

Antitumor activity of *Alangium salvifolium* against Dalton's ascitic lymphoma

R. Venkateshwarlu*, Yerragunta Venu Gopal, Akondi Butchi Raju, K. Bhanu Prasad

St.Peter's Institute of Pharmaceutical Sciences, Vidyanagar, Hanamkonda, Warangal, Andhra Pradesh, India.

*Corresponding author: venky7hill@gmail.com, Tel: 09959096138

Received: 21 June 2012, **Revised:** 1 September 2012, **Accepted:** 2 September 2012

Abstract

Alangium salvifolium (Alangiaceae) was used as folk medicine like anthelmintic, purgative, emetic, febrifuge, and for the treatment of leprosy and other skin diseases. The plant was selected for this investigation based on ethnopharmacological relevance. Anti tumor activity was tested against Dalton's ascitic lymphoma murine cell lines. Antitumor activity of methanolic extract of *Alangium salvifolium* was determined at doses of 200 and 400 mg/kg body weight orally. After 14 days treatment, mice were sacrificed and antitumor effect of methanolic extract of *Alangium salvifolium* (MEAS) assessed by evaluating tumor volume, tumor weight, viable and nonviable tumor cell count, tumor packed cell volume and hematological parameters. Evaluated the direct anticancer activity by normal peritoneal cell count and solid tumor methods. Oral administration of MEAS showed significant decrease in tumor volume, tumor weight, viable cells and increase non-viable cells. Hematological parameters (such as RBC, hemoglobin, WBC and differential count), biochemical parameters (SGPT, SGOT, Albumin, Total protein) were brought to normal values with less side effects. The results showed that methanolic extract of *Alangium salvifolium* has antitumor activity. This activity due to presence of flavonoids, alkaloids and tannins. Deoxytubulosine might be important alkaloid for its antitumor activity. It has less side effects than standard drug.

Keywords: *Alangium salvifolium*; Dalton's ascitic lymphoma; SGPT; SGOT

Introduction

Cancer is the name for a group of more than 100 diseases in which cells in a part of the body begin to grow out of control. Although there are many kinds of cancer, they all start because abnormal cells grow out of control. Untreated cancers can cause serious illness and even death. Several methods exist for the treatment of cancer in modern medicine. These include chemotherapy, radiotherapy and surgery. Chemotherapy is now considered as the most effective method of cancer treatment. However, most cancer chemotheraputants severely affect the host normal cells (Mascarenhas, 1994). Hence, the use of natural products now

has been contemplated of exceptional value in the control of cancer and its eradication program (Suffness and Pezzuto, 1991). There are some well known plant derived anticancer drugs which have passed clinical trials with reasonable efficacy and some levels of safety. They are vinblastin, vincristine (*Cathranthus Roseus*), taxoids (*Taxus brevifolia* and *Taxus baccata*) and Phodophyllotoxin and its derivative etoposide (*Phodophyllum*).

Some of the anticancer plants subjected to some level of ethnopharmacological studies based of ethno medical leads in India have been reported by several researchers; *Nicandra physalodes* (Gunasekara et al., 1981), *Aspergillus sp* (Das and Ray, 1988), *Zingiber officinate* (Unikrishnan and Kuttan, 1998), *Viscum album* (Kuttan et al., 1990), *Alstonia Sclolaris* (Krishnaswami et al, 1991) *Crocera sativus* (Nair et al.,1991), *Hydnocarpus wightiana* (Sharma and Hall 1991). There is long list of plants claimed to have varying levels of anti-cancer properties (Hartwell, 1968).

Alangium salvifolium (L.f) Wang belongs to family Alangiaceae. Locally it called as Aankolam. Alangiaceae is a monogeneric family of trees and shrubs found in tropical and subtropical region. The plant is distributed in dry regions, plains and lower hills in India, Africa, Srilanka, Indochina and China. Plant parts are used in diarrhea, paralysis, piles and vomiting (Sharstri, 1983). They are acrid, astringent, emollient, anthelmintic, thermogenic, diuretic and purgative. Root is useful for external application in acute case of rheumatism, leprosy and inflammation and internal application in cases of bites of rabbit and dogs (Bakhr, 1997).

Alangium salvifolium contains flavonoids, alkaloids, carbohydrates, tannins, phenolic compounds, glycosides and alkaloids, in which important is β -carboline-benzoquinolizidine alkaloid deoxytubulosine. Deoxytubulosine has dihydrofolate reductase inhibition (Rao and Venkatachalam, 1999) and DNA damage activity. Plant is rich in tetrahydroisoquinoline monoterpene glycoside, for e.g., alangiside-1 or ipecoside-2 whose structures are closely related to the ipecac alkaloid (Krishna Rao and Nigam, 1976).

Materials and methods

Plant material

The proposed plant material of fresh *A. Salvifolium* (*Alangium salvifolium*) stem and leaves were collected from Warangal, Andhra Pradesh-India. Authenticated by Dr.Vatsavaya S. Raju, senior professor plant systematics laboratory, Department of Botany, Kakatiya University, Warangal, India. A voucher specimen (No. VSR/DBKU/564) is deposited in the Department of Botany, Kakatiya University, Warangal, India. Preparation of the extract of *A. Salvifolium* Wang (stem, leaves and root) has done using methanol. The shade dried powder of the stem and leaves was packed well in Soxhlet apparatus and was subjected to continuous hot extraction with methanol until the completion of the extraction. The extract was filtered while hot and the resultant extract was distilled in vacuum under reduced pressure in order to remove the methanol completely. It was dried and kept in a desiccator till experimentation. The practical yield found to be 2.14 % w/w.

Acute toxicity study

According to OECD 423 guideline healthy adult male swiss albino mice starved overnight, were divided into groups (n=6) and were orally fed with increased dose of MEAS. Total MEAS administered orally in doses of up to 2000 mg/kg did not produce any sign of toxicity and mortality in mice when observed for 14 days after administration.

Animals

Male Swiss albino mice weighing 25 ± 2 g were taken. They were obtained from Mahaveer enterprises, Hyderabad. The mice were grouped and housed in poly acrylic cages (38 cm \times 23cm \times 10 cm) with not more than six animals per cage and maintained under standard laboratory conditions (temperature $25\pm 2^\circ\text{C}$ and dark/light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Amruth feeds, Pune, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before commencement of the experiment. All procedures described were reviewed and approved by the Institutional Animal Ethical Committee (IAEC No. 1022/SPIPS/Wgl/IAEC/2011).

Chemicals

The following chemicals were obtained from the indicated commercial sources: instead of trypan blue dye (SISCO Research Laboratory, Bombay, India), Thiobarbituric acid, (DTNB) (SISCO Research Laboratory, Bombay, India). All the reagents used were of analytical reagent grade.

Tumor cells

Dalton's Ascitic Lymphoma (DAL) cells were obtained from Amala Cancer Institute, Thrissur, Kerala, India. The DAL cells were maintained in vivo in swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from DAL tumor bearing mouse at the log phase (days 10–12 of tumor bearing) of the tumor cells. Each animal received 0.2 ml of tumor cell suspension containing 2×10^6 tumor cells intraperitoneally.

Treatment schedule

50 Swiss albino mice were divided into five groups (n = 10) and given food and water *ad libitum*. All the animals in each groups except Group-I received DAL cells (2×10^6 cells/mouse i.p.) This was taken as day '0'. Group-I served as normal control (25% Tween 80 per oral) and Group-II served as DAL control. 24-h after DAL transplantation, Group-III and Group-IV received methanol extract of *A. salvifolium* (MEAS) at a dose of 200 and 400 mg/kg/oral for 14 consecutive days, respectively. Group-V received reference drug Cyclophosphamide (CPA) (25 mg/kg/oral for 14 consecutive days (Shrinivas Sharma *et al.*, 2009). 24 hours of last dose, 5 animals of each group were sacrificed to measure tumor growth parameters (mean survival time, viable, non-viable cell, tumor volume, tumor weight and tumor packed cell volume), antioxidant and hematological parameters and the rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor host.

In-vitro-cytotoxicity study

1x10⁶ DLA cells in phosphate buffer saline (PBS) with varying different concentrations of MEAS were incubated at 37° c for 3hrs in 5% CO₂ atmosphere in the filtered cap, flat bottom cell culture flasks. The viability of cells was determined by Trypan Blue exclusion method (Gupta, 2002).

$$\% \text{ Cell viability} = \frac{\text{No. of dead cells}}{\text{No. of viable cells} + \text{No. of dead cells}} \times 100$$

Tumor growth parameters

A. Tumor volume and weight

The mice were dissected and the ascetic fluid was collected from peritoneal cavity. The volume was measured by taking it in a centrifuge tube and weighed immediately (Moul-isha et al., 2010).

B. Viable and non-viable tumor cell count

The viability and nonviability of the cell were checked by trypan blue dye exclusion assay. The cells were stained with trypan blue (0.4% in normal saline) dye. Live (viable) cells actively pump out the dye by efflux mechanism where as dead (non-viable) cells do not. These viable and nonviable cells were counted (Bala et al., 2010).

$$\text{Cell count} = \frac{\text{No. of cells} + \text{Dilution factor}}{\text{Area} \times \text{Thickness of film}} \times 100$$

C. Percentage increased in life span

The effect of MEAS on percentage increases in life span was calculated on the basis of mortality of the experimental mice (Sur and Ganguly, 1994).

$$\text{ILS \%} = \left[\frac{\text{Mean survival time of treated group}}{\text{Mean survival time of treated group}} - 1 \right] \times 100$$

D. Tumor packed cell volume

The tumor fluid was collected into Wintrobe's tube and it was centrifuged at the rate of 3000 rpm for a period of one hour. The volume of packed cells read directly as percentage. (Shrivastava and Das, 1995).

Hematological parameters

At the end of the experimental period, blood was collected from retrorbital pluxes and used for the estimation of hemoglobin (Hb) content (Sharma and Pandey, 2007), red blood

cell (RBC) count (Mohan, 2005), white blood cell (WBC) count (Shrivastava and Das, 1995), packed cell volume (PCV) (Mohan, 2005) and differential count (Sharma and Pandey, 2007) by standard procedures.

Biochemical parameters

The remaining blood was centrifuged and serum was used for the estimation of liver biochemical parameters like Serum Glutamic Pyruvic Transaminase (SGPT) (IFCC Method, 1986), Serum Glutamic-Oxaloacetic Transaminase (SGOT) (IFCC Method, 1986), Albumin (Dumas, 1975), Total Protein (TP) (Gornall, 1949 and Dumas, 1975) by autoanalyzer.

Effect on normal peritoneal cell count

To evaluate effect of MEAS on normal peritoneal cells, 3 groups of normal mice (n=4) were taken. One group was treated with 400 mg/kg/oral of MEAS and the second group received the same treatment for 2 consecutive days. The untreated third group was used as control. Peritoneal exudates of the cells were counted 24 h after treatment for each of the treated groups and compared with those of the untreated groups (Sur and Ganguly, 1994)

Effect on solid tumor

Mice were divided into two groups (n = 4). DAL cell lines (1×10^6 cells/mice) were injected into right hind limb (thigh) of all mice intramuscularly. The Group I mice were DAL tumor control. The Group II treated with MEAS 400 mg/kg/oral for 14 days. Tumor mass was measured from 15th day of tumor induction. The measurement was carried out every 5th day for a period of 30 days. Tumor mass volume was measured from following formula (Rajesh kumar et al., 2002)

$$V=4/3\pi r_1^2r_2$$

Where, r_1 and r_2 were independent radius of tumor mass.

Statistical analysis

All data are expressed as mean \pm S.E.M. Statistical significance (P) calculated by ANOVA. The post hoc test were Dennett's (tumor volume, tumor weight, viable, non viable, mean survival time, tumor PCV) and Benferroni tests (hematological, SGPT, SGOT, TP, albumin). $P < 0.05$ was considered as statistically significant.

Results

In the present study indicates that MEAS showed significant antitumor and alter the biochemical parameters in DAL- mice. The effects of MEAS on survival time, % ILS, tumor volume, packed cell volume, and tumor cell count (viable and nonviable cell) are shown in Table 2.

In-vitro-cytotoxicity study

In vitro study MEAS was incubated in DAL tumor cells with different concentrations

Table 1: Effect of *Alangium salvifolium* on DAL tumor cell lines

Concentration (ug/ml)	%cell death
5	15
10	45
20	46
40	53
50	57
60	65
70	69
80	73
90	76
100	84

Table 2: Effect of methanol extract of *Alangium salvifolium* on following parameters.

Parameters	Group II	Group III	Group IV	Group V
Mean survival time(days)	12.8±0.96	19.2±0.58*	20.6±2.24**	22.6±2.06**
Increased life span (%)	—	42	46	61
Tumor volume(ml)	18±2.2	10.4±2.08*	4.8±0.91***	4.8±1.29***
Tumor weight(g)	16.2±1.93	9.8±2.15*	3.5±0.7***	4.4±1.4***
Tumor packed cell volume (ml)	50.12±2.9	34.68±2.9***	36.7±1.7**	32.7±1.7***
Viable cell count(x10 ⁷ cells/ml)	19.63±0.96	16.91±0.83	12.48±0.7***	12.65±0.67***
Nonviable cell count(x10 ⁷ cells/ml)	0.16±0.017	0.16±0.02	0.53±0.04***	0.72±0.08***

Data are expressed as the mean ± SEM.**P* <0.05, ***P*<0.01,****P*<0.001,extract-treated groups compared with the Group II.Group I is a normal control means no formation of tumor

(5-40 ug/ml). IC₅₀ was determined that is 42.2 ug/ml. The death rate of DAL cells increases with increase in the concentration of the MEAS as shown in Table 1.

Effect of MEAS on mean survival time

The mean survival time of Group II was 12.8±0.96, while it increased to 19.2 ± 0.58 (Group III) and 20.6 ± 2.24 (Group IV) days, respectively in the MEAS-treated groups, whereas the standard drug Cyclophosphamide (25 mg/kg) treated Group V had a mean survival time of 22.6 ± 2.06 days as shown in figure 1.

Effect of MEAS on tumor growth

After treatment with MEAS significantly (*P*<0.001) reduced the tumor volume, tumor packed cell volume, and viable tumor cell count in a dose-dependent manner as compared to that of the Group II as shown in figure 2. Furthermore, nonviable tumor cell count at different doses of MEAS were significantly (*P*<0.001) increased in a dose-dependent manner.

Effect of MEAS on haematological parameters

As shown in Table 3, hemoglobin content (*P*< 0.05) and RBC count in the Group II was decreased as compared to the Group I. After treatment with MEAS significantly increased the hemoglobin content and RBC count to normal levels. The total WBC counts and

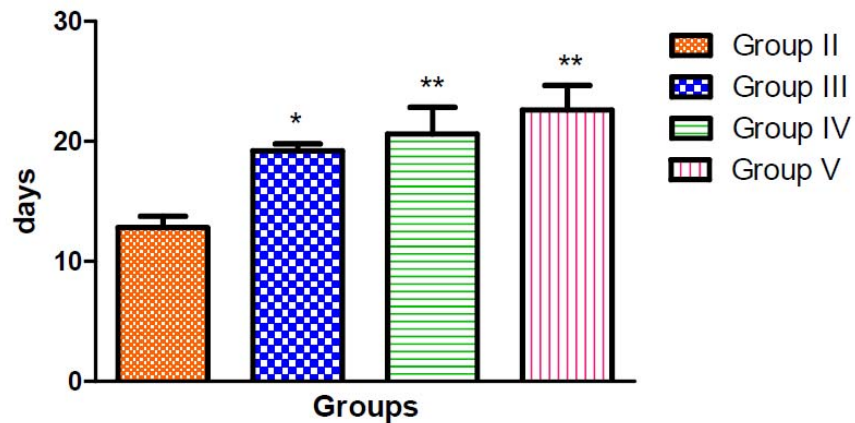


Figure 1: Effect of methanol extract of *Alangium salvifolium* on mean survival time. Data are expressed as the mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, extract-treated groups compared with the Group II.



Figure 2: Normal and tumor bearing mice..

PCV ($P < 0.001$) was found to be increased significantly in the Group II when compared with the normal group. Administration of MEAS in DAL-bearing mice significantly reduced the WBC count and PCV ($P < 0.05$) as compared with the Group II. In a differential count of WBC, the presence of neutrophils increased, while the lymphocyte, eosinophils, monocytes counts decreased in the Group II. Treatment with MEAS at different doses changed these altered parameters more or less to the normal values. Test group IV has been less effect on haemopoietic system to that of standard group V.

Effect of MEAS on biochemical parameters

As shown in Table 4, in the Group II the SGOT, SGPT, albumin levels were increased and total protein level was decreased as compared to the Group I. After treatment with MEAS at the dose of 200 mg/kg, 400mg/kg significantly decreased the SGOT, SGPT, albumin to normal levels and increased total protein level to that of Group V.

Table 3. Effect of methanol extract of *Alangium salvifolium* on haematological parameters.

Parameters	Group I	Group II	Group III	Group IV	Group V
Haemoglobin (%)	14.52±0.46	6.8±1.25 ^a	7.98±0.49	10.54±0.69	7.96±0.99
RBC(x10 ⁶ cell/mm ³)	13.14±0.56	7.5±0.5	8.4±0.5	9.52±0.39	8.24±0.60
PCV (%)	29.84±4.13	43.16±3.61 ^a	35.5±3.45	25.98±1.32	37.62±1.67
WBC(x10 ⁴ cells/mm ³)	0.628±0.13	5.74±0.7	2.58±0.23	1.8±0.19	3.51±0.17
Neutrophils (%)	37.6±1.74	54.8±7.01 ^b	52.2±1.88	40.6±0.7 ^c	48.4±1.69 ^d
Lymphocytes (%)	60.4±2.33	46±5.07 ^a	39.6±1.28	36.2±1.06	46.2±2.43
Eosinophils (%)	3.4±1.03	1.6±0.4	2.6±0.67	3.4±5.0	1.4±1.03
Monocytes (%)	1.8±0.37	0.2±0.2	1.2±0.2	1.6±0.2	0.5±0.37

Data are expressed as the mean of results in 5 mice ± SEM. ^aP<0.05, ^bP<0.01 Group I compared with the Group II. ^cP<0.01 and ^dP<0.001 Group II compared with extract treated groups.

Table 4. Effect of methanolic extract of *Alangium salvifolium* on biochemical parameters.

Parameters	Group I	Group II	Group III	Group IV	Group V
SGPT(U/L)	28.6±2.7	42.8±2.49	37.4±7.9	37.6±0.78	30±0.70
SGOT(U/L)	102.6±27.6	113.8±15.1	66.8±3.2 ^a	77.8±1.24	73.4±1.86
Albumin (gm%)	2.1±0.64	14.2±0.2	3.4±0.32	2.5±0.17	2.32±0.128
Total Protein(gms%)	5.08±0.64	4.7±0.41	13.12±0.95	7.72±0.84	6.3±0.57

Data are expressed as the mean of results in 5 mice ± SEM. ^aP<0.05, ^bP<0.01 Group I compared with the Group II. ^cP<0.01 and ^dP<0.001 Group II compared with extract treated groups.

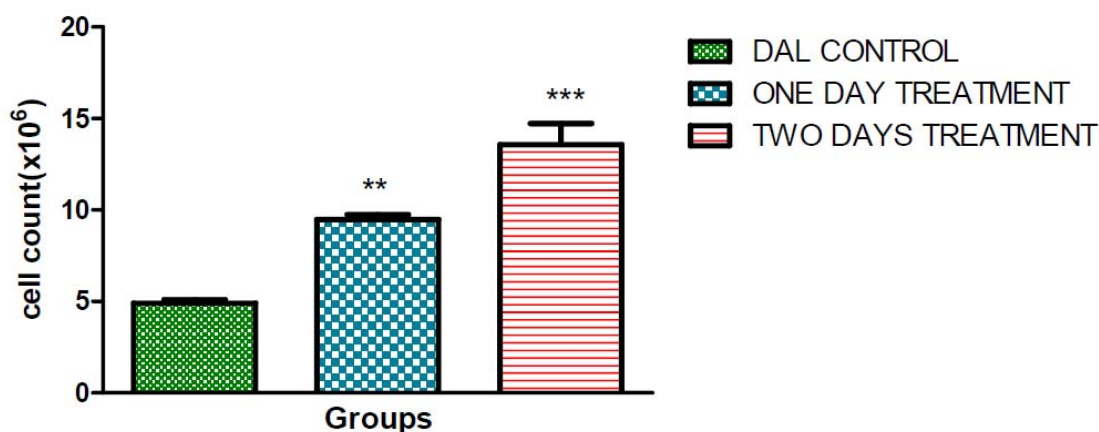


Figure 3: Effect of *Alangium salvifolium* on normal peritoneal cells. Data are expressed as the mean±SEM. *P<0.05, **P<0.01, extract-treated groups compared with the Group II.

Effect of MEAS on normal peritoneal cells

The average number of peritoneal exudate cells per normal mouse was found to be 4.9x10⁶. MEAS (400 mg/kg) single treatment enhanced peritoneal cells to 9.4x10⁶ while two consecutive treatment enhanced the number to 13.57x10⁶ as shown in Figure 3.

Effect of MEAS on solid tumor growth

There was reduction in the tumor volume of mice treated with MEAS from 15th day to 30th day. On 30th day tumor volume of control animals (Group I) was 6.4±1.19 ml,

Table 5. Effect of *Alangium salvifolium* on solid tumor growth.

Groups	Solid tumor volume in ml			
	15 th day	20 th day	25 th day	30 th day
Group I	0.086±0.01	2.0±0.3	3.9±0.7	6.4±1.19
Group II	0.16±.007	0.076±0.16	0.262±0.09**	0.715±0.22***

Data are expressed as the mean of results in 4 mice ± SEM. ** $P < 0.01$, *** $P < 0.001$, Group II compared with the Group I.

whereas for the extract-treated group (Group II) it was 0.715±0.22 ml as shown in Table 5.

Discussion

The present investigation was carried out to evaluate the antitumor effect of MEAS in DAL-bearing mice. The MEAS-treated animals at the doses of 200, 400 mg/kg and Cyclophosphamide (CPA) 25 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor cell count, and brought back the hematological parameters to more or less normal levels.

In vitro cytotoxicity study showed that it has cytotoxicity activity on DAL cell lines. So, we were done *in vivo* antitumor activity. There was regular rapid increase in ascites tumor volume was noted in DAL-bearing mice. Ascites fluid is the nutritional source for tumor cells and a rapid increase in ascitic fluid with tumor growth might be to meet the nutritional requirement of tumor cells (Prasad et al., 1994). Treatment with MEAS inhibited the tumor volume, tumor cell count, and increased the percentage of non-viable cells in tumor bearing mice. The reliable criteria for judging the value of any anticancer drug are the prolongation of the life span of animals (Clarkson et al., 1965). The MEAS decreased the ascites fluid volume, viable cell count, and increased the percentage of life span. It may be concluded that MEAS by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of DAL-bearing mice. These results could indicate either a direct cytotoxic effect of MEAS on tumor cells as evidenced by the *in vitro* studies or an indirectly inhibited tumor cell growth, the effect of MEAS treatment was examined on the peritoneal exudates cells of normal mice.

Normally, each mouse contains about 5×10^6 intraperitoneal cells, 50% of which are macrophages. MEAS treatments were found to enhance peritoneal cell counts. These results demonstrated the indirect effect of MEAS on DAL cells, probably mediated through enhancement and activation of macrophages or through some cytokine product inside the peritoneal cavity produced by MEAS treatment (Kavimani and Manisenthkumar, 2000). Hence, the observed antitumor nature of MEAS may be due to the cytotoxic properties.

To investigate if the inhibitory effect of MEAS on DAL tumor was local or systemic, the effect of MEAS in another experimental system, DAL-induced solid tumor, was tested (Senthil Kumar et al., 2007). The solid tumor was inhibited by treatment with MEAS, suggesting that the inhibitory effect is related not only to a local cytotoxic effect but also with the systemic effect of MEAS.

Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia (Price and Greenfield, 1958 and Hogland, 1982). The ane-

mia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Fenninger and Mider, 1954). Treatment with MEAS brought back the hemoglobin content, RBC, and WBC count more or less to normal levels. This indicates that MEAS possess protective action on the hemopoietic system.

Hepatocellular necrosis leads to high levels of albumin, SGPT and SGOT, which are released from liver into the blood. Increase in its activity is due to increased synthesis in the presence of increased biliary pressure (Moss and Butterworth, 1974). Reduction in the levels of these towards the respective normal values in liver tissues is an indication of stabilization of plasma membrane as well as repair of hepatic tissue damage caused by tumor inoculation.

Previous phytochemical study indicated the presence of flavonoid, alkaloids and tannins in MEAS (Kalarani et al., 2011). Flavonoids have been shown to possess antimalignant and antimutagenic effect (Fotsis et al., 1997). Furthermore, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation, apoptosis and angiogenesis (Wagner et al., 1986). The cytotoxicity and anticancer activity of MEAS are probably due to presence of flavonoids and deoxytubulosine. Deoxytubulosine has dihydrofolate reductase inhibition (Rao and Venkatachalam, 1999) and DNA damage activity.

Finally, based on the results concluded that the methanol extract of *Alangium salvifolium* was effective in inhibiting the tumor growth in ascitic and solid tumor models. Group IV MEAS 400 mg/kg is more effective than standard group (Group V) with less side effects.

Acknowledgement

We are very thankful to the Project guide and Management of St peters Institute of Pharmaceutical sciences, Warangal, for supporting this work. We are also thankful to Amala Cancer Institute, Thrissur, Kerala, India for providing the DAL cell lines.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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