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ORIGINAL RESEARCH

Evaluation of *Ceiba pentandra* (L.) Gaertner bark extracts for in vitro cytotoxicity on cancer cells and in vivo antitumor activity in solid and liquid tumor models

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Abstract The stem bark of *Ceiba pentandra* (L.) Gaertner is claimed to be useful in the treatment of tumors in the southern part of India. This plant possesses a number of sesquiterpenoids and isoflavones which are known for their anticancer properties. The present study was designed to scientifically evaluate the cytotoxic potential of bark extracts in in vitro on Ehrlich ascites carcinoma (EAC), MCF-7 and B16F10 cells and in vivo in EAC (Liquid tumor) model and Dalton's lymphoma ascites (DLA or solid tumor) model. The bark was powdered and extracted successively with solvents viz., petroleum ether (PE), benzene, chloroform, acetone (AC), and ethyl alcohol in the sequential order of polarity. Cytotoxicity of dried extracts was screened on EAC cells by trypan blue assay. Three potent extracts namely petroleum ether, acetone, and ethanol were screened for their cytotoxicity on MCF-7 and B16F10 cells by MTT assay and nucleomorphological alteration by propidium iodide staining. Safe doses of these extracts were evaluated by acute toxicity study in mice. Extracts were found to be safe up to 300 mg/kg in acute toxicity study. Dosage of 1/10th and 1/20th of safe dose i.e., 15 and 30 mg/kg were selected for in vivo study. In the EAC model, both doses of the extracts showed a significant ($P < 0.05$) improvement in mean survival time and a maximum decline in tumor induced increase in body weight (an indirect measure of tumor weight) by the PE and AC treatment at 15 mg/kg compared to control. In the DLA-model, all extracts at both tested dose levels showed >50 % reduction in tumor weight and a significant reduction ($P < 0.05$) in tumor volume on the 30th day compared to control. It can be concluded that these extracts possess cytotoxic and antitumor activity.

Keywords *Ceiba pentandra* (L.) Gaertner · MTT assay · Antioxidant · Anticancer · Dalton lymphoma ascites · Ehrlich ascites carcinoma

Abbreviations

EAC	Ehrlich Ascites carcinoma
IMLS	Increase in mean life span
MST	Mean survival time

Introduction

Cancer is one of the most challenging life-threatening diseases to treat and its incidences is increasing in the 21st century. It is the second most common cause of mortality after cardiovascular diseases (Ferlay et al.

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2015). Excessive scientific research is being conducted in this field to cure this disease, but a safe and effective treatment is still unapproachable. Natural products including plants, marine- and micro-organism, are playing a substantial role in cancer treatment, which cover almost 60 % of the currently used anticancer agents (Cragg et al. 2011). The search for natural products with anticancer activity is mainly based on the traditional use of various plants and their products. The scientific support to the ethnobotanical evidence of the plants have led to the discovery of many known anticancer agents. The scientific supports involve mechanism based studies, which have given strong indications for the interaction between many phytochemicals and cancer cells (Lee et al. 2011). Therefore, there is a rise in interest among the researchers for evaluating the pharmacological activities of various plants used in traditional medicine.

India is a country of diverse cultures and diverse traditions, where along with modern medicine various other traditional medicines are being practiced such as Ayurveda, Siddha and Unani. Apart from these systems of medicines, there is a significant tribal population who follows natural medicines by and large. All these systems of medicines give a lot of scope for seeking information about the medicinal uses of plants. There is diverse climatic condition in various parts of India which supports growth of various types of medicinal plant. *Ceiba pentandra* (L.) Gaertner (Bombacaceae) known as white silk cotton tree or kapok tree, is one such plant whose stem bark is claimed to be useful in the treatment of tumors in the southern part of India (Nandagopalan et al. 2011). It is also widely used in the African traditional medicine (Ueda et al. 2002). Various morphological parts of this plant have been reported to possess medicinal value for the treatment of diabetes, fever, mental trouble, headache, dizziness, hypertension, constipation, peptic ulcer, rheumatism and leprosy (Noumi and Dibakto 2000; Noumi and Tchakonang 2001; Ueda et al. 2002). However, this plant has not been scientifically explored for its claimed antitumor activity. Previous work on *C. pentandra* described the isolation of a number of sesquiterpenoids (Rao et al. 1993) and isoflavones (Ngounou et al. 2000; Noreen et al. 1998). These categories of phytochemicals are known for their anticancer potential. The flower of *Bombax ceiba* (also known as red cotton tree) from the same Bombacaceae family is known to possess anticancer properties (Tundis et al. 2014). Thus, the present study was aimed to explore the anticancer potential of various extracts in vitro on the cell lines and in vivo in EAC-induced liquid tumor model and DLA-induced solid tumor model in mice.

Materials and methods

Materials

Bark of Ceiba pentandra

The *Ceiba pentandra* (L.) Gaertner tree is located in Kunjibettu-ward, Kadiyali, Udupi-district, Karnataka, India. The bark was peeled off from the tree and authenticated by Dr. M. Manjunath Setty, Professor, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka and Dr. G. K. Bhat (a well-known taxonomist of this area), Udupi, Karnataka, India.

Preparation of plant extract

The bark of the tree was peeled off and dried in an oven at 40 °C for 48 h. The dried bark was coarsely powdered in a ball mill. The powdered material was extracted successively using petroleum ether (PE), benzene, chloroform, acetone (AC) and ethyl alcohol (ETOH) in the sequential order of polarity using Soxhlet apparatus until the complete exhaustion of powdered material identified by colourless solvent above the powdered material during extraction (Villarreal et al. 1992). These extracts were concentrated *in vacuo* and stored in a vacuum desiccator for thorough drying.

Animals

Animals were maintained and handled carefully as per the guideline laid down by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Govt. of India. Prior permission was taken from the institutional animal ethics committee for conducting the study (clearance certificate No. IAEC/KMC/12/2008-2009). Studies were performed using Swiss albino mice (male), weighing 26 to 30 g. Animals were maintained in a controlled condition of temperature (23 ± 2 °C), light (14 and

10 h of light and dark cycle, respectively) and humidity (50 ± 5 %) at the Central Animal Research Facility, Manipal University, Manipal-576 104, India. Four animals in polypropylene cage were housed and paddy husk was used as bedding. Animals were provided with sterile food and water ad libitum.

Cell lines

The EAC and DLA cells were originally obtained from Amala Cancer Research Center, Amala Nagar, Thrissur, Kerala, India and were maintained in our animal house by serial intraperitoneal transplantation in Swiss albino mice. The EAC and DLA cells used for the study were collected from the intraperitoneal space of mice with 12–14 days old cell transplantation. Epithelial kidney cells extracted from an African green monkey (Vero), human breast adenocarcinoma (MCF-7) and melanoma cell lines (B16F10) were procured from National Centre for Cell Science, Pune, India. Cells were routinely grown in culture flasks (Techno Plastic Products, Trasadingen, Switzerland) containing Dulbecco's Minimum Essential Medium (DMEM, from Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10 percent fetal calf serum (FCS, from Sigma-Aldrich) and 50 μg/mL gentamycin sulfate (Genticyn®, from Abbott Healthcare Pvt Ltd, Mumbai, India) at 37 °C in CO_2 incubator (5 % CO_2).

Methods

Test drugs preparation

Extracts were dissolved in 0.2 % v/v DMSO for screening of cytotoxicity in EAC, Vero, MCF-7 and B16F10 cells. For animal studies, extracts were suspended in carboxymethylcellulose sodium [CMC (Merck Specialities Pvt. Ltd., Gujarat, India), 0.25 % v/v] and were administered intraperitoneally (*i.p.*) in a volume of 10 mL/kg.

In vitro cytotoxic study in EAC cells

In vitro cytotoxicity of extracts in EAC cells was assessed by trypan blue dye exclusion method. EAC cells were obtained from the ascitic fluid present in the peritoneum of mice containing 12–14 days old transplanted cells. Cells were washed thrice with phosphate buffered saline (pH 7.4) and checked for viability. The cell count was adjusted to ten millions per mL by phosphate buffered saline. One million cells were incubated at 37 °C with various test drug concentrations (10, 50 and 100 μg/mL) of extracts in a final volume of 0.9 mL for 3 h. To the incubation mixture, 0.1 mL of trypan blue was added and total numbers of dead and live cells were counted using haemocytometer. The data were presented as percentage cytotoxicity and CTC_{50} values were calculated using a best fit plot of percentage cytotoxicity versus concentration followed by regression equation (Dhamija et al. 2013).

In vitro cytotoxicity study in Vero, MCF-7 and B16F10 cell lines

In vitro cytotoxicity in Vero, MCF-7 and B16F10 cells was determined using the standard MTT assay method (Mosmann 1983). Briefly, 1×10^5 cells in 100 μL of medium were seeded in each well of 96-well plates and incubated for 24 h. Prior to the experiment, test solutions were prepared by dissolving in 0.2 % DMSO and diluted with medium. The cancer cells (MCF-7 and B16F10) were exposed to different concentrations of extract in a range of 50–500 μg/mL while Vero cells were exposed to a maximum concentration of 250 μg/mL of extracts. Cells in the control wells received the same amount of medium containing 0.2 % DMSO. After 48 and 72 h, the medium was removed and the cells were incubated with 100 μL of 1 mg/ml MTT (Sigma Aldrich) reagent for 4 h at 37 °C. The formazan produced by the viable cells was solubilized with 100 μL DMSO. The plate was placed on a micro-vibrator for 5 min. The absorbance was recorded at 540 nm using an ELISA reader and percentage cytotoxicity was calculated (Kumar et al. 2014).

Nuclear staining

Nuclear staining study was performed using propidium iodide on MCF-7 cells to find out nucleomorphological changes leading to apoptotic or necrotic death. In a 6-well plate, cells were seeded and cultures for 24 h, medium was replaced by extracts containing medium at approximately half of the CTC_{50} value [extract petroleum ether (PE) 75 μg/mL, ethyl alcohol (ETOH) 100 μg/mL, acetone (AC) 125 μg/mL] and

incubated for the next 48 h. Cells in the plate were washed with phosphate buffered saline (PBS), fixed with 1 mL of 90 % methanol and kept at −20 °C for 20 min. Methanol was replaced by1 mL acetone for 10 s, followed by washing with ice-cold PBS thrice. Hundred microliter of propidium iodide (Sigma Aldrich) was added and incubated for 20 min and washed thrice with PBS to remove excess dye. Photographs were taken, 100 cells were analysed for nucleomorphological changes and represented as apoptotic index (Kumar et al. 2012).

In vivo toxicological study

Acute toxicity study for the extracts was conducted in Swiss albino mice through the intraperitoneal route. Dosing was done as per OECD 423 guidelines. Toxicity parameters were assessed based on Irwin's observation test for behavioral and physiological functions (Roux et al. 2005). This test identifies any unwanted CNS effects, helps in understanding the underlying mechanisms of these effects and reveal possible novel therapeutic outcomes (Moser 2011). Safe doses of extracts/fractions were calculated in mice. Selection of test doses for in vivo antitumor study was based on safety profile.

EAC-induced liquid tumor model

Survival study Induction and propagation of tumor were performed as per the method reported by Jagetia and Rao (2006) and Kumar et al. (2014). Known numbers of viable EAC cells (2.5 million cells/mice) were injected intraperitoneally in aseptic conditions. Tumor was inoculated on day zero. Twenty-four hours after EAC cells inoculation (day one), the tumor-bearing animals were randomly divided into desired groups and treated with extracts or vehicle as follows:

Group I	treated with vehicle (CMC, 0.25 % w/v)/EAC control
Group II & III	treated with alcoholic extract (ETOH) (15 and 30 mg/kg, *i.p.*)
Group IV & V	treated with petroleum ether extract (PE) (15 and 30 mg/kg, *i.p.*)
Group VI & VII	treated with acetone extract (AC) (15 and 30 mg/kg, *i.p.*)
Group VIII	treated with Cisplatin (CP) (3.5 mg/kg, *i.p.*)

One group was not inoculation with EAC and was treated with vehicle (CMC, 0.25 % w/v) as follows:

Group IX Sham/Normal control

The dosing of extracts and CMC was done on days 3, 5, 7, 10, 12, and 14 of tumor inoculation at 10 mL/kg. Cisplatin at a single dose of 3.5 mg/kg i.p., was injected on day one, which served as standard drug (Dhamija et al. 2013). Cisplatin is an established drug with potent cytotoxicity and only one dose has been used in this model. Here, test drugs are extracts, a mixture of active components. Thus, multiple dosages were selected. Animals were weighed every 3rd day till the 15th day to assess the tumor development as the intraperitoneal tumor weight adds to animal body weight. The animals were monitored for mortality up to 45 days and mean survival time (MST) and percentage increase in life span (%ILS) were calculated. MST represents average survival time of animals in a particular group. Calculation of % ILS was based on the following formula: % ILS = [(Survival time of animals of treated group−MST of control group) × 100]/MST of the control group (Dhamija et al. 2013).

Hematological profile Hematological profile was evaluated in the experimental design as in the survival study. For whole blood count, the blood was withdrawn on day 15 from retro orbital plexus under diethyl ether (anesthesia). Levels of white blood cells (WBC), red blood cells (RBC), and hemoglobin were evaluated using an automatic blood cell counter (Veterinary Blood cell counter, Model- PEC-21, OVET ERMA INC Tokyo, Japan).

Antioxidant profile Endogenous antioxidant enzymes estimation were performed in the experimental design as in survival study. After blood withdrawal, animals were euthanized by ketamine and the liver was perfused and dissected out. Liver was weighed after drying with tissue paper and homogenated in ice-cold potassium chloride (150 mM) using homogenizer (Yamato LSG LH-21, Tokyo, Japan) (Kumar et al. 2014). The estimation of catalase (Aebi 1984), superoxide dismutase (SOD) (Misra and Fridovich 1972), glutathione (GSH) (Moron et al. 1979), glutathione-s-transferase (GST) (Habig et al. 1974), thiobarbituric acid (TBARS) (Niehaus and Samuelsson 1968) were

performed following standard procedures in supernatant of tissue homogenate (Kumar et al. 2014).

DLA-induced solid tumor model

Dalton's lymphoma ascites cell lines (DLA) were obtained from 15 days old cell transplanted Swiss albino mouse. The ascitic fluid was aspirated and tested for microbial contamination. Viability of cells and cell number was assessed by trypan blue exclusion test. Cell number was adjusted with saline to get a cell suspension of one million cells per mL. 0.1 mL of the cell suspension was injected intramuscularly in the right hind limb of male Swiss albino mice to obtain a solid tumor. Treatments were started 24 h after tumor inoculation. Standard drug, cisplatin was injected on alternate days for 2 days while extracts were administered for seven continuous days from day 1 onwards (Dhamija et al. 2013). Tumor growth was monitored by determining the tumor volume and tumor weight. Tumor volume was measured using the formula $V = 4/3\pi ab^2$; where a and b represented the major and minor diameter. The measurement was made using vernier calipers on every 3rd day. Tumor weight was taken after 4 weeks of study by excising the tumor from euthanized animals. The % inhibition in tumor weight was calculated by the formula, % Inhibition $= (1-B/A) \times 100$; where A is the average tumor weight of the control group, B is the average tumor weight of the treated group. (Dhamija et al. 2013; Kumar et al. 2014).

Statistical analysis

Data were analyzed by one way ANOVA using demo version of GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, followed by Tukey's posttest. A value of $P < 0.05$ was considered to be significant.

Results

In vitro cytotoxicity in EAC cells

All tested extracts showed cytotoxicity in a concentration dependent manner after 3 h of incubation with EAC cells. CTC_{50} (concentration at which fifty percent cells die) value was found to be 53.30, 70.58, 250.48, 67.30 and 56.11 µg/ml, respectively, for PE, benzene, chloroform, AC and ETOH extracts on EAC cells (Fig. 1).

In vitro cytotoxicity in Vero, MCF-7 and B16F10 cells

Extracts were found to be safe till 250 µg/mL on Vero cell line. Percentage cytotoxicity on cancer cell lines (MCF-7 and B16F10) were calculated at 48 and 72 h of incubation of extracts. The three extracts showed significant cytotoxicity in both cell lines in a concentration range of 50–500 µg/mL. After 48 h, PE and ETOH extracts showed highest cytotoxicity in B16F10 cell line (CTC_{50-} 36.28 and 17.07 µg/mL, respectively) compared to MCF-7 cell line (CTC_{50-} 152.17 and 215.96 µg/mL) while after 72 h of incubation the cytotoxicity of these extracts decreased (CTC_{50-} 178.21 and 374.76 µg/mL on B16F10 cell line while 330.85 and 309.98 µg/mL on MCF-7 cell line, respectively). Acetone extract showed a maximum CTC_{50} value of 45.40 µg/mL in B16F10 and of 51.51 µg/mL in MCF-7 cell line after 72 h of incubation (Figs. 2, 3).

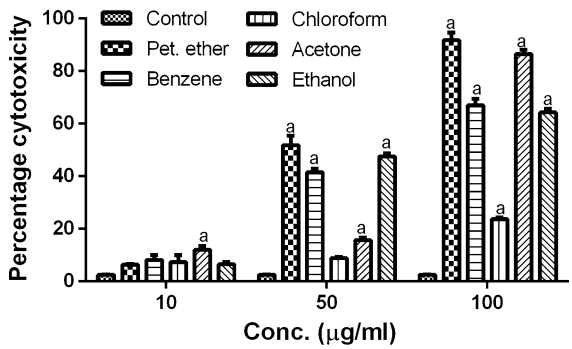

Fig. 1 Short-term in vitro cytotoxicity of extracts by trypan blue exclusion assay on EAC cells. All values are mean ± SEM of three determinations in triplicate, *a* $P < 0.05$ compared to control. EAC cells were incubated with 10, 50 and 100 µg/mL concentrations of various extracts for 3 h in a temperature controlled incubator. CTC_{50} values were calculated from the linear plot of percentage death versus concentration and values for the extracts were: 53.30, 70.58, 250.48, 67.30, 56.11 for Petroleum ether, Benzene, Chloroform, Acetone, and Ethanol extract, respectively, while standard cisplatin CTC_{50} value was 14.26 µg/mL

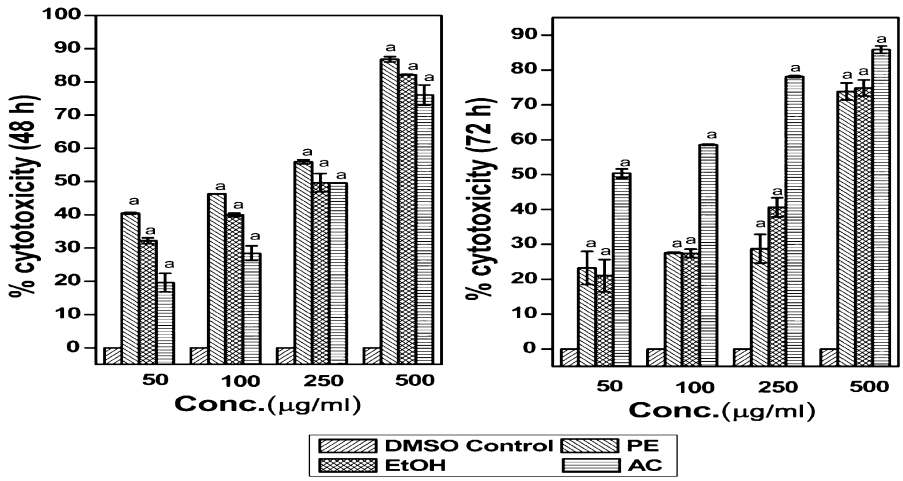

Fig. 2 In vitro cytotoxic activity of various extracts in MCF-7 cells (Human Breast adenocarcinoma cells). All values are mean ± SEM of three determinations in triplicate, *a* $P < 0.05$ compared to control. In vitro cytotoxic activity of various extracts in MCF-7 cells by MTT assay at 48 and 72 h of exposure using four different concentrations 50, 100, 250 and 500 µg/mL. The CTC_{50} values for Petroleum ether, Ethanol, Acetone extracts were 152.17, 215.96, 278.27, respectively, after 48 h of incubation while 330.85, 309.98, 51.51 µg/mL for extracts after 72 h of incubation. The CTC_{50} values for cisplatin after 48 and 74 h incubation were found to be 3.40 and 1.34 µg/mL

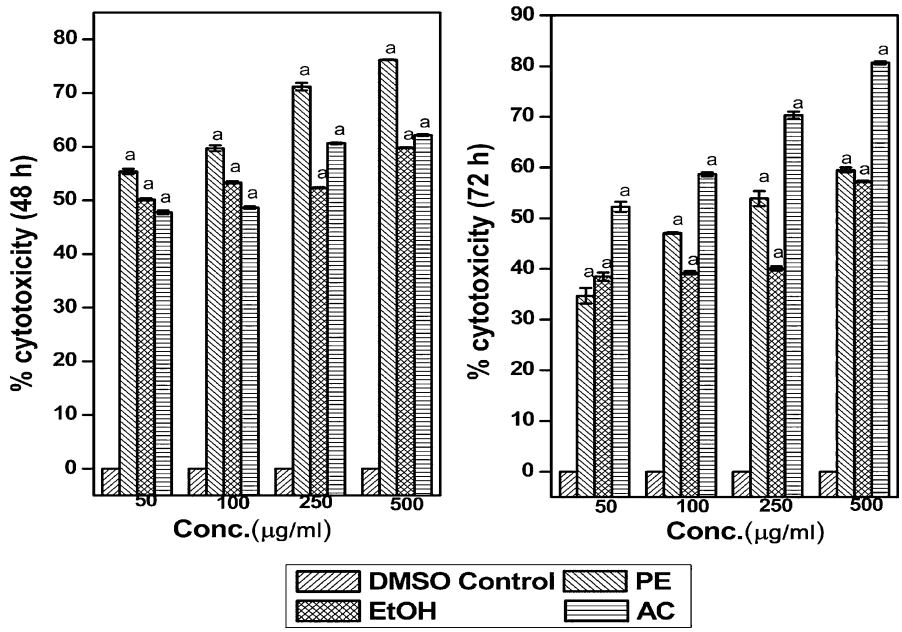

Fig. 3 In vitro cytotoxic activity of various extracts in B16F10 cells (Melanoma cells). All values are mean ± SEM of three determinations in triplicate, *a* $P < 0.05$ compared to control. In vitro cytotoxicity was conducted on B16F10 cells by MTT assay at 48 and 72 h of exposure using four different concentrations 50, 100, 250 and 500 µg/mL. The CTC_{50} values for Petroleum ether, Ethanol, Acetone extracts were 36.28, 17.07, 81.03, respectively, after 48 h of incubation while 178.21, 374.76, 45.40 µg/mL, respectively, after 72 h of incubation. The CTC_{50} values for cisplatin after 48 and 74 h incubation was found to be 5.32 and 2.15 µg/mL

Nuclear staining

All three extracts showed significant ($P < 0.05$) nucleomorphological changes compared to DMSO control on MCF-7 cells counted as apoptotic index (Fig. 4).

Acute toxicity study

Death in mice at a dose of 2000 mg/kg was observed for all tested extracts. However, all tested extracts were found safe up to 300 mg/kg. No signs of neurological and behavioral toxicity were observed at this dose level using Irwin's observation test. Dosage of 1/10th and 1/20th of the safe dose (300 mg/kg) i.e., 15 and 30 mg/kg were selected for study.

Ehrlich ascites tumor model

Body weight

A considerable gain in body weight was observed in EAC inoculated vehicle treated control mice (as the peritoneal tumor grows and the volume increases). Prominent tumor development was observed on the 9th day, which was growing till the end of the study. In EAC control mice (vehicle treated), the maximum gain in body weight was observed on day-15 (44.35 %). Cisplatin administration significantly ($P < 0.05$) prevented the elevation in body weight on every monitored day compared to the EAC control group. PE and AC extracts at 15 mg/kg significantly

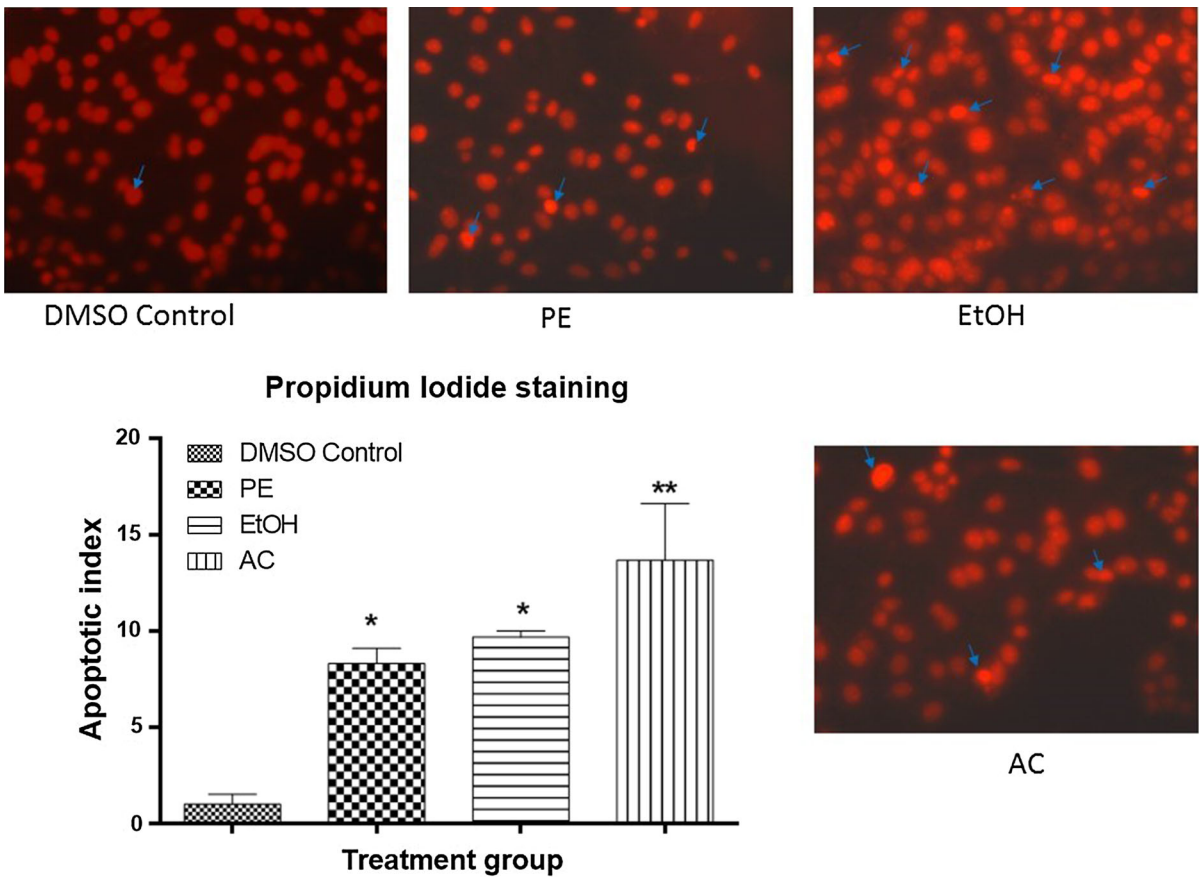

Fig. 4 Nuclear Staining of MCF-7 Cells. All values are mean ± SEM of three determinations in triplicate, $*P < 0.05$ compared to DMSO control and, $**P < 0.01$ compared to DMSO control. Nuclear staining was conducted on MCF-7 cells using propidium iodide staining after 48 h of exposure using approximately half of the CTC_{50} value of extract i.e., PE-75 µg/mL, EtOH-100 µg/mL, AC-125 µg/mL. *Arrows* indicate nucleomorphological changes i.e., shrinkage of cytoplasm and condensed nuclei

Table 1 Effect of various extracts on body weight changes in EAC inoculated mice

Treatment	Percentage increase in body weight as compared to Day-0				
	Day-3	Day-6	Day-9	Day-12	Day-15
Sham	1.28 ± 0.83	5.88 ± 0.49	8.02 ± 0.66	12.44 ± 1.41	15.66 ± 1.73
EAC control	8.53 ± 1.89[#]	5.38 ± 2.29	21.84 ± 3.96[#]	34.95 ± 3.31[#]	44.35 ± 3.69[#]
Cisplatin: 3.5 mg/kg	0.54 ± 0.54[a]	1.17 ± 1.34	10.95 ± 2.34	12.50 ± 2.68[a]	15.81 ± 2.65[a]
Ethanol: 30 mg/kg	9.77 ± 1.47	0.31 ± 1.54	10.36 ± 2.66	24.51 ± 4.74	31.7 ± 5.54
Ethanol 15 mg/kg	6.61 ± 1.30	2.38 ± 1.13	13.54 ± 2.74	19.93 ± 8.28	26.3 ± 8.66
Petroleum ether: 30 mg/kg	−0.96 ± 1.06[a]	2.02 ± 0.77	12.55 ± 2.43	26.8 ± 4.71	29.38 ± 6.41
Petroleum ether: 15 mg/kg	3.88 ± 3.10	0.27 ± 1.52	17.19 ± 2.38	16.78 ± 2.70	18.63 ± 2.98[a]
Acetone: 30 mg/kg	4.54 ± 1.51	−2.69 ± 2.0[a]	20.56 ± 2.52	30.77 ± 5.04	31.54 ± 5.67
Acetone: 15 mg/kg	5.13 ± 1.28	−2.01 ± 1.03[a]	11.99 ± 3.52	17.19 ± 4.11	20.18 ± 4.26[a]

All values are mean ± SEM of six samples

[#] $P < 0.05$ compared to *Sham*

[a] $P < 0.05$ compared to EAC control

($P < 0.05$) prevented the rise in body weight compared to the EAC control group (Table 1).

Mean survival time (MST)

Mean survival time decreased drastically in EAC-inoculated vehicle treated control mice (15.25 days) compared to vehicle treated sham animals, where no death was observed till 45 days. The first death in the control group was observed on day-12, while none of the mice survived >17 days. However, cisplatin significantly increased the MST (38.36 days) and % ILS (151.64) compared to vehicle treatment. All three extracts significantly ($P < 0.05$) improved the MST. PE and AC extracts were found to be more effective at 15 mg/kg than other doses of extracts (Table 2; Fig. 5).

Hematological parameters

WBC count showed a fourfold increase in EAC inoculated vehicle treated control mice. The standard drug (Cisplatin) significantly ($P < 0.05$) prevented the elevation in WBC count compared to the EAC control group. A significant reduction ($P < 0.05$) in WBC count was also observed in the extracts treated groups, namely PE, ETOH, and AC at 30 and 15 mg/kg. In EAC control mice, a drastic fall in RBC count was observed, which was significantly improved by the treatment with the extracts and cisplatin. Significant

Table 2 Effect of various extracts on mean survival time in EAC inoculated mice

Treatment	MST	%ILS
EAC control	15.25 ± 0.70	–
Cisplatin 3.5 mg/kg	38.36 ± 0.46[a]	151.64 ± 0.46[a]
Ethanol 30 mg/kg	17.63 ± 0.82	15.57 ± 0.82[a]
Ethanol 15 mg/kg	20.75 ± 1.05[a]	36.07 ± 1.04[a]
Petroleum ether 30 mg/kg	19.25 ± 0.86	26.23 ± 0.86[a]
Petroleum ether 15 mg/kg	21.75 ± 1.47[a]	42.62 ± 1.47[a]
Acetone 30 mg/kg	20.86 ± 1.39[a]	36.89 ± 1.39[a]
Acetone 15 mg/kg	21.5 ± 1.05[a]	40.98 ± 1.05[a]

None of the animals from the sham group died till 45 days. All values are mean ± SEM of six samples

[a] $P < 0.05$ compared to EAC control. MST represents average survival time of animals in a particular group. MST represents mean survival time. %ILS represents percentage increase in life span compared to mean survival time of EAC control animals

reduction in hemoglobin content was observed in the EAC control group compared to the sham group. Cisplatin and studied extracts (at both doses) significantly ($P < 0.05$) prevented these EAC- induced anemic changes (Fig. 6).

Antioxidant parameters in liver

In EAC control animals, a significant ($P < 0.05$) fall in catalase, SOD and GSH was observed together with a significant elevation in GST and MDA levels

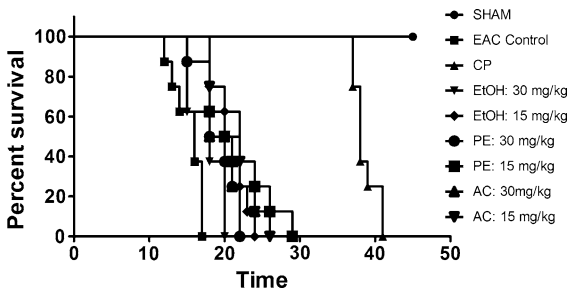

Fig. 5 Survival curve. Survival studies of various extracts were conducted at 15 and 30 mg/kg for 45 days. In each group, six mice were used. First death was observed in the control group and all animals of this group died by the 17th day of study. Among the treatment groups, maximum survival was seen in the CP group followed by the PE 15 mg/kg group

compared to the sham group. Cisplatin and extracts treatments significantly ($P < 0.05$) reversed the EAC-induced changes in catalase, SOD, GSH, GST and MDA content (Table 3).

DLA-induced solid tumor model

Tumor volume

The three extracts showed significant ($P < 0.05$) reduction in tumor development in mice. The maximum reduction in tumor volume was observed in cisplatin-treated mice. Except for EtOH at 15 mg/kg, all tested extracts showed significant prevention in tumor growth on the 30th day of study, which was evident from the significant ($P < 0.05$) reduction in tumor volume compared to the DLA control group (Table 4).

Tumor weight

Tumor formed due to inoculation of DLA cells was found to be prominent on day-30 in the vehicle treated DLA control mice. The extracts treatments significantly ($P < 0.05$) prevented the growth in tumor weight. Among the treatments, PE and AC extracts

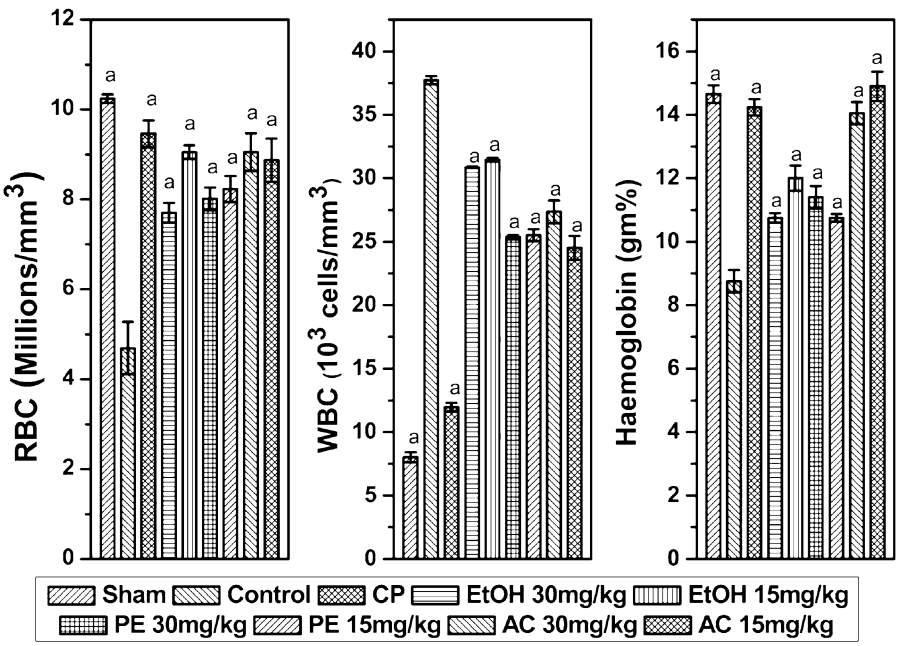

Fig. 6 Effect of various extracts of *Ceiba pentandra* on hematological parameters in EAC inoculated mice. Hematological parameters (RBC, WBC and hemoglobin) were assessed after 14 days of EAC cells inoculation (2.5×10^6 per mouce i.p.). Compared to the EAC control group a significant change was observed in all groups. This indicates a significant decrease in RBC and hemoglobin count after EAC inoculation in the control group on the 14th day which was significantly prevented by all treatments. A reverse trend was observed for WBC, which was increased significantly in the EAC control group compared to the sham treatment and treatments were significantly effective in preventing the rise in WBC levels compared to the control. All values are mean ± SEM of three samples, where *a* $P < 0.05$ compared to control

Table 3 Effect of various extracts on liver antioxidant parameters in EAC inoculated mice

Treatment	Catalase U/mg protein	SOD U/mg protein	GSH μM/mg protein	GST U/mg protein	TBARS [MDA (nM/mg protein)]
Sham	5.90 ± 0.03[a]	12.71 ± 0.41[a]	2.14 ± 0.05[a]	10.64 ± 1.90[a]	63.88 ± 8.37[a]
EAC control	1.88 ± 0.08	0.88 ± 0.06	0.11 ± 0.004	22.36 ± 5.40	238.98 ± 7.92
Cisplatin: 3.5 mg/kg	5.20 ± 0.2[a]	5.87 ± 0.14[a]	1.81 ± 0.02[a]	10.68 ± 0.05[a]	101.63 ± 7.58[a]
Ethanol: 30 mg/kg	2.64 ± 0.34	3.73 ± 0.01[a]	0.85 ± 0.002[a]	10.87 ± 0.23[a]	156.59 ± 9.14[a]
Ethanol 15 mg/kg	3.20 ± 0.24[a]	2.90 ± 0.07	0.72 ± 0.01[a]	5.61 ± 0.63[a]	225.62 ± 4.94
Petroleum ether: 30 mg/kg	2.88 ± 0.07[a]	0.99 ± 0 .01	0.28 ± 0.01[a]	6.72 ± 0.76[a]	159.00 ± 7.06[a]
Petroleum ether: 15 mg/kg	3.85 ± 0.04[a]	27.71 ± 1.28[a]	0.79 ± 0.003[a]	8.61 ± 0.97[a]	228.01 ± 8.36
Acetone: 30 mg/kg	2.84 ± 0.03[a]	3.13 ± 0.02	0.60 ± 0.02[a]	6.69 ± 1.35[a]	137.97 ± 6.17[a]
Acetone: 15 mg/kg	3.55 ± 0.02[a]	20.64 ± 0.56[a]	1.07 ± 0.05[a]	7.07 ± 2.33[a]	145.31 ± 9.58[a]

The values are mean ± SEM of three samples

[a] $P < 0.05$ compared to EAC control

Table 4 Effect of various extracts against DLA induced solid tumor volume (in cm^3) in mice

Treatment	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Sham	0.07 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.00	0.08 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
DLA Control	0.08 ± 0.02	0.2 ± 0.02[#]	0.27 ± 0.01[#]	0.39 ± 0.03[#]	0.50 ± 0.07[#]	0.66 ± 0.09[#]	0.90 ± 0.15[#]
Cisplatin: 3.5 mg/kg	0.10 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.12 ± 0.01[a]	0.12 ± 0.01[a]	0.17 ± 0.01[a]	0.18 ± 0.01[a]
Ethanol: 30 mg/kg	0.06 ± 0.01	0.12 ± 0.01	0.18 ± 0.01	0.20 ± 0.04	0.23 ± 0.05[a]	0.29 ± 0.03[a]	0.39 ± 0.03[a]
Ethanol 15 mg/kg	0.07 ± 0.01	0.12 ± 0.01	0.19 ± 0.01	0.23 ± 0.02	0.24 ± 0.05[a]	0.33 ± 0.12[a]	0.46 ± 0.14[a]
Petroleum ether: 30 mg/kg	0.07 ± 0.01	0.11 ± 0.01	0.19 ± 0.01	0.21 ± 0.04	0.24 ± 0.01[a]	0.27 ± 0.03[a]	0.31 ± 0.05[a]
Petroleum ether: 15 mg/kg	0.06 ± 0.01	0.13 ± 0.01	0.23 ± 0.01	0.24 ± 0.01	0.25 ± 0.06[a]	0.39 ± 0.07[a]	0.39 ± 0.15[a]
Acetone: 30 mg/kg	0.07 ± 0.01	0.11 ± 0.01	0.18 ± 0.01	0.22 ± 0.02	0.26 ± 0.07[a]	0.27 ± 0.09[a]	0.35 ± 0.13[a]
Acetone: 15 mg/kg	0.11 ± 0.01	0.16 ± 0.01	0.21 ± 0.01	0.27 ± 0.01	0.28 ± 0.04	0.33 ± 0.03[a]	0.35 ± 0.06[a]

On day zero, 0.1 mL DLA cells from 1 million cells/mL of the suspension stock were injected into the right hind limb of each mouse. Tumor development was monitored on every 5th day using Vernier caliper till the end of the study (30th day). All values are mean ± SEM of six mice

[#] $P < 0.05$ compared to sham

[a] $P < 0.05$ compared to DLA control

were found to be most effective at 30 mg/kg dose with 61.98 and 56.78 % reduction in tumor weight. The animals treated with cisplatin prevented the increase in solid tumor weight by 71.67 % (Fig. 7).

Discussion

The present study evaluated the anticancer efficacy of the bark of *Ceiba pentandra* in different cancer cell lines and transplantable tumor bearing mice. The powdered bark was successively extracted by five different solvents in a sequential order of polarity viz, petroleum ether (PE), benzene, chloroform, acetone (AC) and ethyl alcohol (EtOH). Petroleum ether was highly non-polar and in the sequence ethyl alcohol was a highly polar solvent used in the present study for extraction. For in vitro screening of cytotoxicity of extracts two types of cell lines were used, EAC cells, which are suspension cells and Vero, MCF-7 and B16F10 which are adherent cells. EAC cells are transplantable tumours, grown in one mouse and transplanted to another mouse. On the other hand continuous cells like Vero, MCF-7 and B16F10, are

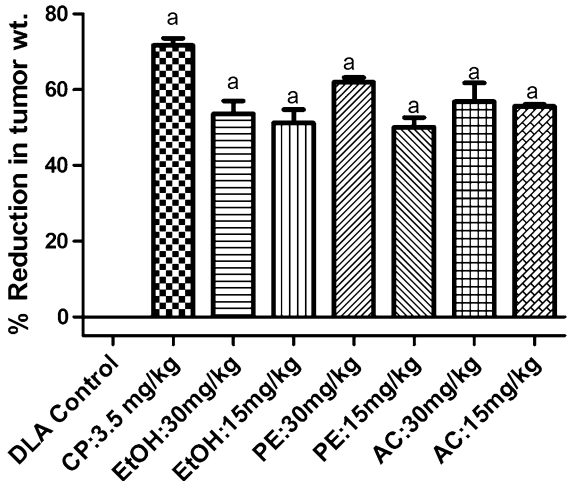

Fig. 7 Effect of various extracts against DLA induced weight of solid tumor in mice. 0.1 mL DLA cells from 1 million cells/mL of suspension stock were injected into the right hind limb of each mouse on day zero. Tumor was excised from euthanized animals on the 30th day, weighed and percentage reduction was calculated compared to the DLA control group. Percentage reduction was found to be 50 % and more by all treatment groups. All values are mean ± SEM of tumor weight of six mice where *a* $P < 0.05$ compared to control

relatively newer and more specific and are grown in cell culture in a CO_2 incubator (Wang et al. 2010). The cytotoxicity on both types of cell lines were evaluated by two methods after two different intervals of incubation. EAC cells were used for screening of extract for their cytotoxicity for 3 h in normal temperature controlled incubators using trypan blue exclusion assay, while Vero, MCF-7, B16F10 cells were used for screening of long term cytotoxicity (48–72 h) in a CO_2 incubator using MTT assay (Kumar et al. 2014).

In the in vitro study, firstly the extracts were evaluated for their cytotoxicity on EAC cells, which were intended to be used later in the in vivo study. Trypan blue exclusion assay, a traditional, relatively inexpensive method (Wang et al. 2010), was used for screening of short term cytotoxicity (3 h) on EAC cells, which do not require cell culture facility such as CO_2 incubator, media for growth and sophisticated technique for culturing and thus minimizes the risks of contamination. Three hours incubation was selected because during this duration no significant changes in viability and morphological alteration in the cells were observed. This time interval is well supported by previous reports (Dhamija et al. 2013; Manjula et al. 2010; Samudrala et al. 2015). Each extract containing a certain number of phytochemicals was screened for cytotoxic potential on EAC cells by trypan blue exclusion assay as a preliminary study to select promising extracts. Our aim was to select the extracts with CTC_{50} value below 100 µg/mL for further study. That is why only three concentrations were selected and performed thrice for repetition. Benzene fractions which showed comparatively lesser yield in extraction process and chloroform fraction which showed CTC_{50} value >100 µg/mL (250.48 µg/mL), were excluded from further screening. Thus, a highly non polar fraction, petroleum ether and two polar fractions, acetone and ethanol extracts, which were found to be more potent with low CTC_{50} value on EAC cells were selected for screening of their long term (48 and 72 h) cytotoxicity on one normal cell line (Vero) and two cancer cell lines [human breast cancer cell line (MCF-7) and mouse melanoma cell line (B16F10)]. These cell lines were selected randomly for identifying cytotoxicity of extracts by MTT assay (a method suitable for screening of cytotoxicity on continuous cell lines). The three extracts did not produce cytotoxicity on Vero cells up to 250 µg/ml, however, these extracts were found to be cytotoxic on MCF-7 and B16F10 cells. This differential activity of extracts showed its selective cytotoxicity towards cancerous cells. Among the tested extracts, acetone extract showed a potent cytotoxic effect after 72 h of incubation in both cancerous cell lines while PE and EtOH extracts showed a reduced cytotoxic potential on MCF-7 and more elevated potential on B16F10 after 48 h of incubation. The reason behind the decrease in CTC_{50} of PE and EtOH extracts after 72 h of incubation is not known. However, the stability of its chemical constituent needs to be studied by fractionation and isolation of active molecule/s, which might be one of the reasons. Nuclear staining of MCF-7 also supported the cytotoxic ability of extract by visualizing nucleomorphological alterations.

Based on the encouraging data obtained from the in vitro study, we further proceeded with screening in tumor bearing mice. Two types of tumor models were selected, a solid tumor and a liquid tumor model. EAC cells were used for the developing liquid tumor and DLA cells were used for the solid tumor. Both cells are undifferentiated suspension tumor cells.

In the EAC model, the three extracts at both dose levels (15 and 30 mg/kg) significantly ($P < 0.05$) improved the MST compared to the EAC control

mice. However, there was a drastic fall in body weight in mice treated with low dose (15 mg/kg) of the PE and AC extracts compared to the EAC control. Conversely, no visible sign of toxicity and change in vital functions was noted in any of these treated animals. In in vitro cytotoxicity study on EAC cells, the same extracts had shown their cytotoxic effect. Thus the effect on the reduction of body weight compared to control might be due to their tumor inhibitory property. Since increasing life span and tumor size/weight are reliable criteria for justifying the anticancer efficacy of any compound (Hollingshead 2008; Mo et al. 2014), our study included survival time analysis along with tumor weight/size. All extracts met with this criterion and prolonged the life span. Previous studies on *Cassia fistula* (Gupta et al. 2000), *Hygrophila spinose* (Mazumdar et al. 1997), *Solanum pseudocapsicum* leaves (Badami et al. 2003), *Careya arborea* (Natesan et al. 2007) also supported the increasing survival time as a criterion for anticancer activity. Thus, the effective reduction in EAC-induced mortality and therefore increase in MST of tumor bearing mice might indicate the potential anticancer activity of *Ceiba pentandra* bark. Ethanol-30 mg/kg and petroleum ether-30 mg/kg showed a percentage increase in life span <30 %. Except for these two tested extracts, remaining extracts showed significant increase in mean survival time compared to control animals.

Myelosuppression and anemia have been observed frequently in ascites carcinoma (Kumar et al. 2014; Queiroz et al. 2004). Anemia in ascites carcinoma mainly occur either due to hemolytic or myeloid abnormality (Justo et al. 2001). In the present study, similar findings were observed in the EAC-inoculated control group, where hemoglobin content and RBC count significantly declined compared to the sham group. *Ceiba pentandra* treatment prevented a reduction in hemoglobin content and RBC count, which suggested its myeloprotective effect. Intraperitoneal administration of EAC cells resulted in inflammatory conditions, reflected by a four-fold increase in WBC count in the control group. The increase in WBC count was also suggested to be because of disparity in the activity of EAC cells in bone marrow and spleen. Queiroz et al. 2004 found that EAC tumor rapidly decreased CFU-GM in bone marrow while progressively increased it in spleen, leading to splenomegaly (Queiroz et al. 2004). Treatment with *Ceiba pentandra* maintained the normal values of WBC, which supported its inhibitory property to EAC cells. The findings on hematological parameters were in accordance with the results obtained for MST. Ethanol-30 mg/kg and petroleum ether-30 mg/kg which could not significantly ($P < 0.05$) increase the mean survival time compared to the EAC control showed significant improvement in hematological parameters. In the previous studies, similar findings were obtained, which suggested the reduction of myeloid toxicity and immune boosting activity by most of the tested anticancer plants (Dhamija et al. 2013; Manjula et al. 2010).

Reactive oxygen species (ROS) are involved in cell proliferation, differentiation and apoptosis (Xia et al. 2007) which are the key steps in tumor formation and control. This involves tumor initiation (also known as carcinogenesis) by damaging DNA leading to genomic instability (Bianchi et al. 2001; Jackson and Loeb 2001). Apart from inducing proapoptotic molecules (p53 and p38 MAP kinase) in tumors in certain conditions (Lee 1998) which are supposed to be tumor controller, they are also involved in activation of certain tumor promoting molecules such as mitogen-activated protein (MAP) kinase, nuclear factor κB (NF-κB), and activator protein 1 (Schreck et al. 1992; Xia et al. 2007). High level of endogenous ROS is important for angiogenesis and tumor growth by increasing expression of hypoxia-inducible factor 1a (HIF 1a) and vascular endothelia growth factors (VEGF) (Xia et al. 2007). Since, the aqueous extract of bark of *Ceiba pentandra* has been reported to possess in vitro (Fofie et al. 2014) and in vivo (Bairwa et al. 2010) antioxidant activity, this antioxidant property along with the cytotoxic activity of extracts might be responsible for anti-angiogenesis and thereby tumor regression. This antioxidant hypothesis for anticancer activity was also supported by report of Radomska-Leśniewska et al. (2016), who found that synthetic and natural antioxidants (vitamin E and C along with antioxidant enzymes inside the cell viz., GSH, catalase, SOD, and glutathione peroxidase) prevented neovascularization promoted by free radicals (Reactive oxygen species-ROS) (Radomska-Leśniewska et al. 2016). These molecules play a protective role through regulation and modulation of genes controlling redox potential inside the cell (Sarsour et al. 2009) and thus enhances endogenous antioxidant parameters and decreases free radical

generation (evaluated by MDA formation) in the EAC model (Manjula et al. 2010; Szatrowski and Nathan 1991). Similar findings were observed in the present study, a significant fall in the hepatic antioxidant level, namely, catalase, SOD, GSH levels while elevation in MDA and GST levels were seen in the EAC control group. Except catalase levels by the treatment of ethanol-30 mg/kg, SOD levels by the treatment of ethanol-15 mg/kg, petroleum ether-30 mg/kg and acetone-30 mg/kg and MDA levels by the treatment of ethanol-15 mg/kg and petroleum ether-15 mg/kg, remaining antioxidant levels were significantly ($P < 0.05$) improved in all treatment groups compared to the EAC control group. The SOD levels by the treatment of petroleum ether-15 mg/kg and acetone-15 mg/kg increased to almost double to the sham value, which might be attributed to its strong antioxidant inducing property. In an ascitic model, the ROS inhibitory activity might have an important role because vascular permeability is one such factor for the increase in body weight along with an increase in the number of the EAC cells in the peritoneum. In the present study, compared to the MST result, the antioxidant trends showed mixed response and thus could not be correlated with each antioxidant parameter.

Antioxidant, anti-inflammatory and anticancer effects of extract have been widely studied for plant extracts in animal models (Davis et al. 2012; Manjula et al. 2010). However, correlation of these parameters was not established which might be due to the variability in the response of various antioxidant markers (Abdel-Rahman et al. 2011) (Aliahmat et al. 2012). In the present study, treatments showed mixed results in reversal of various parameters of oxidative stress. Thus, increase in antioxidant defense cannot be considered as a sole reason for the increase in MST in this study. Even human trails also showed mixed results for few antioxidant compounds in the epidemiological studies for anticancer therapy (Patterson et al. 1997). However the role of antioxidant principle cannot be ruled out as a reason behind anti-inflammatory and antitumor activity of the extracts. Thus, it can be stated that the anticancer potential of extracts might be due to the presence of antioxidant, anti-inflammatory and antitumor principles in the extracts.

For assessing the effect of *Ceiba pentandra* on solid tumors, DLA cells were used as model for development of solid tumor. After tumor transplantation, a progression in tumor volume was observed in vehicle treated DLA control mice. All extracts at both dose levels significantly ($P < 0.05$) prevented the development of tumor by effectively reducing tumor weight and volume. Hence, inhibition of tumor progression and tumor development by the extracts of *Ceiba pentandra* supported its anticancer activity against solid tumor as well. The antitumor potential of extracts was more prominent in the solid tumor model compared to the liquid tumor model.

Conclusion

We observed that bark extracts of *Ceiba pentandra* had significant antitumor activity in both in vitro and in vivo system. At this point, the exact mechanism could not be evaluated behind the antitumor activity of *Ceiba pentandra*. However, the prominent short term cytotoxic effect on EAC cells and long term cytotoxic effect on human breast cancer cell line (MCF-7) and melanoma cell line (B16F10), supported by protection to the hemopoietic system along with elevation of the endogenous antioxidant defense system might play an important role in the antitumor activity of these extracts.

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