively, ASE may also increase the immune surveillance and antimetastatic ability. ASE may have also suppressed the activation of NF-κB and COX-II to exert its antineoplastic action on EAC cells. The anticancer activity of ASE on EAC tumor may be due to the presence of several alkaloids that have been found to kill tumor cells and inhibition of serine protein kinase or immunomodulatory action. Further studies are required to investigate the active principle/s and their mode of action in tumors of different origin in vitro as well as in vivo.

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