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Research Article

ASSESSMENT OF ANTICANCER ACTIVITY OF ETHANOLIC EXTRACT OF *BORASSUS FLABELLIFER* LINN. LEAF AGAINST EHRLICH ASCITES CARCINOMA (EAC) AND DALTONS ASCITES LYMPHOMA (DAL) IN SWISS ALBINO MICE

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ABSTRACT

The medicinal plants have very important role in the health of human being as well as animals. The present study was thus designed to investigate the anticancer activity of ethanolic leaf extract of *Borassus flabellifer* Linn. And evaluated by *in-vitro* and *in-vivo* experimental models. To achieve objectives, the plant was subjected to phytochemical screening and tested for oral toxicity. The *in-vitro* study was carried out by means of MTT assay and Trypan blue dye exclusion assay using EAC cell line and DAL cell lines. The *in-vivo* anticancer activity was evaluated against EAC and DAL tumor bearing mice by liquid tumor models. Preliminary phytochemical screening confirmed the presence of flavanoids, glycosides, Tannins, Phenolic, Steroids and triterpenoids etc. The plant showed good cytotoxic effect on EAC cell line and DAL cell line in both MTT assay and Trypan blue dye exclusion assay. Oral administration of ELBF in tumor bearing mice for 14 days, showed significant reduction in the percent increase in body weight, tumor volume, tumor weight, viable cell count and increased non-viable cell count when compared to the untreated mice of the EAC control group and DAL control group. The restoration of the haematological parameters towards the normal control was also observed. The results suggested that the *Borassus flabellifer* exhibits significant anticancer activity towards both methods.

Keywords: Ethanolic leaf extract of *Borassus flabellifer* Linn, MTT cytotoxicity assay, Trypan blue dye exclusion assay, Ehrlich ascites carcinoma, Daltons ascites lymphoma

INTRODUCTION

In India, Ayurveda has integrated the concept of interconnectedness into its understanding of health and disease. The growing interest in Ayurveda is not only because it is free of synthetic chemicals, but also because of its integrated approach[1]. Cancer is a class of disease in which a group of cells display uncontrolled and invasion that destroy adjacent tissues[2]. Cancer is caused by some environmental factors include; tobacco, obesity, infections, radiations, Lack of physical activity and environmental pollutants[2-5]. These factors cause abnormalities in the genetic materials of cells. Over 50% of the drugs in clinical trials for anticancer properties were isolated from natural sources are related to them. These plants origin have potential value as chemotherapeutic agents. Over 3000 species of plants with anticancer properties have been reported[6-8]. *Borassus flabellifer* is commonly called palmyra palm native of tropical Africa but cultivated and naturalized throughout india. The tree belongs to Arecaceae family. Traditionally the plant is used as antiphlogistic, stimulant, diuretic and anti-leprotic[9-12]. The objective of the present study was to investigate the anticancer activity of ethanolic leaf extract of *Borassus flabellifer* Linn by using *in-vitro* and *in-vivo* methods.

MATERIALS AND METHODS

Chemicals and Reagents

Ethanol (CHEMID), Sodium nitropruside (CHEMCO),
Trypan blue (VETECK),
N-(1-Naphthyl ethylenediaminDihydrochloride (HIMEDIA),
5-Flourouracil (CHEMCO)

Software

Free Word to PDF, Graph pad prism (version 5.0), Instat (version 7.00)

Instruments

Water bath (NAVYUG), Vacuum Oven (Rotek), Hot plate (KEM), Electronic balance (HERCULES), UV chamber (Rotek)

Collection of plant

The whole plant was collected in the month of October and authenticated (Specimen 148212) by A.k Pradeep, Herbarium curator, Department of Botany, University of Calicut, Thenhipalam, Kerala, India. The specimen voucher was deposited in the Department of Botany, University of Calicut, itself.

Extraction

The leaves were shade dried for seven days and powder was prepared by using grinder (coarse powder). The powdered dried leaves were extracted with ethanol by using Soxhlet apparatus. After extraction the solvent was evaporated and concentrated extract was obtained.

PHARMACOLOGICAL STUDIES

Animals

Albino mice of Swiss strains were used for the pharmacological and toxicological studies. These animals were purchased and stock maintained in the animal house of Devaki Amma Memorial College of pharmacy, Chelembra, Malappuram District of Kerala, India. Female mice selected were nulliparous and non-pregnant. Female mice weighing 25-30 g were used for the study. Each animal at the commencement of its dosing between 8-12 weeks old and thus weight variation was within ±20% of the mean weight of any previously dosed animals. The temperature in the experimental animal room was 22℃ (±3℃) and the relative humidity was between 50-60%. These animals fed with pellet diet manufactured by Amrut Laboratory, Animal Fed Company, Sangli, Maharashtra and drinking water and *Ad libitum.* They were kept in 12 hr light/dark cycle and maintained for at least 5 days prior to dosing to allow for acclimatization to the laboratory condition. The animals experimental protocol has been approved by our Institutional Animal Ethics Committee (IAEC) vide registration no:DAMCOP/IAEC/021

Acute Oral Toxicity Study

Limit test

The acute oral toxicity study was carried out on Swiss Albino mice as per the guidelines No: 423 given by the organization for Economic Co- operations and Development (OECD 423, 1988). A limit test at one dose level of 2000 mg/kg.b.wt was carried out with six animals (Three animals per step) were fasted overnight. Animals were observed individually after dosing at least once during the first 30 minutes periodically during the first 24 hrs, with special attention given during first 4 hrs and daily thereafter for 14 days[13].

***IN-VITRO* ANTICANCER STUDY**

MTT assay

MTT[3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide] is a pale yellow substrate that is cleaved by living cells to yield a dark blue formazan product. This process requires active mitochondria, and even freshly dead cells do not cleave significant amount of MTT. Thus the amount of MTT cleaved is directly proportional to the number of viable cells present, which is qualified by colorimetric methods. Briefly, the compounds were dissolved in DMSO and serially diluted with complete medium to get the concentration a range of test concentration. DMSO concentration was kept <0.1% in all the samples. Cell line maintained in appropriate conditions were seeded in 96 well plates and treated with different concentration of the test samples, and incubated at 37℃, 5% CO_2 for 96 hrs. MTT reagent was added to the wells and incubated four 4 hrs; the dark blue formazan product formed by the cells was dissolved in DMSO under a safety cabinet and read at 550 nm. Percentage inhibition were calculated and plotted with the concentrations and Graph (4.42 version) software used to calculate the IC_{50} values.

Trypan Blue Dye Exclusion Assay

Trypan blue is a vital stain leaves non- viable cells with a distinctive blue colour when observed under microscope ,viable cells have intact cell membrane and hence do not take in dye from the surrounding medium. The non viable cells do not have an intact and functional membrane and hence do take up dye from their surroundings. From this easily distinguish between viable and non viable cells, so the former are unstained, small, and round, while the latter are stained and swollen. Cell suspensions were prepared by incubating for 24 hrs in 50% CO_2 300µl of extract were added. After incubating for 48hr, 100µl of cell suspension and 100µl of 0.4% trypan blue solution were taken in an eppendorf tube, mixed thoroughly and allowed to stand for 15 min. Cover slip was placed and Pasteur pipette was used to transfer a small amount of trypan blue- cell suspension Mixture to both chambers of a haemocytometer. The edge of the cover slip was carefully touched with the pipette tip and allowed each chamber to fill by capillary action. Starting with a chamber of the haemocytometer all the cells in the 1mm center square and four 1mm corner squares were counted and percentage inhibition was calculated and plotted with concentrations and Graph Pad Prism(5.0 version) software to calculate IC_{50} values[14-15].

***IN-VIVO* ANTICANCER STUDY**

Ehrlich Ascites Carcinoma (EAC) Tumor Cells Induced Anti Cancer Study

Group I: Normal control (oral dose of 10 ml/kg.b.wt. sodium CMC suspension0.5%)
Group II: EAC control (oral dose of 10 ml/kg.b.wt. sodium CMC suspension 0.5%)
Group III: EAC induced + Reference drug (oral dose of 20 mg/kg.b.wt. 5-Flourouracil)
Group IV: EAC induced + ethanolic extract (200 mg/kg.b.wt) treated mice
Group V: EAC induced + ethanolic extract (400 mg/kg.b.wt) treated mice

Thirty mice were divided into 5 groups, group 1 containing 6 animals, served as normal control, for which inoculation of tumor cells is not done. The remaining animals are inoculated with EAC tumor cells (1×10^6 cells/mouse) intraperitoneally and divided into 4 groups containing 5 mice in each group. Group II, served as the EAC control. Group I (Normal control) and Group II (EAC control) received an oral dose of 10 ml/kg.b.wt. SodiumCMC suspension (0.3%). Group III, served as a reference drug, is treated orally with 5-Fourouracil (5-FU) at the dose of 20 mg/kg.b.wt. GroupIV and Group V are treated with extract at 200 and 400 mg/kg.b.wt. respectively. All the treatments are given orally at 24hr after tumor inoculation and continued once daily for 14 days.

On the 15th day, half of the animals from each group are anesthetized and blood is collected by retro-orbital puncture for the evaluation of hematological parameters, which include Hemoglobin (Hb) content, Red Blood cell count (RBC) and White Blood Cell count (WBC). The remaining animals in each of the groups are kept to check the Mean Survival Time (MST) and percent increase in life span of the tumor bearing hosts.

$$\text{Death MST} = \frac{\text{Day of first death} + \text{Day of last death}}{2}$$

Statistical Analysis

The experimental results were expressed as mean±SEM. Data is assessed by ANOVA followed by the Tukey test, Value of $p<0.05$ was considered as statistically significant. The IC_{50} concentration were calculated using Graph software (version 5.0).The statistical analysis were performed using Graph prism software (version 5.0)

Dalton's Ascites Lymphoma cells induced anticancer study (method same as that of EAC) [16-17]

IN-VITRO ANTIOXIDANT STUDY

Nitric Oxide Free Radical Scavenging Activity

2 ml of 10Mm sodium nitroprusside in 0.5 ml phosphate buffer saline (p^h 7.4) was mixed with 0.5 ml of extract at various concentrations and the mixture incubated at 25℃ for150 min. From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulphanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 ml naphthylethylenediamine dihydrochloride, 0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radical scavenging activity was calculated.

$$S\% = [(A_{control}-A_{sample}) / A_{control}] \times 100$$

Where $A_{control}$ = absorbance of the blank control (containing all reagents except the extract solution) and A_{sample}= absorbance of test sample.

DPPH Assay

Step 1 (Preparation of stock solution): Prepare 0.05Mm solution of DPPH by mixing 9.8 mg of DPPH in 50 ml ethanol and incubate it at normal room temperature for 2-3 hrs.

Step 2 (Preparation of Drug dilution): Stock solution of 1 mg/ml of the plant extract is prepared in distilled water and diluted to get various concentrations.

Step 3 (Reaction mixture and analysis): a) Working solution: Take out 10 ml of stock solution and dissolve it in 40ml of ethanol. This makes the working solution.
b) Reaction mixture: Mix 1 ml of working solution with 1ml various extract concentration (100-1000µg/ml) Prepare in final volume of 1ml incubate the mixture for 30 min at room temperature. After 20 min absorbance of reaction mixture was recorded at 517 nm. Reference compound used here was ascorbic acid. All the tests were performed in triplicate in order to get the mean values. The percentage inhibition was calculated by comparing the absorbance values of the control and test samples. Antiradical activity was expressed as percentage inhibition (I %) and calculated using the following equation[18-23].

$$\text{Percentage inhibition (I \%)} = (Abs_{control}- Abs_{samle} /Abs_{control}$$

RESULTS

Phytochemical Screening

The percentage yield of the ethanolic extract of the leaves of *Borassus flabellifer* Linn was found to be 18.87µg/ml. The phytochemical identification was carried out as per the standard procedure and the ELBF showed the presence of alkaloids, glycosides, flavanoids, carbohydrates, steroids and triterpeniods, saponins, Tannins, fat and fixed oils.

Pharmacological Studies

Acute Oral Toxicity Study

The ELBF was showed no mortality even at 2000 mg/kg. All the animals were found to be normal and there were no gross behavioral and no weight changes till the end of the observation period of 14 days.

In-Vitro Anticancer Study

The ELBF was subjected to MTT assay and Trypan blue dye exclusion assay against EAC cell lines and DAL cell lines. ELBF showed an IC50 concentration at 140.95µg/ml against EAC cell line and 167.58µg/ml against DAL cell lines in MTT assay. In Trypan blue dye exclusion assay ELBF showed an IC50 concentration 150.01µg/ml and 167.59µg/ml respectively.

In- Vivo Anticancer Study

Ehrlich Ascites Carcinoma (Eac) Cells Induced Anti-Cancer Study

Effect on Tumor Growth

The average life span of EAC tumor control animals was found to be days. When EAC control groups compared to other groups, ELBF showed a significant change in average life span animals than the EAC control groups. The average life span of 5-FU treated animals was found to be days. Which indicate its potential anticancer nature. . The anticancer nature of ELBF was evidenced by the significant reduction in the body weight of animals than EAC control mice. The ELBF treated groups showed a significant decrease in the tumor volume, tumor weight, viable cell count and a significant increase in the non- viable cell count as compared to EAC bearing mice.

Effect on Haematological Parameters

EAC tumor bearing mice showed a significant increase in the Hb, RBC count and a significant increase in the WBC count as compared to the normal groups. Treatment with ELBF group significantly reversed EAC tumor induced changes in the haematological profiles. The standard 5-FU showed the similar observations.

In-Vitro Antioxidant Study

Nitric Oxide Free Radical Scavenging Activity: The IC50 values for ELBF and Ascorbic acid were found to be 48.65µg/ml and 69.17µg/ml respectively. ELBF significantly inhibited nitric oxide in a dose dependent manner[15-16].

DPPH Assay

The IC50 values for ELBF and Ascorbic acid were found to be 54.03µg/ml and 113.98µg/ml respectively. The ELBF significantly inhibited the DPPH in a dose dependent manner[17-21].

Table 1: Percentage inhibition of ELBF in *in-vitro* anticancer study (MTT assay)

Cell line	Samples	% inhibition					IC_{50} (µg/ml)
		Concentration (µg/ml)					
		10	20	50	100	200	
EAC cell line	5-FU	23.57±0.0208	34.2±0.395	56.14±0.431	81.70±0.333	87.51±0.395	55.60
	ELBF	8.056±0.084	15.22±0.193	31.32±0.706	40.54±0.328	64.20±0.061	140.95
DAL cell line	5-FU	22.07±0.021	30.76±0.042	56.01±0.012	81.39±0.562	86.99±0.11	55.72
	ELBF	6.783±0.287	10.48±0.352	26.58±0.34	33.50±0.328	56.73±0.329	167.58

Table 2: Percentage inhibition of ELBF in *in-vitro* anticancer study (Trypan blue dye exclusion assay)

Cell line	Samples	% inhibition					IC_{50} (µg/ml)
		Concentration (µg/ml)					
		10	20	50	100	200	
EAC cell line	5-FU	23.57±0.208	34.2±0.399	56.14±0.43	81.70±0.33	87.51±0.395	50.80
	ELBF	8.14±0.101	15.12±0.080	30.1±0.070	36.1±0.0230	62.07±0.058	150.01
DAL cell line	5-FU	22.07±0.021	30.76±0.042	56.01±0.012	81.39±0.562	86.99±0.011	55.72
	ELBF	6.196±0.038	10.16±0.069	26.176±0.038	32.24±0.037	58.01±0.066	167.59

Table 3: Effect of survival time of tumour bearing mice in *in-vivo* anticancer study

Parameter	EAC control	5-FU20 mg/kg	ELBF200 mg/kg	ELBF400mg/kg
Mean survival time	21 days	28 days	23 days	25 days
Percentage life span	_	33.33%	9.52%	19.04%

Table 4: Effect of body weight and other parameters on in vivo anticancer study

Parameter	EAC control	5-FU20 mg/kg	ELBF200 mg/kg	ELBF400mg/kg
Body weight(g)	40.10±0.860 ***	32.80±0.839 ***	37.15±1.181 *	35.37±0.662 **
Tumour weight (g)	21.93±0.0666	9.16±0.161 ***	18.66±0.166 ***	14.66±0.166 ***
Tumour volume (ml)	22.93±0.2404	8.70±0.093 ***	20.10±0.385 ***	16.70±0.451 ***
Viable cells(x10^{7}cells ml^{-1})	5.975±0.068	2.045±0.025 ***	4.731±0.094 ***	3.236±0.079 ***
Non-viable cells (x10^{7}cells ml^{-1})	0.2176±0.011 ***	1.634±0.017 ***	0.564±0.004 ***	0.744±0.0182 ***

Table 5: Effect of haematological parameters in *in-vivo* anticancer study

Parameter	Normal control	EAC control	5-FU 20mg/kg	ELBF 200mg/kg	ELBF 400mg/kg
WBC	8734±16.992	26920±41.92 ***	1150±6.640 ***	203118±2.0067 ***	18460±59.566 ***
RBC	7.851±0.0501	4.166±0.02211 ***	6.901±0.0157 ***	5.098±0.0297 ***	5.328±0.01833 ***
Hb	15.63±0.0614	10.47±0.0666 ***	14.57±0.0954 ***	11.37±0.0614 ***	12.11±0.0733 ***

Table 6: Effect of survival time of tumor bearing mice in *in-vivo* anticancer study

Parameter	DAL control	5-FU20 mg/kg	ELBF200 mg/kg	ELBF400mg/kg
Mean survival time	19 days	27days	20days	22days
Percentage life span	-	42.10%	5.26%	15.78%

Table 7: Effect of body weight and other parameters on in vivo anticancer study

Parameter	DAL control	5-FU20 mg/kg	ELBF200 mg/kg	ELBF400mg/kg
Body weight(g)	40.53±0.836	32.70±0.827 ***	37.52±1.208 Ns	35.46±0.678 ***
Tumour weight (g)	23.08±0.582	8.8±0.435 ***	18.59±0.274 ***	15.7±0.1562 ***
Tumour volume (ml)	19.80±0.341	7.89±0.035 ***	16.02±0.4599 **	16.93±0.7954 *
Viable cells (x10^{7}cells ml^{-1})	9.71±0.0175	8.81±0.059 ***	4.54±0.009 ***	4.09±0.057 ***
Non-viable cells (x10^{7}cells ml^{-1})	0.815±0.007	5.61±0.0493 ***	4.66±0.035 ***	3.60±0.011 ***

Table 8: Effect of haematological parameters in *in-vivo* anticancer study

Parameter	Normal control	DAL control	5-FU 20mg/kg	ELBF 200mg/kg	ELBF 400mg/kg
WBC	8734±14.84	21919±0.386 ***	11785±15.811 ***	20399±15.88 ***	13038±18.85 ***
RBC	8.85±0.062	3.583±0.038 ***	8.035±0.07201 ***	6.76±0.171 ***	7.06±0.140 ***
Hb	14.68±0.081	6.101±0.030 ***	15.23±0.0410 ***	12.77±0.096 ***	8.745±0.0534 ***

Table 9: IC_{50} Values of samples in Nitric oxide free radical and DPPH scavenging assay

Samples	IC_{50} Values (µg/ml)	
	Nitric oxide free radical scavenging activity	DPPH assay
Ascorbic acid	48.65	54.03
ELBF	69.17	113.98

DISCUSSION

The present study was designed to evaluate anticancer activity of *Borassus flabellifer* leaves. The collected plant specimen was authenticated by a botanist and ethanol extract was prepared from the dried powdered leaves sample using Soxhlet extraction technique. The percentage yield of ethanol extract was found to be 18.57% w/w. The preliminary phytochemical screening of ethanolic leaf extract of *Borassus flabellifer* was performed and revealed the presence of Flavanoids, Glycosides, Saponins, Tannins, Steroids and Triterpenoids and carbohydrates.

The acute toxicity study of ethanol extract was carried out as per OECD guidelines (423). The results showed that there was no significant behavioral as well as weight change in the animals. It can be conducted that the extract was safe up to a 2000 mg/kg. *Borassus flabellifer* was performed the *in-vitro* antioxidant studied by Nitric Oxide Scavenging Assay and DPPH assay. The results showed a good antioxidant capacity. Plant was performed the *in-vitro* anticancer activity against EAC cell line and DAL cell line by MTT assay and Trypan blue dye exclusion assay. The results suggested that ELBF showed moderate activity against in both cell lines.

Borassus flabellifer showed good cytotoxic effect on EAC cell line and DAL cell line in both MTT assay and Trypan blue dye exclusion assay. The plant showed significant reduction in the percent increase in body weight, tumor volume, tumur weight, viable cell count and increased non- viable cell count. These results suggested that *Borassus flabellifer* exhibit significant anticancer activity towards both methods. Further study is needed to determine to find out the active component responsible for its activity.

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