Medicinal and Aromatic Plant Science and Biotechnology ©2012 Global Science Books

Preclinical Determination of the Anticancer Activity of Rohituka (*Aphanamixis polystachya*) in Ehrlich Ascites Tumor-Bearing Mice

Ganesh Chandra Jagetia[1*] • V. A. Venkatesha[2]

[1] Department of Zoology, Mizoram University, Aizawl-796 004, India
[2] Oncology R&D, Piramal Life Sciences Ltd., 1, Nirlon Complex, behind The Hub, Goregaon-East, Mumbai – 400 063, India
Corresponding author: * **gc.jagetia@gmail.com**

ABSTRACT

Anticancer activity of various doses of alcoholic extract of rohituka, *Aphanamixis polystachya* (APE) was studied in mice transplanted with Ehrlich ascites carcinoma (EAC). Administration of 0, 0.125, 0.25, 0.5, 0.75, 1, 1.25 or 1.5 g/Kg body weight APE once daily for consecutive 9 days resulted in a dose dependent regression in the tumor mass and increase in tumor-free survivors. The greatest anticancer activity was observed for 1 g/Kg APE as is evident by a maximum number of tumor-free survivors by 120 days post APE administration. Administrations of split dose of 0.5 g/Kg APE twice daily for nine consecutive days resulted in a greater number of tumor free-survivors than the single administration of 1 g/Kg APE concomitantly or 1 mg/Kg doxorubicin (positive control). The stage specific evaluation revealed that APE treatment was effective in regressing the tumors at all the stages and the most pronounced effect was observed up to stage III that lessened when the APE was administered during late stages of tumor development. Biochemical estimation revealed that APE administration increased lipid peroxidation by two folds accompanied by a two-fold decline in the glutathione contents at 8 h post-APE treatment. Similarly, APE treatment reduced the activities of glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase, and catalase at 8 h by 2.1, 2.3, 2, and 3.5 folds, respectively. Our study indicates that APE treatment caused a dose dependent retardation in the tumor mass and regressed tumors even in the late stages of tumor development, which may be due to increased lipid peroxidation and reduction in the activities of antioxidant enzymes.

Keywords: Antioxidant enzymes, Ehrlich ascites carcinoma, mice, survival

INTRODUCTION

Cancer is the second largest killer disease in the world and one in four patients in the United State die due to cancer. Despite several advances made in the treatment of cancer, the cancer mortality rates have not come down and remains at the level it was fifty years ago specifically for solid tumors (Jemal *et al.* 2011). This indicates that new approaches are required to treat cancer. Natural products including plants have been used by mankind for healthcare since the advent of human history and several traditional medicinal systems solely depend on natural products for medicament. Plants in particular have been used for more than 3,500 years in the treatment of 'cancer,' it was only in the late 1950s that the evaluation of crude plant extracts for their antiproliferative potential was initiated (Mann 2002; Cragg and Newman 2005; Bailly 2009) and subsequently, several anticancer lead molecules including vincristine, vinblastine, vinorelbine, etoposide, teniposide, paclitaxel, irinotecan, topotecan and camptothecin were initially isolated from plants (Cragg and Newman 2005; Harvey 2008; Kinghorn *et al.* 2009) before their chemical synthesis. These agents have been used successfully to treat various neoplastic diseases in humans. However, medicinal use of these active molecules has been associated with various undesired side effects and induction of second malignancies in the survivors (Swerdlow *et al.* 2011). The plant based drugs may be more compatible, less toxic and better tolerated by humans than synthetic drugs due to their biologic origin (Jagetia 2007). The herbal products therefore, may provide an alternate strategy to control cancer. Moreover, 80% of world population as such relies on alternative and complimentary medicines for their healthcare needs and cancer is no exception. Therefore, screening of plants could provide newer paradigms for the treatment of neoplastic disorders with lesser toxic side effects.

Aphanamixis polystachya Wall. Parker (*Amoora rohituka* (Roxb.) Wight & Arn.) known as rohituka in Sanskrit is distributed throughout India in evergreen forests and is a member of the family Meliaceae. It has been reported to possess several medicinal properties. The stem bark is acrid, astringent, bitter, vulnerary, digestive, anthelmintic, depurative, urinary astringent, ophthalmic and refrigerant. It is useful in the treatment of splenomegaly, liver disorders, tumors, ulcers, dyspepsia, intestinal worms, skin diseases, leprosy, diabetes, ophthalmopathy, jaundice, haemorrhoids, burning sensation, rheumatoid arthritis and leucorrhoea (Chopra *et al.* 1956). The seeds are useful in ulcers, ophthalmopathy, otopathy, myalgia, skin diseases, intestinal worms, burning sensation and vitiated conditions of vata (Warrier 1994). Rohituka has been reported to be an insect repellant, insecticidal, antifeedant, toxicant, and protectant against many species of insects and fungi (Talukder and Howse 1995). It has also been found to be active against various bacterial strains (Ripa *et al.* 2012; Saklani *et al.* 2012). The leaf extract of rohituka has been found to be an antidepressant and analgesic in mice (Hossain *et al.* 2009). The stem bark has been found to be useful in certain tumors including liver, spleen and leukemia in mice (Dhar *et al.* 1968; Srivastava and Agnihotri 1985; Rabi and Gupta 1995). The leaf extract of rohituka has been found to be hepatoprotective against carbon tetrachloride-induced hepatotoxicity (Gole and Dasgupta 2002). Various extracts of rohituka have been reported to be antioxidant and membrane stabilizing (Sikder *et al.* 2010; Saklani *et al.* 2011). The fruits and leaves of rohituka have been reported to contain labdane diterpenoid, aphanamixol (Chandrasekharan and Chakraborty 1968). The seeds and stem bark contain an

Received: 31 January, 2012. Accepted: 21 August, 2012.

Original Research Paper

alkaloid rohitukin (Harmon *et al.* 1979; Kundu *et al.* 1985; Zhang *et al.* 2007) and a range of complex limonoids like, polystachin, prieurianin and hispidin C (Connolly *et al.* 1979; Mulholland and Naidoo 1999) and flavonone glycosides viz., anthraquinone and naringenin glycosides and 8-*C*-methyl-quercetin-3-*O*-β-D-xylopyranoside (Srivastava and Agnihotri 1985; Jain and Srivastava 1985). Limonoids have been reported to possess anticancer activity in various human cancer cell lines (Ahn 1994). It is noteworthy that most of limonoids are structurally so complex that they would never have emerged from a synthetic program alone or from a combinatorial approach to new drug discovery (Gunatilaka 1998). Several activities attributed to rohituka and its frequent use in India stimulated us to conceptualize and investigate the anticancer activity of alcoholic extract of the stem bark of rohituka (*Aphanamixis polystachya*) in mice bearing Ehrlich ascites carcinoma.

MATERIALS AND METHODS

Chemicals

5,5'-dithiobis-2-nitrobenzoic acid (DTNB), reduced nicotinamide adenine dinucleotide (NADPH), epinephrine, 1-chloro-2, and 4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co., St. Louis, USA; Trichloroacetic acid (TCA) was purchased from Acros Organics, Geel, Belgium, while all other routine chemicals were procured from Ranbaxy Fine Chemicals, Mumbai, India.

Preparation of the extract

Rohituka, *Aphanamixis polystachya* R. Br. (family: *Meliaceae*) was identified by Dr. G. K. Bhat (a well-known taxonomist) Department of Botany, Poorna Prajna College, Udupi, India and the herbarium specimen has been stored with us. The stem bark of the tree was carefully peeled off, shade dried, and coarsely powdered in a ball mill. The powdered material was extracted with petroleum ether in a Soxhlet apparatus at 40°C for 30 cycles and dried at 40°C overnight. The petroleum ether free powdered material was further extracted with 95% ethanol in a Soxhlet apparatus at 60°C extensively for 3 days. The cooled liquid extract was concentrated by evaporating its liquid contents under reduced pressure at room temperature and finally freeze dried so as to obtain a fine powder of the extract. Henceforth the extract of rohituka will be called as APE.

Experimental

1. Determination of heavy metal contamination

Rohituka extract was subjected to atomic absorption spectrometry (AAS) for presence of cadmium and lead, the two most prevalent heavy metals according to WHO guidelines (WHO 1994) before undertaking the detailed investigation of its antineoplastic activity.

2. Animal care and handling

The animal care and handling were done according to the guidelines issued by the World Health Organization, Geneva, Switzerland and the INSA (Indian National Science Academy, New Delhi, India). Ten to twelve weeks old female Swiss albino mice weighing 30 to 36 g were selected from an inbred colony maintained under the controlled conditions of temperature (23 ± 2°C), humidity (50 ± 5%) and light (10 and 14 h of light and dark, respectively). The animals had free access to sterile food and water. Four animals were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. The study was approved by the Institutional Animal Ethical Committee (IAEC) of the Kasturba Medical College, Manipal, India, where the study was performed.

3. Tumor model

Ehrlich ascites carcinoma (EAC) procured from the Cancer Research Institute (ACTREC), Mumbai, India was used throughout the study.

4. Preparation of drug and mode of administration

The APE was freshly dissolved in 0.5% CMC (carboxy methylcellulose) in sterile phosphate buffered saline (SPS) before use. The APE was administered intraperitoneally in the tumor bearing mice.

5. Acute drug toxicity studies (OECD guidelines 420-425)

The acute toxicity (Prieur *et al.* 1972; Ghosh 1984) of 0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 3 g/Kg body weight of APE was determined according to OECD guidelines 420-425. Various behavioural and neurological responses in the mice fed with APE were recorded as per the standard criteria (Ghosh 1984). The responses were scored with a scale of 0 to 8 and scoring was done at the time of the peak effect.

6. Influence of APE on sleeping time in mice

The influence of APE on sleeping time in mice was assessed (Lovell 1986) to test for the proper functioning of central and peripheral nervous systems in the form of sleep induction as follows:

Female mice (25-35 g) fasted for 18 h were divided into three groups of eight each. Animals of group I were injected intraperitoneally with 40 mg/Kg pentobarbitone, and the animals of group II were pretreated with APE 1g/Kg body weight intraperitoneally 1 h before the injection of pentobarbitone, whereas the animals of group III were pretreated with APE 1 g/Kg body weight orally 1 h before the injection of pentobarbitone. A total of 24 animals were used for this study.

The period between the loss of righting reflex and regaining of righting reflex was considered as the sleeping time.

7. Antineoplastic activity

The optimum dose of APE for its antineoplastic activity was selected following the standard protocol recommended by Cancer Chemotherapy National Service Center (CCNSC) (Geran *et al.* 1972). Ten animals were administered with 0, 0.125, 0.25, 0.5, 0.75, 1, 1.25 or 1.5 g/Kg body weight APE once daily for 9 consecutive days 24 h after tumor inoculation. Concomitantly, a group of ten animals received 1 mg/Kg body weight doxorubicin (DOX) intraperitoneally and served as a positive control. A total of 90 animals were used for this experiment.

8. Altered administration schedule

A separate experiment was designed to evaluate the effect of alterations in the drug administration schedule on the antineoplastic activity of APE in the tumor bearing mice. A group of ten EAC mice were treated with 1 g/Kg body weight of APE once daily for 9 consecutive days or once every alternate day or once every alternate two days intraperitoneally. A still another group of ten EAC mice were treated with 0.5 g/Kg body weight APE (1 g/Kg APE was split into two parts) twice daily for nine consecutive days with a gap of 12 h between two fractions. A group of 10 matching saline treated animals served as control. A total of 50 animals were utilized for this experiment.

9. Stage-specific evaluation of the anticancer activity

The tumors were inoculated and allowed to grow for 1, 3, 6, 9, 12 or 15 days before APE treatment and for reasons of clarity these days have been designated as stage I, II, III, IV, V and VI, respectively. A group of 10 tumor bearing animals were treated with 1 g/Kg APE (optimum dose, split into 0.5 mg/Kg) or 1 mg/Kg doxorubicin intraperitoneally at 1, 3, 6, 9, 12 and 15 days after tumor inoculation (stage I, II, III, IV, V and VI, respectively), consecutively for nine days. A total of 130 animals were used for this experiment.

The animals of all experiments, except acute toxicity studies were monitored regularly for body weight changes, signs of

toxicity and mortality. The weight of animals was recorded every third day up to 120 days or until the animal survival after tumor inoculation in all the groups. A 33% of drug related deaths or a weight loss of 5 g per mouse was considered as an index of toxicity. 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 (Nias 1990). The tumor response was assessed on the basis of median survival time (MST) and the average survival time (AST). The MST and AST were calculated from the animals dying within 120 days and those surviving 120 days were excluded from it. The increase in median life span (% IMLS) and the increase in average life span (% IALS) were calculated using the following formulae (Geran *et al.* 1972):

$$MST = (X + Y)/2$$

where X is the earliest day when the cumulative number of deaths is $\geq N/2$, and Y is the earliest day when the cumulative number of deaths is $\geq (N/2) + 1$. $N = 10$ (total number of animals including survivors).

AST = (Sum of animal death on different days) / (No. of animals)

IMLS (%) = [(MST of treated mice - MST of control) / (MST of control)] × 100

IALS (%) = [(AST of treated mice - AST of control) / (AST of control)] × 100

Table 1.1 Effect of different doses of rohituka stem extract (APE) on the behavioural responses in the normal mice.

	Behavioral responses												
	Awareness					Mood				Motor activity			
APE (g/Kg) body weight	Alertness	Visual placing	Passivity	Stereotypy	Grooming	Vocalization	Restlessness	Irritability	Fearfulness	Reactivity	Spontaneous	Touch response	Pain response
0.0	4	4	0	0	4	0	0	0	0	4	4	4	4
0.05	4	4	0	0	4	0	0	0	0	4	4	4	4
0.1	4	4	0	0	4	0	0	0	0	4	4	4	4
0.25	4	4	0	0	4	0	0	0	0	4	4	4	4
0.5	4	4	0	0	4	0	0	0	0	4	4	4	4
1	4	4	0	0	4	0	0	0	0	4	4	4	4
2	4	4	0	0	4	0	0	0	1	5	4	4	5
3	5	4	0	0	4	0	1	0	0	4	5	4	4

Ten animals were used for each APE dose

Table 1.2 Effect of different doses of *Aphanamixis polystachya* extract (APE) on the neurological responses in the normal mice.

	Neurological profile															
	CNS excitation				Posture		Motor incoordination			Muscle tone				Reflexes		
APE (g/Kg) body weight	Startle response	Straubs response	Tremors	Convulsions	Body posture	Limb posture	Staggering gait	Abnormal gait	Righting reflex	Limb tone	Grip strength	Body tone	Abdominal tone	Pinna	Corneal	ISR
0.0	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
0.05	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
0.1	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
0.25	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
0.5	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
1	0	0	0	0	4	4	0	0	0	4	4	4	4	4	4	4
2	0	0	0	0	5	4	0	0	0	4	4	4	4	4	4	4
3	0	0	0	0	5	4	0	0	0	5	4	4	4	4	5	4

ISR = Ispilateral reflex

Ten animals were used for each APE dose

Table 1.3 Effect of different doses of *Aphanamixis polystachya* extract (APE) on the autonomic responses in the normal mice.

	Autonomic profile						
APE (g/Kg) body weight	Writhing	Pupil size	Salivation	Piloerection	Hypothermia	Lacrimation	Acute death
0.0	0	4	0	0	0	0	0
0.05	0	4	0	0	0	0	0
0.1	0	4	0	0	0	0	0
0.25	0	4	0	0	0	0	0
0.5	0	4	0	0	0	0	0
1	0	4	0	0	0	0	0
2	0	4	0	0	0	0	0
3	0	4	1	0	0	0	0

Ten animals were used for each APE dose

10. Biochemical estimations

A separate experiment was conducted to estimate the antioxidant enzyme and lipid peroxidation in the EAC cells after treatment with APE or DOX. The tumor bearing animals were administered with an intraperitoneal single injection of 1 g/Kg body weight of the freshly prepared APE in SPS containing 1% CMC or 1 mg/Kg doxorubicin once daily for 9 consecutive days. Four animals from each group were sacrificed at 0, 1.5, 3, 6, 9, 12, 18 or 24 h post last drug administration (PLDA). A total of 96 animals were used for this study.

The tumor cells were aspirated in an aseptic manner and were washed with SPS thrice. The cells were counted under an inverted microscope (Leica Microsystems, Wetzlar, Germany) and 1×10^6 cells from each EAC mice were suspended in 1 ml of 0.15 M Tris–HCl (pH 7.4) with 50 µl of Triton X 100 and sonicated on ice for 20 sec using a microprobe sonicator (Sonics Vibra-cell, CT, USA). The sonicated cell suspension was centrifuged at 13,000 rpm for 15 min. The supernatant (S) thus obtained was immediately used for the estimation of lipid peroxidation (LOO) (Ohkawa *et al.* 1979), glutathione (GSH) (Moron *et al.* 1979), glutathione peroxidase (GSHPx) (Ho 1994), glutathione-*S*-transferase (GST) (Habig *et al.* 1974), superoxide dismutase (SOD) (Misra and Fridovich 1972), and catalase (CAT) (Abei 1974).

Statistical analyses

The statistical significance between the treatments for survival studies was determined using the *Z*-test (Abramowitz and Stegun 1972), whereas the student's *t*-test was used for biochemical estimations. Solo 4 statistical package (BMDP Inc., USA and Ireland) was used for statistical analyses.

RESULTS

The results are presented in **Tables 1-7**. All the data are expressed as mean ± SEM (standard error of the mean).

Determination of heavy metal contamination

Atomic absorption spectrometry did not show any detectable amounts of lead and cadmium in the APE sample as well as corresponding blank. Therefore, the APE was considered free from heavy metal contamination.

Determination of acute toxicity

The acute toxicity of APE was determined by administering a single dose of 0, 0.05, 0.1, 0.25, 0.5, 1, 2 or 3 g/Kg body weight of APE. The APE was found to be non-toxic up to 3 g/Kg as no mortality could be observed up to 14 days post APE administration. The evaluation of higher doses was precluded owing to the problem in drug dissolution.

The experimental animals did not show neurological and behavioural toxic symptoms and signs up to 3 g/Kg body weight APE the highest dose that could be tested (**Table 1**). Similarly, the animals administered with APE did not show significant alteration in the sleeping time induced by pentobarbitone when compared with the inducer alone (**Table 2**).

Antineoplastic activity of APE

The transplanted EAC cells grow very rapidly in the peritoneum of the mouse, which was reflected in the rapid weight gain and increase in the abdominal size in the SPS group (**Table 4**). All animals of this group died within 15 days of tumor inoculation due to increased tumor burden. The MST and AST of this group were found to be 16 and 15 days, respectively.

Administration of 1 mg/Kg body weight of doxorubicin to EAC mice caused a marked increase in the survival time, where 50% of the EAC animals survived up to day 40 and 10% survived beyond 120 days post-tumor transplantation (**Table 3**). The MST and AST were 29.5 and 29 days, respectively. The increase in median life span (%IMLS) 90.3% and average life span (%IALS) was 93.3% (**Table 3**).

Table 2 Alteration in the sleeping time (Mean ± SEM) induced by pentobarbitone sodium in the normal mice treated with rohituka stem extract (APE).

Treatment	Sleeping time (h) ±SEM
Pentobarbitone alone	2.37 ± 0.077
APE (IP) + pentobarbitone	2.275 ± 0.065
APE (Oral) + pentobarbitone	2.25 ± 0.059

SEM = Standard error of the mean
Eight animals were used for each group

Treatment of EAC mice with various doses of APE resulted in a dose dependent enhancement in cell killing effect and remission of tumor up to 1 g/Kg body weight and a further increase in the APE dose caused a decline in its antineoplastic activity (**Table 3**). Treatment of EAC mice with 0.125 g/Kg body weight APE increased the survival by 2 days when compared to the concurrent SPS treatment. The MST and AST were 18 and 17 days, respectively and showed no protection against tumor growth (**Table 3**). Doubling the dose of APE to 0.25 g/Kg body weight had some ameliorating effect and all the animals died within 21 days. The MST and AST increased to 21 and 20.3 days, respectively, when compared to SPS treatment. A further increase in the dose of APE up to 0.5 or 0.75 g/Kg body weight did not show improvement in reducing the tumor growth and weight gain, where almost all animals succumbed to death by 45 days (**Table 3**). The MST and AST were found to be 23 and 22 days, respectively for 0.5 g/Kg group and 26 and 25.6 days, respectively for 0.75 g/Kg group. The administration of 0.5 and 0.75 g APE increased the life span of EAC mice up to 40 days (**Table 3**). When the APE dose was raised up to 1 g/Kg the 40 day survival was almost doubled where, 90% of the animals did survive (**Table 3**). The average weight gain was inhibited by 75% when compared with SPS control group (**Table 4**). The MST and AST also increased to 35 days approximately and the increase in median life span (%IMLS) and increase in average life span (%IALS) were greater than 120%. APE at 1 g/Kg body weight exhibited the best anti-tumor activity amongst all the doses tested as 40% of tumor free survivors were observed beyond 120 days (**Table 3**). Therefore, this dose of APE was considered as an optimum dose for its anticancer activity and further studies were carried out using this dose. The antineoplastic activity of APE declined when the dose of administration was increased to 1.25 or 1.5 g/Kg. However, the life span of 30 and 10% mice was increased up to 40 day for 1.25 and 1.5 g/Kg APE, respectively. The MST and AST increased by an almost 10 days and 7 days for 1.25 and 1.5 g APE, respectively when compared with the SPS treatment.

Altered administration schedule

Alteration in the nine-day administration schedule of 1 g/Kg APE once every day, once every alternate day or once every two alternate days resulted in an elevation in the survival of mice. The greatest effect was observed when a single dose of APE was administered for 9 consecutive days. The MST and AST were found to be 32.5 days each. Administration of 1 g/Kg body weight APE on alternate days and on every two alternate days also resulted in the increase in the MST and AST. However this schedule of administration was not as potent as split dose regimen (**Table 5**). Even though changes in single administration of drug to alternate day and alternate two day reduced the survival when compared to daily administration, it was higher than the SPS treated groups (**Table 5**).

Since alteration in single administration did not improve the antineoplastic activity of APE, it was decided to split 1 g/Kg APE into two equal doses and test the effect of this schedule on the EAC tumor cell killing. The EAC animals receiving 0.5 g/Kg of APE twice a day with a gap of 12 h

Table 3 Alteration in the survival of EAC mice treated with rohituka stem extract or doxorubicin.

Dose (g/Kg)	MST	IMLS	AST	IALS	Percent survivors on day			
					30	40	60	120
SPS	16	-	15	-	0	0	0	0
APE 0.125	18	12.5	17	13.33	0	0	0	0
APE 0.25	21	31.25	20.3	35.33	0	0	0	0
APE 0.5	23	43.75	22	46.66	50[b]	20	0	0
APE 0.75	26	62.5	25.6	70.66	70[c]	30[a]	0	0
APE 1	35.5	121.8	35	133.33	100[d]	90[d]	40[b]	40[b]
APE 1.25	26.5	65.62	25.7	71.33	60[b]	30[a]	0	0
APE 1.5	23.5	46.87	22.5	50	30[c]	10	0	0
DOX 1 mg/Kg	29.5	90.32	29	93.37	100[d]	50[b]	10	10

P < a = 0.05; b = 0.002; c = 0.001; d = 0.0001 and no symbols = non-significant when compared with SPS.
SPS = Sterile physiological saline; APE = Alcoholic extract of *Aphanamixis polystachya* and DOX = Doxorubicin hydrochloride
Ten animals were used for each group. Significance between the treatments was determined by the *Z*-test.

Table 4.1 Body weight changes (mean ± SEM) at various post-treatment days of tumorized mice treated with different concentrations of rohituka stem extract (APE) 24 h post-tumorization.

Days	APE dose mg/kg body weight							
	0	125	250	500	750	1000	1250	1500
0	30.33 ± 1.09	26.17 ± 0.68	30.80 ± 1.09	24.31 ± 0.58	35.71 ± 1.16	29.35 ± 0.47	37.41 ± 1.18	29.64 ± 0.35
3	31.43 ± 1.06	25.74 ± 0.67	30.57 ± 1.09	25.17 ± 1.00	34.21 ± 1.35	30.03 ± 0.51	35.60 ± 1.11	27.99 ± 0.34
6	27.77 ± 6.04	27.12 ± 0.81	31.48 ± 0.97	26.15 ± 0.75	32.05 ± 1.30	29.47 ± 0.44	32.76 ± 1.06	28.84 ± 0.54
9	32.71 ± 1.66	29.82 ± 0.90	33.63 ± 0.94	28.28 ± 0.86	34.05 ± 0.88	29.37 ± 0.75	33.10 ± 1.40	28.31 ± 1.04
12	45.01 ± 1.12	36.04 ± 0.67	36.71 ± 1.65	37.93 ± 1.19	36.70 ± 1.16	31.19 ± 0.83	33.34 ± 1.49	29.39 ± 2.30
15	49.47 ± 0.96	36.55 ± 0.67	39.29 ± 1.69	34.68 ± 0.22	38.18 ± 0.96	32.88 ± 0.78	38.17 ± 1.71	28.64 ± 0.21
18	-	37.04 ± 0.69	40.54 ± 1.72	36.06 ± 0.44	44.94 ± 1.49	33.40 ± 0.70	40.12 ± 4.05	29.63 ± 0.00
21	-	-	40.37 ± 2.30	36.47 ± 0.17	45.62 ± 0.00	34.17 ± 0.70	47.00 ± 0.00	-
24	-	-	-	-	-	34.52 ± 0.75	-	-
27	-	-	-	-	-	34.96 ± 0.73	-	-
30	-	-	-	-	-	35.50 ± 0.72	-	-
33	-	-	-	-	-	35.61 ± 0.74	-	-
36	-	-	-	-	-	35.83 ± 0.71	-	-
39	-	-	-	-	-	36.07 ± 0.70	-	-
42	-	-	-	-	-	36.35 ± 0.73	-	-
60	-	-	-	-	-	41.01 ± 0.55	-	-
120	-	-	-	-	-	47.05 ± 0.68	-	-

Ten animals were used for each APE dose.

Table 4.2 Body weight changes (mean ± SEM) at different days of tumorized mice treated with different concentrations doxorubicin 24 h post-tumorization.

Days	Doxorubicin mg/kg body weight					
	0.75	1	1.5	2	2.5	5
0	32.14 ± 0.51	34.18 ± 1.47	33.13 ± 0.80	31.65 ± 0.61	29.05 ± 0.43	30.32 ± 0.99
3	32.09 ± 0.69	33.63 ± 0.86	34.81 ± 1.37	32.83 ± 0.62	29.17 ± 0.54	28.40 ± 0.74
6	32.62 ± 0.58	34.59 ± 1.70	32.51 ± 0.73	31.19 ± 0.40	29.78 ± 0.49	28.23 ± 0.92
9	31.64 ± 0.94	34.36 ± 1.90	30.37 ± 1.23	30.63 ± 0.45	26.99 ± 0.74	-
12	28.61 ± 0.76	29.72 ± 1.28	30.92 ± 1.26	29.49 ± 0.84	26.08 ± 0.68	-
15	32.22 ± 0.74	32.27 ± 1.98	24.13 ± 1.37	-	-	-
18	31.38 ± 0.66	31.74 ± 1.65	-	-	-	-
21	31.35 ± 0.59	32.03 ± 1.97	-	-	-	-
24	32.34 ± 1.04	32.33 ± 2.05	-	-	-	-
27	31.90 ± 0.66	32.74 ± 5.03	-	-	-	-
30	32.11 ± 0.60	33.98 ± 2.76	-	-	-	-
33	37.77 ± 3.17	35.52 ± 4.38	-	-	-	-
36	33.78 ± 0.00	35.71 ± 4.37	-	-	-	-
39	34.39 ± 0.00	35.95 ± 4.41	-	-	-	-
42	34.47 ± 0.00	36.14 ± 4.43	-	-	-	-
60	37.03 ± 0.00	36.30 ± 4.41	-	-	-	-
120	-	-	-	-	-	-

SEM = Standard error of the mean.
Ten animals were used for each APE dose.

for nine consecutive days showed a significant tumor remission as was evident by retardation in the body weight gain of EAC mice and increase in the life span, where 50% of the animals survived beyond 120 days, a 10% elevation when compared with the single administration regimen (**Table 5**).

Stage-specific evaluation

Stage-specific evaluation of the antineoplastic activity of APE was carried out in EAC animals at 1, 3, 6, 9, 12 or 15 days (stages I, II, III, IV, V and VI, respectively), where two split doses of 0.5 g/Kg of APE or a single dose of 1 mg/Kg of DOX was administered every day for nine days, consecutively at stages I, II, III, IV, V and VI (**Table 6**). The APE was effective in increasing the life span of EAC mice up to stage III, where the animals did survive beyond 120 days, which is reflected in the body weight changes. The APE treatment caused higher survival (**Table 6**) and tumor remission during stages I, II and III.

Administration of APE at stage IV also lengthened the life span of 40% animals up to 40 days. The administration

Table 5 The effect of altered administration schedule on the survival of EAC mice treated with 1000 mg/kg body weight rohituka stem extract.

Dose (g/Kg)	MST	IMLS	AST	IALS	Percent survivors on day			
					30	40	60	120
SPS	16	-	15.5	-	0	0	0	0
APE 1000	35	118.75	34	119.35	100^{d}	90^{d}	40^{b}	40^{b}
APE 1000-AD	27	68.75	26.5	70.96	80^{d}	60^{c}	30^{a}	0
APE 1000-A2D	22	37.5	21.75	40.32	60^{c}	50^{b}	20	0
APE 500+500-D	48	200	47.25	204.8	100^{d}	90^{d}	60^{c}	50^{b}

APE administered on AD = alternate days; A2D = alternate two days; SD = two equal split doses of 0.5 g/Kg daily.
P < a = 0.05; b = 0.002; c = 0.001; d = 0.0001 and no symbols = non-significant when compared with SPS treatment.
SPS = Sterile physiological saline; APE = Alcoholic extract of *Aphanamixis polystachya* and DOX = Doxorubicin hydrochloride
Ten animals were used for each group. Significance between the treatments was calculated using the *Z*-test.

Table 6 Survival of EAC mice treated with rohituka stem extract or doxorubicin at various stages of tumor development.

Stages of tumor	MST	IMLS	AST	IALS	Percent survivors on day			
					30	40	60	120
SPS	16	-	15	-	-	-	-	-
APE I	49	206.25	48.5	223.33	100^{d}	90^{d}	60^{d}	50^{d}
APE II	41	156.25	41.5	176.66	100^{d}	90^{d}	50^{d}	40^{c}
APE III	34	112.5	34	120	100^{d}	70^{d}	40^{c}	20
APE IV	28	75	26.25	75	60^{c}	40^{b}	0	0
APE V	22	37.5	21.5	43.33	20	0	0	0
APE VI	16.5	3.125	15.5	3.33	0	0	0	0
DOX I	31	106.66	30	106.89	100^{d}	50^{c}	10	10
DOX II	29	81.25	27.5	83.33	90^{d}	40^{b}	10	10
DOX III	26.5	65.62	25.5	70	60^{c}	20	10	0
DOX IV	23.5	46.87	23	53.33	30^{a}	10	0	0
DOX V	19.5	21.875	18	20	0	0	0	0
DOX VI	17	6.25	16	6.66	0	0	0	0

P < a = 0.05; b = 0.002; c = 0.001; d = 0.0001 and no symbols = non-significant when compared with SPS treatment.
SPS = Sterile physiological saline; APE = Alcoholic extract of *Aphanamixis polystachya* and DOX = Doxorubicin hydrochloride
The tumors were inoculated and allowed to grow for 1, 3, 6, 9, 12 or 15 days before APE treatment and these days have been designated as stage I, II, III, IV, V and VI, respectively.
Ten animals were used for each group. Significance between the treatments was determined by the *Z*-test.

of APE at stage V and VI also increased the life span of EAC mice when compared to SPS treatment. The effect of APE dwindled especially after stage III of tumor development (**Table 6**). The treatment of mice with 1 g/Kg (two doses of 0.5 g each) APE at various stages of tumor development resulted in an increase in the MST up to 49, 41, 34, 28, 22 and 16.5 days at stage I, II, III, IV, V and VI, respectively when compared with the DOX treatment (**Table 6**). Similarly, the IMLS and IALS also increased after APE treatment depending on the stage of drug inoculation. The IMLS and IALS declined depending on the treatment stage and the lowest values were observed for stage VI. The IMLS of 206.25, 156.25, 112.5, 75, 37.5 and 3.1%, whereas the IALS of 223.23, 176.66, 120, 75, 43.33 and 3.33% were recorded for stage I, II, III, IV, V and VI, respectively (**Table 6**).

Administration of APE resulted in 50, 40 and 30% survivors by 120 days at stage I, II and III respectively of tumor development. The MST and AST increased by 3-, 2.6-, 2-, 1.7- and 1.4-fold at stage I, II, III, IV and V, respectively after APE treatment when compared with the DOX treatment. The IMLS was 1.9-, 1.9-, 1.7-, 1.6- and 1.7-fold greater for stage I, II, III, IV and V, respectively, while the IALS was elevated by 2.1-, 2.1-, 1.7-, 1.4- and 2.2-fold for stage I, II, III, IV and V, respectively, when compared with concurrent DOX group (**Table 6**).

Biochemical estimations

The spontaneous values of LOO, GSH, GSHPx, GST, SOD and CAT did not change significantly in EAC cells with assay time (**Table 7**).

1. Lipid peroxidation

Treatment of EAC mice with APE caused a time dependent elevation in the LOO and a peak level was reached at 8 h (2-fold; $P < 0.05$) that remained unaltered up to 12 h ($P < 0.05$). The LOO declined steadily thereafter without reaching to normal level even up to 24 h PLDA (1.5-fold more when compared with 0 h PLDA). The lipid peroxidation was significantly higher up to 16 h PLDA in APE treatment group ($P < 0.05$). The pattern of lipid peroxidation in DOX-treated group was almost similar to that of APE treatment, except that the highest level was observed at 4 h that remained unaltered up to 12 h PLDA (1.8-fold; $P < 0.05$). However, magnitude of LOO increase was lesser in the DOX-treated group than that of APE-treated group (**Table 7**).

2. Glutathione

Treatment of EAC mice with APE or DOX resulted in a time-dependent decline in the GSH concentrations in the EAC cells that reached a nadir at 8 h for the APE or DOX treatment ($P < 0.01$). Thereafter, GSH concentrations steadily elevated up to 24 h PLDA (**Table 7**). The decline in the concentrations of GSH in APE and DOX treated groups was 2- and 1.7-fold, respectively at 8 h PLDA ($P < 0.01$). The reduction in GSH concentration in APE-treated group was 1.2-fold higher when compared with the DOX-treated group at 8 h PLDA. The GSH concentrations ($P < 0.05$) did not reach control level even at 24 h PLDA in both APE- and DOX-treated groups (**Table 7**).

3. Glutathione peroxidase

APE or DOX treatment significantly reduced the GSHPx activity ($P < 0.05$) when compared with the untreated control at all PLDAs (**Table 7**). The activity of GSHPx in EAC cells declined steadily up to 8 h, where a maximum reduction of 2-fold was observed for APE and DOX treatment. Thereafter, GSHpx activity increased steadily up to 24 h PLDA in the EAC cells treated with APE or DOX. The GSHPx activity was 1.6- and 1.5-fold lower in APE- ($P < 0.05$) and DOX-treated groups when compared with the concurrent non-drug treated group at 24 h PLDA (**Table 7**).

Table 7 Alteration in the antioxidant enzymes and lipid peroxidation in the Ehrlich ascites carcinoma (EAC) cells treated with rohituka stem extract or doxorubicin.

Treatment	PLDA (h)	LOO	GSH	GSHPx	GST	SOD	CAT
SPS	0	4.09 ± 0.52	2.63 ± 0.09	8.63 ± 0.67	0.247 ± 0.023	2.92 ± 0.051	0.37 ± 0.026
	1	4.08 ± 0.51	2.62 ± 0.07	8.49 ± 0.52	0.252 ± 0.024	2.89 ± 0.053	0.368 ± 0.032
	2	4.08 ± 0.37	2.63 ± 0.07	8.60 ± 0.56	0.250 ± 0.020	2.91 ± 0.041	0.352 ± 0.032
	4	4.09 ± 0.44	2.62 ± 0.08	8.54 ± 0.61	0.249 ± 0.021	2.92 ± 0.05	0.36 ± 0.028
	8	4.11 ± 0.56	2.62 ± 0.06	8.64 ± 0.24	0.251 ± 0.019	2.90 ± 0.042	0.37 ± 0.024
	12	4.11 ± 0.50	2.62 ± 0.08	8.60 ± 0.58	0.250 ± 0.022	2.88 ± 0.043	0.36 ± 0.029
	16	4.13 ± 0.48	2.63 ± 0.09	8.58 ± 0.42	0.248 ± 0.021	2.91 ± 0.040	0.377 ± 0.031
	24	4.13 ± 0.67	2.62 ± 0.09	8.62 ± 0.49	0.249 ± 0.023	2.90 ± 0.049	0.36 ± 0.024
APE	0	4.09 ± 0.44	2.62 ± 0.08	7.67 ± 0.51	0.196 ± 0.017	2.75 ± 0.044	0.24 ± 0.022
	1	6.17 ± 0.46	2.18 ± 0.06[a]	5.10 ± 0.45	0.16 ± 0.011	2.21 ± 0.047[b]	0.203 ± 0.02
	2	7.29 ± 0.41[a]	1.86 ± 0.05[b]	4.29 ± 0.50[a]	0.107 ± 0.01[a]	2.06 ± 0.043[b]	0.126 ± 0.017[a]
	4	7.70 ± 0.42[a]	1.52 ± 0.05[b]	4.16 ± 0.43[a]	0.083 ± 0.01[a]	1.75 ± 0.041[c]	0.105 ± 0.019[b]
	8	8.45 ± 0.46[a]	1.31 ± 0.06[b]	4.08 ± 0.42[b]	0.109 ± 0.013[a]	1.43 ± 0.048[c]	0.105 ± 0.018[a]
	12	8.38 ± 0.36[a]	1.49 ± 0.05[b]	4.13 ± 0.46[a]	0.116 ± 0.014[a]	1.90 ± 0.040[c]	0.128 ± 0.02[a]
	16	7.33 ± 0.37[a]	1.64 ± 0.04[b]	4.79 ± 0.51[a]	0.124 ± 0.015[a]	2.08 ± 0.040[b]	0.17 ± 0.021[a]
	24	6.38 ± 0.41	2.11 ± 0.06[a]	5.21 ± 0.38[a]	0.13 ± 0.012[a]	2.21 ± 0.041[b]	0.208 ± 0.018[a]
DOX	0	4.09 ± 0.39	2.62 ± 0.08	7.93 ± 0.67	0.20 ± 0.016	2.71 ± 0.053	0.26 ± 0.021
	1	6.12 ± 0.42	2.1 ± 0.07[a]	5.14 ± 0.65	0.162 ± 0.016	2.07 ± 0.051[b]	0.21 ± 0.021
	2	7.18 ± 0.49[a]	1.92 ± 0.05[a]	4.5 ± 0.54[a]	0.117 ± 0.01[a]	1.94 ± 0.052[b]	0.14 ± 0.02[a]
	4	7.41 ± 0.59[a]	1.74 ± 0.05[b]	4.32 ± 0.40[a]	0.101 ± 0.01[a]	1.58 ± 0.043[c]	0.13 ± 0.019[a]
	8	7.37 ± 0.68[a]	1.58 ± 0.04[b]	4.26 ± 0.48[a]	0.115 ± 0.011[a]	1.86 ± 0.043[b]	0.12 ± 0.017[b]
	12	7.30 ± 0.61[a]	1.71 ± 0.05[b]	4.52 ± 0.49[a]	0.13 ± 0.013[a]	2.10 ± 0.042[b]	0.127 ± 0.021[a]
	16	6.23 ± 0.58	2.04 ± 0.05[a]	5.11 ± 0.57[a]	0.149 ± 0.014	2.12 ± 0.041[b]	0.192 ± 0.021[a]
	24	5.87 ± 0.49	2.17 ± 0.06[a]	5.53 ± 0.61	0.16 ± 0.015	2.40 ± 0.042[a]	0.227 ± 0.023

P < a = 0.05; b = 0.01; c = 0.005 and No symbols = non-significant when compared concurrent SPS-treated group.
DOX = Doxorubicin 1 mg/kg; APE = alcoholic extract of *Aphanamixis polystachya* 1g/kg body weight administered intraperitoneally 24 h after tumorization of mouse once daily for 9 consecutive days, and PLDA = post last drug administration.
LOO = nM TBARS/10^6 EAC cells; GSH = nM/10^6 EAC cells; CAT = nM H_2O_2 decomposed/min/10^6 EAC cells; SOD = U/mg protein/10^6 EAC cells; GSHPx = nM NADPH consumed/min/10^6 EAC cells; GST = mM GSH-CDNB conjugate formed/min/10^6 EAC cells.
Four animals were used for each group at each assessment time. Significance between the treatments was calculated using student's *t*-test.

4. Glutathione-S-transferase

The GST activity declined steadily and significantly ($P < 0.05$) with assay time up to 4 h PLDA in both APE (3-fold) and DOX (2.5-fold) treated groups, thereafter a steady elevation was observed in the GST activity in both APE- or DOX-treated groups up to 24 h PLDA (**Table 7**). However, the GST activity was below normal in APE group ($P < 0.05$). The activity of GST was 1.9-fold lower at 24 h after APE treatment ($P < 0.05$), while it was 1.5-fold for DOX treatment when compared with untreated control (**Table 7**).

5. Superoxide dismutase

Administration of mice with APE or DOX resulted in a time dependent decline in the activity of superoxide dismutase in EAC cells up to 8 h PLDA (2-fold) and 4 h (1.7-fold) and increased steadily up to 24 h, which was significantly higher in the DOX-treated group when compared with the 4 h PLDA (**Table 7**). The SOD activity in APE or DOX treatment group did not reach control level even by 24 h PLDA (**Table 7**). The decline in SOD activity was 1.3-fold higher in the APE treated group ($P < 0.01$) at 24 h PLDA when compared with SOD activity of the concurrent untreated control (**Table 7**).

6. Catalase

The activity of catalase enzyme declined in a time-dependent manner in EAC cells treated with APE or DOX. The greatest decline in CAT activity was observed at 8 h for APE treatment or DOX treatment. This reduction in CAT activity was approximately 3.5- and 3-fold for APE and DOX treatments, respectively. The reduction in CAT activity was significantly ($P < 0.02$) higher at 12 h PLDA in both the groups (**Table 7**). The decline in the CAT activity was greater in APE treatment than the DOX treatment (**Table 7**). However, the CAT activity did not reach control level in both DOX and APE groups (**Table 7**). The decline in catalase activity was almost 1.7- and 1.6-fold greater in the APE- ($P < 0.05$) and DOX-treated groups at 24 h PLDA when compared with the concurrent untreated control (**Table 7**).

DISCUSSION

Nature has been generous to bestow a variety of plants and other resources for the benefit of humans. Plants perform all the living functions including communication and defense. Plants synthesize and make use of a variety of chemicals including terpenes, alkaloids, tannins, phenols etc. for their defense and survival. That is why different plants contain different types of one or more of these chemicals. Humans have employed plants and herbs for various purposes including healthcare since the time immemorial, and plant-derived chemicals have played an important role in the treatment of various human diseases including cancer in the modern system of medicine (Mann 2002; Newman and Cragg 2007). The systemic drug discovery and development have established a reasonable armamentarium of useful chemotherapeutic agents during the last six decades (DeVita and Chu 2008; Harvey 2008; Bailey 2009). Further, the treatment with most modern chemotherapeutic drugs leads to acquired chemoresistance as a result tumors become non-responsive to the treatment (Tan *et al*. 2010; Chang 2011). The success with most of the chemotherapeutic agents has been limited due to their high toxicity, lack of tumor selectivity, low efficacy, recurrence and ineffectiveness against single or multi-drug resistant cancers (Mellor and Callaghan 2008; Walko and McLeod 2009). Therefore, the continued commitment to the arduous tasks involved in the discovery of new anticancer therapeutic agents with less toxicity, higher efficacy and better selectivity remains critically important. This target can only be achieved by screening newer paradigms and agents for the management of neoplastic diseases. Therefore, the present study was undertaken to evaluate the antineoplastic activity of *Aphanamixis polystachya* in Ehrlich ascites tumor bearing mice.

Many medicinal plants including *Alstonia scholaris*, *Ervatamia heyncana*, *Rubia cordifolia*, *Withania somnifera*,

Hygrophila spinosa, *Podyphyllum hexandrum*, *Tylophora indica*, *Nigella sativa*, *Annona glabra*, *Sansevieria roxburghiana*, *Dysoxylum caulostachyum*, *Eugenia aquea*, *Garcinia celebica*, *Psychotria valentonic* and *Tinospora cordifolia* have been reported to possess antineoplastic activity in different tumor models *in vitro* and *in vivo* (Chitnis *et al.* 1972; Adwankar *et al.* 1980; Devi *et al.* 1992; Mazumdar *et al.* 1997; Goel *et al.* 1998; Jagetia *et al.* 1998, 2005; Jagetia and Baliga 2005; Jagetia and Rao 2006; Cochrane *et al.* 2008; Patel *et al.* 2010; Philip *et al*. 2011; Subarnas *et al*. 2012). APE treatment caused regression of EAC tumor in a dose dependent manner up to 1 g/Kg body weight as is evident by the maximum number of tumor free survivors at the end of 120 days. Increase in APE dose beyond 1 g resulted in a decline in its antineoplastic activity. As far as authors are aware this is probably the first systematic report on the anticancer properties of APE in tumor bearing mice. Flavopiridol, a derivative of plant alkaloid rohitukin (extracted from the leaves and stems of rohituka and *Dyoxylum binectariferum*) has been found to be active against various cancers (Senderowicz *et al.* 1998).

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 1 g/Kg APE was evaluated at different stages of the tumor development. The results from the stage specific evaluation show that the APE inhibited increase in the body weight gain in animals due to the retardation of tumor cell proliferation during various stages effectively and it was very effective until stage III, which may be due to an efficient tumor cell killing. The studies of the anticancer activity of plants at different stages of tumor development are scanty, however, *Alstonia scholaris* has been reported to inhibit tumor growth in mice when administered in mid stages of tumor development (Jagetia and Baliga 2004). APE was effective in increasing the median survival of EAC mice by 18, 12, 7.5, 4.5 and 2.5 days and average survival time by 18.5, 14, 9.5, 3 and 3.5 days at stage I, II, III, IV and V of tumor development when compared with the concurrent positive DOX control. The efficacy of APE lies in that, it even killed the tumor cells, when administered during the late stages of tumor development, where APE treatment lengthened the life span of 40 and 20% animals beyond 120 days, especially during II and III stages, respectively. APE is found to be active even at stage IV and V where a tumor free survivors of 40% beyond 40 days and 20% up to 30 days, respectively was recorded. Ehrlich ascites carcinoma (EAC) is an undifferentiated malignancy. Penetration of EAC to tissues and tumor development is due to the absence of tumor-associated transplantation antigens (Lettre *et al.* 1972). APE might have inhibited penetration of EAC cells into other organs of EAC mice as the spleens of APE-administered mice were found to be absolutely normal in size and color.

The exact mechanism of action of APE is not known. The cells killing effect of APE may not be due to single mechanism but it may be due to the interplay of several mechanisms. APE has reduced glutathione and its related enzymes including GSHpx and GST by 2- and 2.3-fold accompanied by a two-fold increase in the lipid peroxidation at 8 h PLDA, which may be one of the reasons of its cytotoxic effect on EAC tumor in the present study. This contention is supported by the observation that increased concentration of GSH and GSH related enzymes like GST in the tumor cells have been reported to make the tumor refractory to the treatment, while their depletion has been reported to enhance the cell death and apoptosis of the tumor cells along with the loss of essential sulfhydryl groups that result in an alteration of the calcium homeostasis and eventually loss of cell viability (Tew 1994; Townsend and Tew 2003). Lipid peroxidation is another important event related to cell death and has been reported to cause severe impairment of membrane function through increased membrane permeability and membrane protein oxidation, DNA damage, cytotoxicity and eventually cell death (Linden *et al.* 2008; Chen and Niki 2011). A similar mechanism seems to be operational in the present study, where APE has increased the LOO and reduced GSH. This may have subsequently damaged the DNA of EAC cells and killed them effectively. APE treatment also decreased the inventory levels of CAT, and SOD by 3.5- and 2-fold at 8 h PLDA in EAC cells, and disturbed the redox status. This reduction in CAT and SOD may have made the tumor cells more vulnerable to death. APE treatment may have also arrested the cell cycle in G_2+M phase and killed the tumor cells effectively. Most of the tumors express nuclear factor (NF)-κB and suppression of NF-κB by APE may have been also responsible for the effective tumor cell kill in the present study. APE has been reported to inhibit NF-κB activity in various leukemic cells lines *in vitro* (Lampronti *et al.* 2005). The cell killing effect of APE may be attributed to the presence of alkaloids including rohitukin, amoorastatin and 12α-hydroxyamoorastatin and limonoids like, polystachin, prieurianin, hispidin C, aphanamixin and aphananin (Connolly *et al.* 1976; Harmon *et al.* 1979; Polonsky *et al.* 1979; Mulholland and Naidoo 1999). It has also been reported to contain a lignin, polystachyol and lignin glycosides including lyoniside and nudiposide (Sadhu *et al*. 2006). Recently, a few new liminoids have been isolated from rohituka including rohituka-7, dregeana-1, rohituka-15, Tr-B, rohituka-3, rohituka-5, rohituka-14, and aphanamolides A and B and Aphanamixoid A (Zhang *et al*. 2007; Yang *et al*. 2011; Cai *et al*. 2012).

Natural medicines have gained popularity over synthetic drugs in recent years with the belief that they are much safer and have led to the tremendous growth of phytopharmaceutical usage. While it is known that plant extracts are active against cancer, the standard approach has been to isolate, synthesize and administer the single chemical compound thought to be responsible for this effect. This may also be the reason for higher toxicity and low efficacy of single components. However, different components in a botanical may have synergistic activities and there is also some evidence that the presence of multiple compounds in a plant extract can buffer the toxic effects of a single constituent (Williamson 2001).

CONCLUSIONS

The treatment of APE has killed EAC cells and increased the life span of EAC mice when compared with the control. APE has also been effective in treating the tumor during the different stages of development. However, the effect dwindled with the advanced stages of tumor development. Cytotoxic effect of APE may be due to increased lipid peroxidation, depletion in GSH, GSHpx, GST, SOD and CAT and suppression in the transactivation of NF-κB.

ACKNOWLEDGEMENTS

The financial assistance from the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi in the form of senior research fellowship awarded to Dr. Venkatesha VA to carry out this study is gratefully acknowledged. We are thankful to Dr. G.K. Bhat (Poorna Pragna College, Udupi, India) for his expert help in identifying *Aphanamixis polystachya*.

REFERENCES

Abramowitz M, Stegun IA (1972) *Handbook of Mathematical Functions*, Dover Publications Inc., New York, 925 pp

Adwankar MK, Chitnis MP, Khandalekar DD, Bhadsavale CG (1980) Anticancer activity of the extracts of *Rubia cordifolia* Linn. (NSC b668893). *Indian Journal of Experimental Biology* **18**, 102

Aebi H (1974) Catalase. In: Ulhrich Bergmeyer H (Ed) *Methods of Enzymatic Analysis*, Verlag Chemie International, Florida, pp 673-682

Ahn JW (1994) Cytotoxic limonoids from *Melia azedarachta* var. Japonica. *Phytochemistry* **36**, 1493-1496

Bailly C (2009) Ready for a comeback of natural products in oncology. *Biochemical Pharmacology* **77 (9)**, 1447-1457

Cai JY, Zhang Y, Luo SH, Chen DZ, Tang GH, Yuan CM, Di YT, Li SH, Hao XJ, He HP (2012) Aphanamixoid A, a potent defensive limonoid,

with a new carbon skeleton from *Aphanamixis polystachya*. *Organic Letters* **14 (12)**, 2524-2527

Chandrasekharan S, Chakraborty T (1968) Aphanamixol, a diterpene alcohol from *Aphanamixis polystachya* (Wall) Parker. *Journal of Indian Chemical Society* **45**, 208

Chang A (2011) Chemotherapy, chemoresistance and the changing treatment landscape for NSCLC. *Lung Cancer* **71**, 3-10

Chen Z-H, Niki E (2011) Two faces of lipid peroxidation products: the 'Yin and Yang' principles of oxidative stress. *Journal of Experimental and Integrative Medicine* **1 (4)**, 215-219

Chitnis MP, Khandalekar DD, Adwankar MK, Sahasrabudhe MB (1972) Anti-cancer activity of the extracts of stem and leaf of *Tylophora indica*. *Indian Journal of Medical Research* **60**, 359-362

Cochrane CB, Nair PKR, Melnick SJ, Resek AP, Ramachandran C (2008) Anticancer effects of *Annona glabra* plant extracts in human leukemia cell lines. *Anticancer Research* **28**, 965-972

Chopra RN, Nayar SL, Chopra IC (1956) *Glossary of Indian Medicinal Plants*, Council of Scientific & Industrial Research, New Delhi, India

Connolly JD, Okorie DA, de Wit DL, Taylor DAH (1976) Structure of dregeanin and rohitukin. *Journal of the Chemical Society, Chemical Communications* **22**, 909-910

Cragg GM, Newman DJ (2005) Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology* **100**, 72-79

Devi PU, Sharada AC, Solomon FE, Kamath MS (1992) *In vivo* growth inhibitory effect of *Withania somnifera* (Ashwagandha) in a transplantable mouse tumor, sarcoma 180. *Indian Journal of Experimental Biology* **30**, 169-172

DeVita VT, Chu E (2008) A history of cancer chemotherapy. *Cancer Research* **68 (21)**, 8643-8653

Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Ray C (1968) Screening of Indian plants for biological activity. Part I. *Indian Journal of Experimental Biology* **6**, 232-247

Geran RI, Greenberg NH, MacDonald MM, Schumacher AM, Abbott BJ (1972) Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemotherapy Reports* **3 (3)**, 25-27

Ghosh MN (1984) *Fundamentals of Experimental Pharmacology*, Scientific book Agency, Kolkata, India

Goel HC, Prasad J, Sharma A, Singh B (1998) Antitumour and radioprotective action of *Podophyllum hexandrum*. *Indian Journal of Experimental Biology* **36**, 583-587

Gole MK, Dasgupta S (2002) Role of plant metabolites in toxic liver injury. *Asia Pacific Journal of Clinical Nutrition* **11 (1)**, 48-50

Gunatilaka AAL, Bolzani V da S, Dagne E, Hofmann GA, Johnson RK, McCabe FL, Mattern MR, Kingston DG (1998) Limonoids showing selective toxicity to DNA repair-deficient yeast and other constituents of *Trichelia emetica*. *Journal of Natural Products* **61**, 179-184

Habig WH, Pabst MJ, Jacoby WS (1974) Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry* **249**, 7130-7139

Harmon AD, Weiss U, Silverton JV (1979) The structure of rohitukine, the main alkaloid of *Amoora rohituka* (Syn. *Aphanamixis polystachya*) (Maliaceae). *Tetrahedron Letters* **8**, 721-724

Harvey A (2008) Natural product in drug discovery. *Drug Discovery Today* **13**, 894-901

Ho YS, Magnenat JL, Roderick T, Cao BJ, Gargano M, Sugawara M, Funk CD (1997) Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. *Journal of Biological Chemistry* **272**, 16644-16651

Hossain MM, Biva IJ, Jahangir R, Vhuiyan MMI B (2009) Central nervous system depressant and analgesic activity of *Aphanamixis polystachya* (Wall.) Parker leaf extract in mice. *African Journal of Pharmacy and Pharmacology* **3 (5)**, 282-286

Jagetia GC (2007) Radioprotective potential of plants and herbs against the effects of ionizing radiations. *Journal of Clinical Biochemistry and Nutrition* **40 (2)**, 74-81

Jagetia GC, Baliga MS (2004) Effect of *Alstonia scholaris* in enhancing the anticancer activity of berberine in the Ehrlich ascites carcinoma bearing mice. *Journal of Medicinal Food* **7 (2)**, 235-244

Jagetia GC, Baliga MS (2005) The effect of seasonal variation on the antineoplastic activity of *Alstonia scholaris* R. Br. in HeLa cells. *Journal of Ethnopharmacology* **96 (1-2)**, 37-42

Jagetia GC, Nayak V, Vidyasagar MS (1998) Evaluation of the antineoplastic activity of guduchi (*Tinospora cordifolia*) in cultured HeLa cells. *Cancer Letters* **127**, 71-82

Jagetia GC, Rao SK (2006) Evaluation of cytotoxic effects of dichloromethane extract of guduchi (*Tinospora cordifolia* Miers ex Hook F & THOMS) on cultured HeLa cells. *Evidenced-Based Complementary and Alternative Medicine* **3**, 267-272

Jagetia GC, Venkatesh, P, Baliga MS (2005) *Aegle marmelos* (L.) Correa inhibits the proliferation of transplanted Ehrlich ascites carcinoma in mice. *Biological and Pharmaceutical Bulletin* **28 (1)**, 58-64

Jain SA, Srivastava SK (1985) 8-*C*-methyl-quercetin-3-*O*-β-D-xylopyranoside, a new flavone glycoside from the roots of *Amoora rohituka*. *Journal of Natural Products* **48**, 299-301

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D (2011) Global cancer statistics. *CA Cancer Journal for Clinicians* **61 (2)**, 69-90

Kinghorn AD, Carcache de Blanco EJ, Chai HB, Orjala J, Farnsworth NR, Soejarto DD, Oberlies NH, Wani MC, Kroll DJ, Pearce CJ, Swanson SM, Kramer RA, Rose WC, Fairchild CR, Vite GD, Emanuel S, Jarjoura D, Cope FO (2009) Discovery of anticancer agents of diverse natural origin. *Pure and Applied Chemistry* **81 (6)**, 1051-1063

Kundu AB, Sanghamitra R, Chatterjee A (1985) Aphananin, a triterpene from *Aphanamixis polystachya*. *Phytochemistry* **24**, 2123-2125

Lampronti I, Khan MT, Bianchi N, Ather A, Borgatti M, Vizziello L, Fabbri E, Gambari R (2005) Bangladeshi medicinal plant extracts inhibiting molecular interactions between nuclear factors and target DNA sequences mimicking NF-kappa B binding sites. *Medicinal Chemistry* **1 (4)**, 327-333

Lettre R, Paweletz D, Werner D, Granzow C (1972) Sublines of the Ehrlich-Lettre mouse ascites tumor - a new tool for experimental cell research. *Naturwissenschaften* **59**, 59-63

Linden A, Gülden M, Martin HJ, Maser E, Seibert H (2008) Peroxide-induced cell death and lipid peroxidation in C6 glioma cells. *Toxicology in Vitro* **22 (5)**, 1371-1376

Lovell DP (1986) Variation in barbiturate sleeping time in mice: 1. Strain and sex differences. *Laboratory Animals* **20**, 85-90

Mann J (2002) Natural products in cancer chemoptherapy: past, present and future. *Nature Reviews Cancer* **2**, 143-148

Mazumdar UK, Gupta M, Maiti S, Mukherjee D (1997) Antitumor activity of *Hygrophila spinosa* on Ehrlich ascites carcinoma and sarcoma-180 induced mice. *Indian Journal of Experimental Biology* **35**, 473-477

Mellor HR, Callaghan R (2008) Resistance to chemotherapy in cancer: A complex and integrated cellular response. *Pharmacology* **81**, 275-300

Misra HP, Fridovich I (1972) The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. *Journal of Biological Chemistry* **247**, 3170-3175

Moron MS, Pierre JN, Mannervick B (1979) Levels of glutathione, glutathione reductase and glutathione-*S*-transferase activities in rat lung and liver. *Biochimica et Biophysica Acta* **582**, 67-71

Mulholland DA, Naidoo N (1999) Limonoids from *Aphanamixis polystachya*. *Phytochemistry* **51**, 927-930

Newman DJ, Cragg G (2007) Natural products as sources of new drugs over the last 25 years. *Journal of Natural Products* **70**, 461-477

Nias AHW (1990) Radiation biology. In: Sikora K, Halnan KE (Eds) *Treatment of Cancer*, Chapman and Hall Medical, London, pp 53-92

Ohkawa H, Ohishi N, Yagi K (1979) Assay of lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry* **95**, 351-358

Patel PN, Suthar M, Shah TG, Patel LJ (2010) Anticancer activity of *Nigella sativa* seeds against HL-60, U-937 and HEK-293T cell line. *Pharm Expt 01*

Philip D, Kaleena PK, Valivittan K (2012) *In vitro* cytotoxicity and anticancer activity of *Sansevieria roxburghiana. International Journal of Current Pharmaceutical Research* **3 (3)**, 71-73

Polonsky J, Varon Z, Marazano C, Arnoux B, Pettit GR, Schmid JM (1979) The structure of amoorastatone and the cytotoxic limonoid 12-hydroxyamoorastatin. *Experientia* **35**, 987-989

Prieur DJ, Young DM, Davis RD, Cooney DA, Homan ER, Dixon RL, Guarino AM (1972) Procedures for preclinical toxicologic evaluation of cancer chemotherapeutic agents: Protocols of the laboratory of toxicology. *Cancer Chemotherapy Reports* **4**, 1-28

Rabi T, Gupta RC (1995) Antitumor and cytotoxic investigation of *Amoora rohituka*. *International Journal of Pharmacognosy* **33**, 359-361

Ripa FA, Nahar L, Fazal A, Khatun MH (2012) Antibacterial and phytochemical evaluation of three medicinal plants of Bangladesh. International *Journal of Pharmaceutical Sciences and Research* **3 (3)**, 788-792

Sadhu SK, Phattanwasin P, Choudhuri MSK, Ohtsuki T, Ishibashi M (2006) A new lignan from *Aphanamixis polystachya*. *Journal of Natural Medicines* **60 (3)**, 258-260

Saklani S, Mishra AP, Sati B, Sati H (2012) Pharmacognostic, phytochemical and antimicrobial screening of *aphanamixis polystachya*, an endangered medicinal tree. *International Journal of Pharmacy and Pharmaceutical Sciences* **4 (3)**, 235-240

Saklani S, Mishra AP, Rawat A, Chandra S (2011) Free radical scavenging (DPPH) and ferric reducing ability (FRAP) of *Aphanamixis polystachya* (Wall) Parker. *International Journal of Drug Development and Research* **3 (4)**, 271-274

Senderowicz AM, Headlee D, Stinson SF, Lush RM, Kalil N, Villalba L, Hill K, Steinberg SM, Figg WD, Tompkins A, Arbuck SG, Sausville EA (1998) Phase I trial of continuous infusion flavopiridol, a novel cyclin-dependent kinase inhibitor, in patients with refractory neoplasms. *Journal of Clinical Oncology* **16**, 2986-2999

Sikder MA, Kuddus MR, Kaisar MA, Karna S, Rashid MA (2010) *In vitro* membrane stabilizing activity, total phenolic content, free radical scavenging and cytotoxic properties of *Aphanamixis polystachya* (Wall.). *Bangladesh Pharmaceutical Journal* **13 (2)**, 55-59

Srivastava SK, Agnihotri VK (1985) New glycosides from the stem bark of

Aphanamixis polystachya. *Current Science* **54**, 38-40

Subarnas A, Diantini A, Abdulah R, Zuhrotun A, Yamazaki C, Nakazawa M, Koyama H (2012) Antiproliferative activity of primates-consumed plants against MCF-7 human breast cancer cell lines. *Journal of Medical Research* **1 (4)**, 38-43

Swerdlow AJ, Higgins CD, Smith P, Cunningham D, Hancock BW, Horwich A, Hoskin PJ, Lister TA, Radford JA, Rohatiner AZ, Linch DC (2011) Second cancer risk after chemotherapy for Hodgkin's lymphoma: A collaborative British cohort study. *Journal of Clinical Oncology* **29 (31)**, 4096-4104

Talukder FA, Howse PE (1995) Evaluation of *Aphanamixis polystachya* as repellants, antifeedants, toxicants and protectants in storage against *Tribolium castaneum*, (Herbst). *Journal of Stored Product Research* **31**, 55-61

Tan DS-W, Gerlinger M, Teh B-T, Swanton C (2010) Anti-cancer drug resistance: Understanding the mechanisms through the use of integrative genomics and functional RNA interference. *European Journal of Cancer* **46**, 2166-2177

Tew KD (1994) Glutathione-associated enzymes in anticancer drug resistance. *Cancer Research* **54 (16)**, 4313-4320

Townsend DM, Tew KD (2003) The role of glutathione-*S*-transferase in anti-cancer drug resistance. *Oncogene* **22**, 7369-7375

Walko CM, McLeod H (2009) Pharmacogenomic progress in individualized dosing of key drugs for cancer patients. *Nature Clinical Practice Oncology* **3**, 153-162

Warrier PS (1994) *Indian Medicinal Plants*, Orient Longman Ltd., New Delhi, India

Williamson EM (2001) Synergy and other interactions in phytomedicines. *Phytomedicine* **8**, 401-409

World Health Organization (1998) Quality control methods for medicinal plants. In: *Determination of Arsenic and Heavy Metals*, UK, pp 62-63

Yang SP, Chen HD, Liao SG, Xie BJ, Miao ZH, Yue JM (2011) Aphanamolide A, a new limonoid from *Aphanamixis polystachya*. *Organic Letters* **13 (1)**, 150-153

Zhang H, Chen F, Wang X, Wu D, Chen Q (2007) Complete assignments of ^{1}H and ^{13}C NMR data for rings A,B-seco limonoids from the seed of *Aphanamixis polystachya*. *Magnetic Resonance Chemistry* **45 (2)**, 189-192