Phytopharmacology

Inforesights Publishing

Anti-tumor effect of acetone extract of *Madhuca longifolia* against Ehrlich Ascites Carcinoma (EAC) in mice

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Abstract

Present study was performed to explore the protective effect of acetone extract of *Madhuca longifolia* leaves (*AEML*) against Ehrlich Ascites Carcinoma (EAC) in mice. The activity was assessed using mean survival time (MST), tumor volume, tumor weight, tumor cell count, body weight, haematological studies and *In-vitro* cytotoxicity. Results found that oral administration of *AEML* increased the mean survival time. tumor volume, tumor weight, body weight and tumor cell count were also significantly reduced. haematological parameters including protein and PCV, which were altered by tumor inoculation, were restored. The effects of *AEML* was comparable with standard drug 5-Flourourasil.

Keywords Anti-tumor activity; Ehrlich Ascites Carcinoma; EAC; *Madhuca longifolia*

Introduction

It is well established fact that plants have been a useful source of clinically relevant antitumor 69 compounds (Kamuhabwa et al.*,* 2000). Indeed there have been worldwide efforts to discover new anticancer agents from plants. There are different approaches for the selection of plants that may contain new biologically active compounds (Cordell *et al.*1991). One of the approaches used is the ethnomedical data approach, in which the selection of a plant is based on the prior information on the folk medicinal use of the plant. It is generally known that ethnomedical data provides substantially increased chance of finding active plants relative to random approach (Chapuis *et al.,* 1988). However, as for cancer, the disease is complicated and heterogeneous, which makes it difficult to be well diagnosed, especially by traditional healers. The ethnomedical information obtained for a plant extract that is used to treat cancer might therefore not be reliable (Kamuhabwa *et al.,* 2000). Traditional Indian and Chinese medicinal herbs have been used in the treatment of different diseases in the country for centuries. There have been claims that some traditional healers can successfully treat cancer using herbal drugs. Indeed, some traditional healers who were interviewed recently in the

country stressed that they have successfully treated patients presented with cancer or cancer related diseases.

Madhuca commonly known as the Butter nut tree is a medium to large sized deciduous tree distributed in Nepal, India and Sri Lanka. The flowers are used as tonic, analgesic and diuretic. The bark is used for rheumatism, chronic bronchitis and diabetes mellitus. *Madhuca longifolia* leaves are expectorant and also used for chronic bronchitis and Cushing's disease (Prajapati *et al.,* 2003; Chandra *et al.,* 2001).

Method and Materials

Collection and Authentication of the leaves

The leaves of *Madhuca longifolia* was collected from Garden of National Botanical Research Institute, Lucknow, India in month of July 2009. The leaves were authenticated by Dr. Sayeeda Khatoon, chemotaxonomist and the voucher specimens were deposited in the departmental herbarium for future reference.

Preparation of extracts of Madhuca longifolia

The powdered leaves (500 g) were sequentially extracted using petroleum ether, chloroform, acetone and aqueous solution in Soxhlet apparatus. After about forty siphons of each solvent extraction step, the materials were concentrated by evaporation (Trease *et al.,* 2003).

Pharmacological evaluation

Animals

Swiss Albino mice (20-25gm) of either sex and of approximately the same age, procured from Institute of Animal Health and Veterinary Biological, Mhow, Indore, Madhya Pradesh were used for Anticancer study and Wistar albino rats (150-200 g) was procured from Central Drug Research Institute, Lucknow, were used for acute toxicity studies. They were housed in polypropylene cages and fed with standard rodent pellet diet (Hindustan Lever Limited, Bangalore) and water *ad libitum*. The animals are exposed to alternate cycle of 12 hrs of darkness and light each. Before each test, the animals are fasted for at least 12 hrs. The experimental protocols were subjected to the securitization of the Institutional Animals Ethical Committee and were cleared by the same. All experiments were performed during morning according to CPCSEA guidelines for care of laboratory animals and the ethical guideline for investigations of experimental pain in conscious animals.

Experimental design

EAC cells were obtained through the courtesy of Amala Cancer Research Center, Thrissur. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse (Kuttan *et al.,* 1990; Mazumder *et al.,* 1997).

The animals (Swiss albino mice weighing 20-25 g) were divided into 4 groups consisting of 12 animals. Animals were fed with basal diet and water throughout the experimental period. All the groups were injected with EAC cells except the group I. This was taken as day zero. From day 1st, normal saline (5 ml/kg) was given in group I, 5-fluorouracil (20mg/kg) and *AEML* (500 mg/kg) were given to group III and group IV respectively, for 14 consecutive days, whereas group II was serve as a tumor control group and normal saline (5 ml/kg) was given to this group also, on day 15th half of the mice from each group were sacrificed, 24h after last dose, for the determination of tumor volume, tumor weight, haematological parameters etc, and rest were kept with food and water *ad libitum* to check the increase in the life span of the tumor hosts and body weight.

Effect of AEML on mean survival time

Animals were inoculated with 1×10^6 cells/mouse on day '0' and treatment with *AEML* was started 24 h after inoculation, at a dose of 500 mg/kg/day, p.o. The control group was treated with the same volume of 0.9% sodium chloride solution. All the treatments were given for 14 days. The mean survival time (MST) of each group was noted. The anticancer efficacy of *AEML* was compared with that of 5- fluorouracil (Dabur Pharmaceutical Ltd, India; 5-FU, 20 mg/ kg/day, i.p. for 14 days). The MST of the treated groups was compared with that of the control group using the following calculation (results are given in table 1): (Kuttan *et al.,* 1990; Rajkapoor *et al.,* 2003; Mazumder *et al.,* 1997; Sur *et al.,* 1994)

ILS (%) = [(Mean survival of treated group/ Mean survival of control group)-1]x100

Mean survival time = [1st Death + Last Death] / 2

Effect of AEML on tumor volume and tumor weight

On 15th day, after 24h of dose, 6 mice from each group were dissected and the ascites fluid was collected from peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube. The tumor weight was measured by taking the weight of mice before and after collection of ascites fluid from peritoneal cavity (Kuttan *et al.,* 1990; Mazumder *et al.,* 1997). The result was shown in table 1

Effect of *AEML* on tumor cell count

To determine the effect of *AEML* on tumor cell count, the ascites fluid withdrawn from the peritoneal cavity of the mice was taken in WBC pipette and diluted 100 times with normal saline. A drop of a diluted cell suspension was placed on the neubauers chamber and the number of cells in the 64 square was counted. The viability and non viability of cells was

Table 1. Effect of *AEML* treatment on the survival of tumor bearing mice.

S No	Treatment	Mean Survival Time (Days)	Increase in life span (%)
1	Tumor Control	21.50 ± 2.73	-
2	5- FU (20mg/kg, i.p)	40.16 ± 2.13*	86.79 %
3	*AEML* (500 mg/kg, p.o)	30.33± 4.7*	41.06 %

n=6 animals in each group, *P<0.01 Vs control. Days of treatment = 14, Values are expressed as mean ± SEM

Table 2. Effect of *AEML* on tumor volume, tumor weight and tumor cell count.

S No	Treatment	Tumor Volume (ml)	Tumor weight (gm)	Tumor cell count	
				Viable cells[1]	Nonviable cells[2]
1	Tumor Control	6.70 ± 0.16	6.87 ±0.21	9.83 ±0.3	0.33 ±0.21
2	5- FU (20mg/kg, i.p)	1.01 ± 0.10*	1.1 ±0.06*	0.83 ±0.3*	1.67 ±0.33**
3	*AEML* (500 mg/kg, p.o)	3.46 ± 0.07*	3.54 ±0.31*	3.66 ±0.21*	2.5 ±0.22*

n=6 animals in each group, *P<0.01 Vs control. Days of treatment = 14, Values are expressed as mean ± SEM, Viable cells[1]x 10^7/ml, [2]Nonviable cells[2] x 10^7/ml

checked by tryphan blue method. On staining viable cells did not take the dye whereas the non viable cells were stained blue (Kuttan *et al.,* 1990; Mazumder *et al.,* 1997; Rajkapoor *et al.,* 2003). The result was shown in table 2.

Effect of AEML on body weight

Body weights were recorded every 7th day till 40 days of treatment or till the death of the animal (Kuttan *et al.,* 1990; Mazumder *et al.,* 1997). The result was shown in table 3.

Effect of AEML on haematological parameters

On the 15th day, blood was drawn by retro orbital plexus method. WBC count, RBC count, haemoglobin, protein and packed cell volume were determined (D'Amour *et al.,* 1965; Lowry *et al.,* 1951). Cells smear was prepared in slide and stained with Lieshman stain solution (Docie *et al.,* 1958). Red blood cells (RBC), White blood cells (WBC) and Heamoglobin (Hb) were estimated with the help of MS-09 heamatology analyzer (France).The result was shown in table no 4.

Statistical analysis

All the values were expressed as mean ± SEM (standard error of mean) for six rats. Statistical analysis was carried out by using PRISM software package (version 3.0). Statistical significance of differences between the control and experimental groups was assessed by One-way ANOVA followed by Newman-Keuls Multiple Comparision Test. The value of probability less than 5% ($P < 0.05$) was considered statistically significant (Rajkapoor *et al.,* 2003).

Results

The results of the present study show an protective effect of *AEML* against EAC in Swiss albino mice. There was no mortality amongst the graded dose groups of animals and

Table 3. Effect of *AEML* treatment on body weight of tumor bearing mice

Treatment/dose	7th Day	14th Day	21st Day	28th Day	35th Day
Normal	22.00±0.77	23.00±0.51	24.16±0.54	27.66±0.66	31.83±0.83
Tumor Control	27.83±0.79*	40.33±0.76*	50.16±0.65*	-	-
5-FU (20mg/kg, i.p)	23.33±0.61	24.50±0.34$	26.83±0.6 $	29.33±0.66	32.83±0.47
AEML(500 mg/kg, p.o)	24.33±0.33**	29.50±0.76#	30.5±0.88$,#	31.00±0.25#	35.83±0.30#

n= 6 in each group, * P< 0.001 Vs Normal control, $ P< 0.001 Vs Tumor control, # P<0.001 Vs Standard, ** P<0.01Vs Tumor control, Values were expressed as mean± SEM.

Table 4. Effect of *AEML* treatment on Hematological Parameters of tumor bearing mice.

Parameter	Normal	Tumor control	5 FU (20 mg/kg)	*AEML*	Parameter
Hb(g/dl)	14.3±0.10	8.35±0.09*	14.0±0.05*,$	12.4±0.4*,$	Hb(g/dl)
RBC (million/mm^3)	4.68±0.06	2.6±0.07*	4.11±0.04*,$	3.18±0.3*,$	RBC (million/mm^3)
WBC(million/mm^3)	7.48±0.03	27.19±0.07*	8.23±0.02*,$	9.6±0.7*,$	WBC(million/mm^3)
Proteing %	8.21±0.06	13.95±0.2*	8.65±0.04*,$	9.2±0.1*,$	Proteing %
PCV (mm)	16.5±0.42	31.5±0.42*	19.5±0.42*,$	26.2±0.1*,$	PCV (mm)
Neutrophils %	30.83±0.60	68.83±0.60*	31.83±0.47*,$	38.1±2.2*,$	Neutrophils %
Lymphocytes %	68.5±0.42	30±0.57*	64.66±0.42*,$	50.3±2.1*,$	Lymphocytes %
Monocytes %	1.16±0.16	2.16±0.16[#]	1.33±0.21 [ns]	1.8±0.3[ns]	Monocytes %

n= 6 in each group, * P< 0.001 Vs Normal control, $ P< 0.001 Vs Tumor control, # P<0.05Vs Normal Control, ns – not significant, Values are expressed as Mean ± SEM

they did not show any toxicity or behavioral changes at a dose level of 5000 mg/kg. This finding suggests that the *AEML* was safe in or non-toxic to rats and hence doses of 500 mg/kg, po were selected for the study.

A significant enhancement of MST was observed. The MST for the control group was 21.50±2.73 days, whereas it was 30.33± 4.7 and 40.16 ± 2.13 days for the groups treated with *AEML* (500 mg/kg/day, p.o.) and 5-FU (20 mg/kg/day, i.p.) respectively. The % increase se in the lifespan of tumor-bearing mice treated with *AEML* and 5-FU was found to be 41.06 and 86.79% respectively (P< 0.01) as compared to the control group. The result was showed in table 1.

There was reduction in the tumor volume, tumor weight and tumor cell count of mice treated with *AEML* (P<0.001). as showed in table 2. Tumor volume of control animals was 6.70 ± 0.16, whereas for the extract-treated group it was 3.46 ± 0.07 for *AEML*. Tumor weight of control animals was 6.87 ±0.21 g, whereas for the extract-treated group it was 3.54 ±0.31g.

There was a significant decrease in the weight gain by *AEML* treated mice when compare with tumor control as showed in table 3. The analysis of the haematological parameters showed minimum toxic effect in mice treated with *AEML*. After 14 days of transplantation, *AEML* were able to reverse the changes in the haematological parameters consequent to tumor inoculation. The total WBC count, proteins and PCV were found to increase with a reduction in the haemoglobin content of RBC. The differential count of WBC showed that the percentage of neutrophils increased (P<0.001) while that of lymphocytes decreased (P<0.001). At the same time interval, *AEML* (500 mg/kg/day, p.o.) treatment could change these altered parameters to near normal as showed in table 4.

Discussion

The reliable criteria for judging the value of any anticancer drug are prolongation of lifespan and decrease of WBC from blood (Clarkson *et al.,* 1965; Oberling *et al.,* 1954). The *AEML* treated animals at the doses 500 mg/ kg significantly decrease the tumor volume, tumor weight, tumor cell count, body weight, and brought back the haematological parameters to more or less normal levels. In EAC-bearing mice, a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid in-

crease in ascites fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad *et al.,* 1994).

The *AEML* treatment decreased the ascites fluid volume, viable cell count, and increased the percentage of life span. It may be concluded by decreasing the nutritional fluid volume and arresting the tumor growth, increases the life span of EAC-bearing mice. Usually, in cancer chemotherapy the major problems that are being encountered are of myelosuppression and anemia (Price *et al.,.* 1958; Hogland *et al.,* 1982). The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or haemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions (Fenninger *et al.,* 1954). In EAC control group, a differential count the presence of neutrophils increased, while the lymphocyte count decreased, the observed leucocytopenia indicates a common symptom of immunosuppression in many types of cancers (Rashid et al., 2010; Ropponen et al*.,* 1997) and one of the causes of neutrophilia is myeloid growth factors which are produced in malignant process as part of a paraneoplastic syndrome. In addition to this another factor granulocyte colony stimulating factor produced by the malignant cells has also been attributed to be the cause of neutrophilia because of its action on bone marrow granulocytic cells in cancer. After the repeated treatment, *AEML* able to reverse the changes in altered neutrophils and lymphocytes count (Ulich *et al.,* 1990; Uchida *et al.,* 1992). Treatment with *AEML* brought back the haemoglobin content, RBC, and WBC count more or less to normal levels and this indicates that *AEML* posses protective action on the haematopoietic system. From above studies it was concluded that acetone extract of *M. longifolia* is very much effective in preventing EAC in mice and possess significant anticancer activity.

Acknowledgment

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