

Anticancer Activity of Extract Derived From the Leaves of *Careya Arborea* on RatsRamesh HA^{1,2*}, and Dinesh B Shenoy²¹ Department of Pharmacology, Priyadarsini College of Pharmacy, Siluvepura, Koratagere, Tumkur (D), Karnataka (S), India.² NIMS University, Jaipur, Rajasthan, India.

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ABSTRACT

One of the largest causes of mortality worldwide is cancer. Increasing interest and research on herbal medicine have revealed its importance in treating many diseases including cancer. In the present study anticancer activity of the methanolic extract of leaves of *Careya arborea* Roxb was on DAL model in rats was evaluated. After inoculation of DAL cells into rats, treatment with *Careya arborea* Roxb extract (200 and 400 mg/kg) and standard drug 5-Fluorouracil (20 mg/kg) were continued for 9 days. Evaluation of the effect of drug response was made by the study of tumor growth response including increase in life span, study of hematological parameters, biochemical estimations, antioxidant assay of liver tissue and *in vitro* cytotoxicity. Experimental results revealed that *Careya arborea* Roxb extract possesses significant anticancer activity which may be due to its cytotoxicity and antioxidant properties. Further research is going on to find out the active principles of *Careya arborea* Roxb extract for its anti-cancer activity.

INTRODUCTION

Cancer is a serious clinical problem that possesses significant social and economic challenges to the healthcare system. Despite improved imaging and molecular diagnostic techniques, cancer continues to affect millions of people globally [1]. In many countries, cancer is the second leading cause of death after heart diseases [2]. Lung, colorectal and stomach cancer are among the five most common cancers in the world for both men and women [3]. Over the past few decades, cancer has remained as the largest cause of mortality worldwide and the number of individuals living with cancer is steadily expanding. Hence, a major portion of the current pharmacological research is involved with the anticancer drug design customized to fit new molecular targets [4]. Due to the enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities. Traditionally various plants have long been used in the treatment of cancer [5, 6, 7, 8]. Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer. *Careya arborea* Roxb. Family Lecythidaceae, is named 'the slow match tree'. It is a large tree found throughout India in deciduous forests and grassland. Stem bark of *Careya arborea* is traditionally used in the treatment of bronchitis, epileptic fits, astringents, antidote to snake-venom and skin disease. It is also used as remedy for diarrhea, dysentery with bloody stools and ear pain [9]. The leaves are useful in ulcers. The flowers are useful in healing vaginal ruptures caused by child birth. The fruits are acrid, astringent, and aromatic and are useful in dyspepsia [10]. However, so far no anticancer activity has been reported from this plant. Hence, in the present study is to investigate the antitumor properties of the methanol extract of *Careya arborea* Roxb against Dalton's lymphoma ascites (DLA) induced tumor in rats.

MATERIALS AND METHODS**Collection of Plant Material and Extraction**

The fresh leaves were collected from South Karnataka and authenticated at Department of Botany. The leaves were cleaned with water and dried later. Then they were cut into small pieces. 10g of these pieces were extracted for half an hrs with 50 ml methanol. After

pressing the marc, filtrate (henceforth called as leaf extract) was collected and concentrated to its 1/4th volume and preserved in a tightly closed glass container and stored away from direct sunlight. The experiments were carried by using same set of glass wares for all type evaluation.

Experimental Animals

Albino Wistar rats of either sex weighing 200–225g were used. Animals were maintained under hygienic conditions and they were provided with commercial food pellets and tap water *ad libitum*. Cleaning and sanitation work were done on alternate days. Paddy husk was provided as bedding material, which was changed every day. The cages were maintained clean and all experiments were conducted between 9 am to 5 pm.

Acute Toxicity Study

Healthy Albino Wistar rats of either sex, starved overnight, were divided into five groups (n=4). Group I–IV animals were orally fed with the *Careya arborea* Roxb extract in increasing dose levels of 0.5, 1.0, 1.5 and 2.0 g/kg b. wt, while group V (untreated) served as control. The animals were observed continuously for first 2h for any gross change in behavioral, neurological and autonomic profiles or any other symptoms of toxicity and mortality if any, and intermittently for the next 6h and then again after 24h, 48h and 72h for any lethality or death. One-tenth and one-fifth of the maximum safe dose of the extract tested for acute toxicity were selected for the experiment ^[11].

Tumor Cells

DLA cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The DLA cells were maintained in Albino Wistar rats, by intra peritoneal (i.p.) transplantation on every 9th days ^[12]. The ascitic fluid was collected by syringe and the tumor cell count was per-formed in a Neubauer hemocytometer and 2×10^7 cells/ml was obtained by dilution with normal saline ^[13]. Tumor cell suspension showing more than 90% viability (checked by trypan blue dye (0.4%) exclusion assay) was used for transplantation.

Experimental Protocol

Albino Wistar rats were weighed and divided into five groups (n=6). DLA cells (2×10^6 cells/rat) were injected i.p to each rat of each group except control group. This was taken as Day 0. MECA and standard drug (5-fluorouracil) treatment were continued for subsequent 9 days starting from Day 1. On 10th day, 24h after the last dose six rats were sacrificed from each group and the rest were kept for the life span study of the tumor hosts. After sacrificing the animals, blood was collected to evaluate the hematological and biochemical parameters. Liver tissue was collected from the animals for the evaluation of antioxidant activity.

The groups and the design of the experiment were as follows ^[14]:

Group I: 2% Tween-80 (5ml/kg b. wt, i.p.)

Group II: DLA (2×10^6 cells/rat) + 2% Tween-80 (5ml/kg b. wt, i.p.)

Group III: DLA (2×10^6 cells/rats) + MECA (200mg/kg b. wt, i.p.)

Group IV: DLA (2×10^6 cells/rats) + MECA (400mg/kg b. wt, i.p.)

Group V: DLA (2×10^6 cells/rat) + 5-fluorouracil (20mg/kg b. wt, i.p.)

Antitumor activity of *Careya arborea* Roxb extract was assessed by observation of changes with respect to the following parameters.

Tumor Growth Response

The effect of *Careya arborea* Roxb extract on tumor growth and host's survival time were examined by studying the following parameters such as tumor volume, packed cell volume, tumor cell count, viable tumor cell count, nonviable tumor cell count, median survival time and increase in lifespan.

Tumor Volume and Packed Cell Volume

The rats were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. Packed cell volume was determined by centrifuging the ascetic fluid at 1000 rpm for 5min.

Tumor Cell Count

The ascetic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the numbers of cells in the 64 small squares were counted.

Viable and Nonviable Tumor Cell Count

The cells were then stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those which took the stain were nonviable. These viable and nonviable cells were counted.

Percentage Increase in Life Span

The effect of *Careya arborea* Roxb extract on tumor growth was monitored by recording the mortality daily for 6 weeks and percentage increase in life span (% IMST) was calculated. An enhancement of life span by 25% or more was considered as effective antitumor response [14, 15] $IMST (\%) = [(Median\ survival\ time\ of\ treated\ group / Median\ survival\ time\ of\ control\ group) - 1] \times 100$. Median Survival Time (MST) = [Day of first death + Day of last death] / 2

Hematological studies

RBC, WBC counts and estimation of hemoglobin was done by standard procedures from the blood obtained intracardially [16, 17].

Hemoglobin Estimation

0.1ml of heparinized blood was taken in Sahli's Hemoglobin meter and diluted with 0.1N HCl until the color matched with standard. The reading was then taken from the graduated cylinder and expressed as g/100ml of blood.

Counting of Erythrocytes

The blood sample was diluted (1:200) with the diluting fluid using Thoma pipette. After vigorous mixing, a drop of resultant mixture was discharged under the cover glass of Neubauer hemocytometer and the corpuscles were allowed to settle for 3 minutes. The number of erythrocytes in 80 small squares was counted under light microscope. The number of cells in 1 cumm of undiluted blood was calculated.

Total Count of Leukocytes

Blood was diluted 1:20 with a diluting fluid. The Neuberger hemocytometers were filled with the mixture and the number of cells in four corner blocks (each block subdivided into 16 squares) was determined and the total leukocyte count / cumm of blood was calculated.

Biochemical Estimation

Blood samples were collected from the animals' intracardially and serum was separated for the biochemical estimations of serum glutamic pyruvate transaminase (SGPT), serum glutamic oxaloacetate transaminase (SGOT) [18] and alkaline phosphatase (ALP) [19].

In vivo antioxidant assay

The antioxidant assay was performed with liver tissue and evaluation was carried out by measuring the level of lipid peroxidation [20] and the amount of enzymatic (catalase; CAT) [21] and nonenzymatic antioxidant system (reduced glutathione; GSH) [22] is measured.

Cytotoxic Activity

Stock cells of HEp-2, RD and Vero cell lines were cultured in RPMI-1640 and DMEM supplemented with 10% sheep serum, penicillin (100IU/ml) and streptomycin (100µg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with 0.2% trypsin, 0.02% EDTA in PBS. The cytotoxic assay was carried out by adding 0.1ml of cell suspension containing 10,000 cells to each well of a 96 well microtitre plate (Tarson) and fresh medium containing different concentrations of the extracts was added at 24h after seeding. Control cells were incubated without the test extract and with DMSO (solvent). The very small % of DMSO present in the wells (maximal 0.2%) was proved not to affect the experiment. The microtitre plates were incubated at 37°C in a humidified

incubator with 5% CO₂ for a period of 3 days. Twelve wells were used for each concentration of the extracts. The cells were observed at different time intervals during incubation in the presence of the extracts. Cellular viability was determined by the standard MTT and SRB assay methods [23].

DLA-Induced Solid Tumor Studies

DLA cells / mouse were injected subcutaneously to the right hind limb of mice and divided into 4 groups containing 6 mice in each group. All the treatments were given I.P. at 24 h after DLA tumor inoculation and continued once daily for 14 days. Initial diameter of the right hind limb was noted using vernier calipers. From the 7th day onwards, tumor diameter was measured every third day and recorded up to 34 days. The tumor volume was calculated by the following formula: $V = 4 / 3 \pi r_1 r_2 r_2$, where r₁ and r₂ are the radii of tumors at two different planes [24].

Statistical Analysis

Values were presented as mean ± S.E.M. Data were statistically evaluated by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. P<0.05 was considered as statistically significant.

RESULTS

Acute toxicity study, *Careya arborea* Roxb extract did not show any toxic effect up to the dose of 2g/kg b. wt, accordingly 200 and 400mg/kg b. wt were taken as low and high dose of *Careya arborea* Roxb extract for the experiment.

In case of tumor growth response study, *Careya arborea* Roxb extract treatment significantly reduced tumor volume, packed cell volume and viable cell count compared to those of DLA control rats, while nonviable cell count was found to be increased significantly in the treated groups. These results were summarized in Table.1. Table.2 depicts the effects of *Careya arborea* Roxb extract on prolongation of life span. In life span study, the median survival time for the control group was 10.5 days, whereas it was 17 and 23 days for low dose and high dose of *Careya arborea* Roxb extract treated groups. The median survival time for the group treated with standard drug 5-FU was 29 days. Increase in life span at low dose and high dose of *Careya arborea* Roxb extract treated groups (38.46% and 69.23% increase) as well as for the standard drug treated group (74.36%) were found to be significant with respect to the DLA control rats and it reflects the antitumor property of the extract *Careya arborea* Roxb extract. Administration of *Careya arborea* Roxb extract significantly reduced WBC count in both the groups of III and IV with respect to that of DLA control group. RBC count and hemoglobin content, which were decreased after DLA inoculation, were found to be significantly restored to the normal levels in the animals treated with *Careya arborea* Roxb extract of both 200 and 400mg/kg b. wt, as well as standard drug 5-FU. The results (Fig.1) implies the protective role of *Careya arborea* Roxb extract on the hematological profile of DLA bearing rats.

Table 1: Effect of *Careya arborea* Roxb extract on Tumor growth response of DLA bearing rats

Groups	Ascitic tumor volume (ml)	Packed cell Volume (ml)	Viable [% cell count]	Non viable [% cell count]	Total cell count (X107 /ml)
DLA Control	5.51±0.35	1.17±0.22	24.00±0.53 [98.18%]	0.46±0.18 [1.82%]	12.26±0.45
MECA (200 mg/kg)	2.82±0.10*	0.70±0.14*	11.92±0.50* [87.64%]	1.98±0.58* [12.36%]	8.90±0.55*
MECA (400 mg/kg)	2.04±0.08*	0.30±0.08*	7.25±0.75* [62.62%]	3.54±0.98* [37.38%]	5.79±0.94*
5Fluorouracil (20 mg/kg)	1.83±0.16*	0.45±0.06*	4.15±0.46* [60.10%]	3.75±0.32* [39.90%]	5.90±0.30*

Values are Mean ± SEM.; n=6 in each group. Drug treatment was done for 9 days. * p< 0.01 for treated groups vs DLA control group; where the significance was performed by One way ANOVA followed by Tukey-Kramer multiple comparison tests

Biochemical estimation as shown in Table.3 indicates the elevated level of liver functional enzymes in serum in DLA treated group with respect to normal animals, while these were significantly reduced to near normal value in the drug treated groups.

Fig.2 illustrates the effects of *Careya arborea* Roxb extract on the antioxidant status of DLA bearing rats. The level of lipid peroxide in liver tissue (expressed as nM lipid peroxide/mg of wet liver tissue) was significantly increased in DLA control rats when compared to normal control animals. After administration of *Careya arborea* Roxb extract (200 and 400mg/kg b.wt), lipid peroxide levels

were significantly reduced when compared with DLA control rats. Similarly, reduced glutathione level which was GSH level which was reduced in DLA control group was restored to the near normal values by treatment with *Careya arborea* Roxb extract as well as 5-FU. In DLA control rats there were significant reduction in antioxidant enzyme catalase activity, which was significantly improved by the treatment with *Careya arborea* Roxb extract, as was observed in standard drug treated mice in group V as well.

Table 2: Effect of *Careya arborea* Roxb extract on prolongation of life span of DLA bearing rats

Groups	MST (days)	%IMST
DLA Control	10.5	-
MECA (200 mg/kg)	17.0	38.46
MECA (400 mg/kg)	23.0	69.23
5Fluorouracil (20 mg/kg)	29.0	74.36

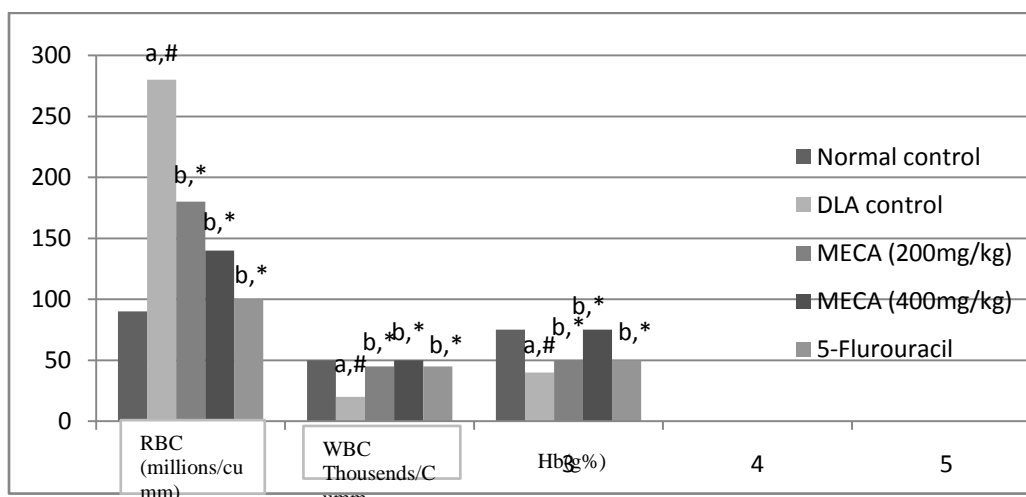
MST: Median survival time; % IMST: % Increase in MST= [(T/C)-1] x100 where T is median survival time of treated group and C that of control group

Table 3: Effect of *Careya arborea* Roxb extract on biochemical parameters of DLA treated rats

Groups	SGPT (IU/L)	SGOT (IU/L)	SALP (IU/L)
Normal Control	111.001.65	97.792.99	59.502.00
DLA Control	229.704.52a,#	210.546.01a,#	149.124.05a,#
MECA (200 mg/kg)	169.905.60b,*	120.557.12b,*	72.507.12b,*
MECA (400 mg/kg)	146.005.00b,*	95.504.50b,*	60.005.80b,*
5-Fluorouracil (20 mg/kg)	143.153.39b,*	99.964.16b,*	61.053.20b,*

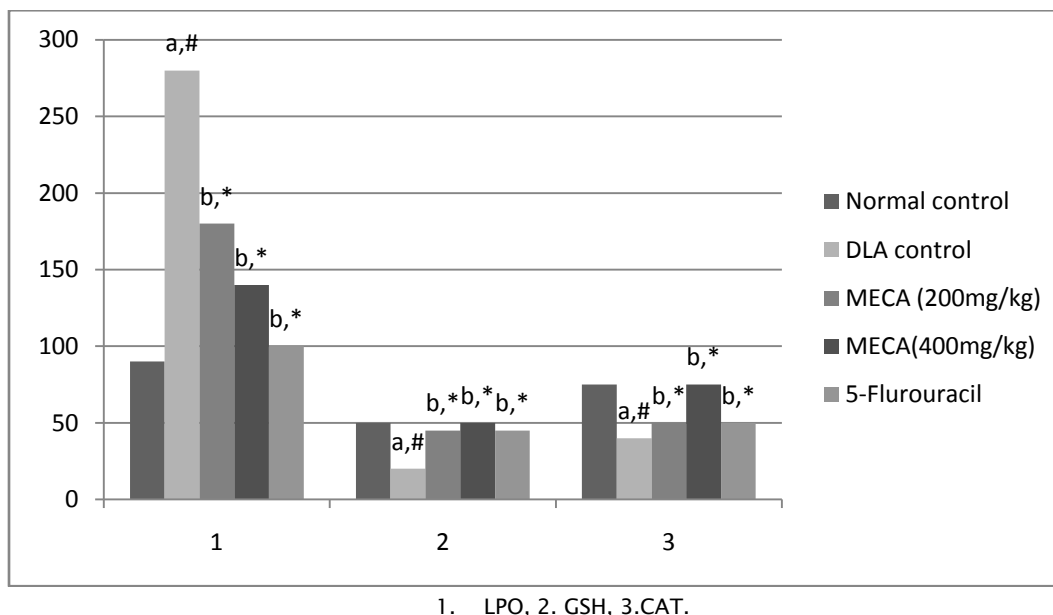
Values are Mean±SEM.; n=6 in each group. Drug treatment was done for 9 days. DLA control group vs normal control group, # p < 0.01; b Treated groups vs EAC control group, * p < 0.01; where the significance was per-formed by Oneway ANOVA followed by Tukey-Kramer multiple comparison tests

Figure 1: Effect of *Careya arborea* Roxb extract on hematological parameters of DLA bearing rats.



Values are Mean±SEM; n=6 in each group. Drug treatment was done for 9 days. a DLA control group vs normal control group, # p < 0.01; b Treated groups vs DLA control group, * p < 0.01; where the significance was performed by Oneway ANOVA followed by Tukey-Kramer multiple comparison tests

Figure 2: Effect of *Careya arborea* Roxb extract on antioxidant status of DLA bearing rats



Values are Mean±SEM; n=6 in each group. Drug treatment was done for 9 days. aDLA control group vs normal control group, # $p < 0.01$; b Treated groups vs DLA control group, * $p < 0.01$; where the significance was performed by Oneway ANOVA followed Tukey-Kramer multiple comparison tests

DISCUSSION

Cancer is uncontrolled proliferation of tumor cells. The present study was carried out to investigate the antitumor potential of *Careya arborea* Roxb extract against DLA bearing rats. DLA is a very rapidly growing carcinoma with very aggressive behavior [25]. It is able to grow in almost all strains of rats. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells [26]. *Careya arborea* Roxb extract treatment significantly reduced tumor volume probably by lowering the ascitic nutritional fluid volume. Further, the packed cell volume and the number of viable DLA tumor cells in peritoneum were significantly lower in the mice treated with *Careya arborea* Roxb extract when compared to the tumor control group. These results could indicate either a direct cytotoxic effect of *Careya arborea* Roxb extract on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

Prolongation of life span of the animals is a reliable criterion for judging the value of any anticancer drug [27]. The increase of life span of tumor bearing mice by *Careya arborea* Roxb extract treatment is a positive result and supports the antitumor effect of *Careya arborea* Roxb extract.

In cancer chemotherapy the major problem are myelosuppression and anemia [28, 29]. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions [30]. Treatment with *Careya arborea* Roxb extract brought back the hemoglobin content, RBC and WBC cell count near to normal values. This indicates that *Careya arborea* Roxb extract possesses protective action on the hemopoietic system.

Significant elevation in the levels of SGOT, SGPT, ALP reflects the hepatocellular damages caused by a number of agents. Biochemical measurements of these parameters showed that to some extent hepatotoxicity was associated after 9 days of inoculation with DAL. Treatment with the *Careya arborea* Roxb extract restored the elevated biochemical parameters more or less to normal range, indicating the protection of the tumor cell induced hepatotoxicity by *Careya arborea* Roxb extract.

The improper balance between ROMs (Reactive Oxygen Metabolites) and antioxidant defences results in 'oxidative stress', which deregulates the cellular functions leading to various pathological conditions including cancer [31, 32]. The oxidative stress may lead to damage of the macromolecules such as lipids and can induce lipid peroxidation [33]. In DLA bearing rats the level of lipid peroxide in liver was significantly elevated, which was however reduced to near normal level in the *Careya arborea* Roxb extract treated group animals. This reflects the decrease in free radical production and the subsequent reduction in oxidative stress, one of the main risk factors for the disease. Glutathione, a potent inhibitor of neoplastic process plays an important role as an endogenous antioxidant system that is found particularly in high concentration in liver and is known to have key function in the protective process [34]. The level of reduced glutathione

was depleted in cancer bearing mice which may be due to its utilization by the excessive amount of free radicals. Treatment with *Careya arborea* Roxb extract was found to increase the GSH content in the liver as compared to DLA control animals.

On the other hand the free radical scavenging enzyme catalase is present in all oxygen metabolizing cells and its function is to provide a direct defense against the potentially damaging reactivities of superoxide and hydrogen peroxide. The inhibition of CAT activities as a result of tumor growth has also been reported [35]. Similar findings were observed in the present investigation with DLA bearing mice. The administration of *Careya arborea* Roxb extract at different doses increased the CAT levels in a dose dependent manner, which along with the restoration of lipid peroxide and GSH content to near normal indicates the antioxidant and free radical scavenging property of *Careya arborea* Roxb extract.

Preliminary phytochemical study showed the presence of flavonoid, polyphenolics, saponin, protein and carbohydrate in *Careya arborea* Roxb extract. Many such compounds are known to possess potent antitumor properties. Hence the potent anticancer activity of *Careya arborea* Roxb extract may be due to any of these phytoconstituents. Further research is ongoing in our laboratory to find out the bioactive principle(s) for the anticancer activity of the extract.

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