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Antitumour activity of *Amoora rohituka* Roxb. stem against Ehrlich ascites carcinoma in mice

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Abstract. The present study was designed to evaluate the antitumour activity of ethyl acetate (EAEAR) and dichloromethane (DMEAR) extracts, prepared successively from *Amoora rohituka* Roxb. stem, against Ehrlich's Ascites Carcinoma (EAC) in mice. Administration of EAEAR and DMEAR in EAC cell bearing mice at 20 and 40 mg/kg body weight significantly decreased viable tumour cells and increased mean survival time. Altered hematological parameters and serum enzymes (i.e., ALP and SGOT) were reverted more or less to normal levels in extracts-treated mice. The results obtained, were compared with the corresponding results for a standard anticancer drug *bleomycin* (0.3 mg/kg). The dichloromethane extract (DMEAR) of *Amoora rohituka* stem exhibited better antitumour effect than ethyl acetate extract (EAEAR) on EAC bearing mice.

Keywords: *Amoora rohituka*, stem bark, Ehrlich's carcinoma, antitumour, mice.

Introduction

Cancer represents the largest cause of mortality in the world and claims over 6 million lives every year (Abdullaev et al. 2000). Today an extremely promising strategy for cancer prevention is chemoprevention, which is defined as the use of synthetic or natural agents (alone or combination) to block the development of cancer in humans. A survey on medicinal plants reported that over 3000 species of plants have alleged anticancer properties (Graham et al. 2000). *Amoora rohituka* Roxb (Benagali: Pithraj) is one of such plants that grows in forests and roadsides of many districts of Bangladesh (Ghani 2003). The stem bark of *Amoora rohituka* is used in spleen and liver diseases, tumours and abdominal complaints. The seeds have a folkloric reputation to exhibit laxative, anthelmintic and antiulcer properties (Kirtikar & Basu 1980). The plant contains limonoids (Mulholland & Naidoo 1999), triterpenes (Chatterjee et al. 1970), amooramin (a triterpene acid) (Rabi et al., 2002), alkaloid (Harmon et al. 1979), flavonoid glycosides (Jain & Srivastava 1985) and sesquiterpenes (Chowdhury et al. 2003b). In addition, seed oils and plant extracts have been reported to possess multiple therapeutic properties like hepatoprotective (Chatterjee & Das 1999), antibacterial (Chowdhury et al. 2003a), antiviral (Connolly et al. 1976) and laxative (Chowdhury & Rashid 2003) activities. Ethyl acetate extract of the stem bark of *Amoora rohituka* showed anti-tumour activity against Dalton's lymphoma ascites cells (DLA) in mice (Rabi & Cupta 1995).

From this viewpoint the present study was designed to evaluate the antitumour activity of ethyl acetate and dichloromethane extracts, prepared successively from stem bark of *Amoora rohituka*, against Ehrlich ascites carcinoma (EAC) in mice.

Materials and methods

Plant materials

Stem bark of *Amoora rohituka* Roxb. (Family: Meliaceae) collected from Rajshahi district of Bangladesh, were taxonomically identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi and a voucher specimen was deposited under the accession number DACB-28927 at the Bangladesh National Herbarium.

Extraction

The shade dried and powdered stem bark of *Amoora rohituka* was successively extracted with ethyl acetate and dichloromethane at room temperature and after filtration, filtrates were evaporated under reduced pressure at 40°C using a rotary evaporator to have ethyl acetate (EAEAR) and dichloromethane (DMEAR) extracts.

Animals

For this study, Swiss albino mice of either sex, 3-4 weeks of age, weighing between 20-25 g were collected from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). The mice were grouped and housed in iron cages and maintained under standard laboratory conditions (temperature 25±2 °C; humidity 55±5 %) with 12 dark/light cycle. They were allowed free access to standard dry pellet diet (Collected from ICDDR,B) and water *ad libitum*. Permission and approval for animal studies were obtained from Animal Ethics committee of Science Faculty, University of Rajshahi.

Acute Toxicity Study

The acute toxicity study was conducted by the method of Lorke (1983) to determine the LD_{50} value of EAEAR and DMEAR in mice. For each extract, this method was carried out by a single intraperitoneal injection in twenty five animals (5 in each group) at different doses (100, 200, 400, 800 and 1600 mg/kg body weight). LD_{50} was evaluated by recording mortality after 24 hours.

Tumour Cells

EAC cells were obtained by the courtesy of Indian Institute for Chemical Biology (IICB), Kolkata, India and were maintained by weekly intraperitoneal (i.p.) inoculation of 10^5 cells/mouse in the laboratory.

Cell growth inhibition

In vivo cell growth inhibition was carried out by the method as described by Sur and Ganguly (1994). For therapeutic evaluation $2x10^5$ cells/mouse were inoculated into the mice of six groups (6 mice in each group) on the first day. Treatment was started after 24 hours of tumour inoculation and continued for 5 days. Group 1, 2, 3, 4, 5 and 6 received the vehicle (2% v/v Dimethylsulfoxide; DMSO), EAEAR (20 and 40 mg/kg body weight), DMEAR (20 and 40 mg/kg body weight) and standard drug, *bleomycin* (Biochem Pharmaceutical, India; 0.3 mg/kg body weight). The mice were sacrificed on the 6th day after transplantation and viable tumor cells per mouse of the treated group were compared with those of control.

Studies on Survival Time

Animals were divided into six groups, consisting of 6 mice and inoculated with $1x10^5$ cells/mouse on day '0'. The control group was treated with 2% DMSO solution. Treatment (i.p.) with EAEAR (20

and 40 mg/kg body weight), DMEAR (20 and 40 mg/kg body weight) and *bleomycin* (0.3 mg/kg body weight) were started after 24 hours of inoculation and continued for 10 days. Then average body weight gain and mean survival time (MST) of each group were noted (Halder et al. 2010).

Studies on Heamatological and Biochemical Parameters

Forty two mice in seven groups (6 mice in each group) were injected with EAC cells (2 x 10^5 cells/mouse) intraperitoneally except the normal group at the day "0". From the first day normal saline and 2% DMSO were intraperitoneally administered to normal (group 1) and EAC control (group 2), respectively for 10 days. Similarly both EAEAR and DMEAR at 20 and 40 mg/kg/mouse/day doses were administered in groups 3, 4, 5 and 6, respectively. Mice in group 7 were treated with standard *bleomycin* (0.3 mg/kg/mouse/day). Heamatological parameters were measured on 14th day after tumour inoculation from freely flowing tail vein blood of each mice of each group (Ruisa & Sood 1988). Then every mouse was sacrificed and blood was collected by heart puncture. Serum was separated and analyzed for alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) in an Bioanalyzer (Microlab 200) using commercial kits (Atlas Medica, UK).

Statistical Analysis

All values were expressed as mean ± SD (Standard deviation). Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's 't' test using SPSS statistical software of 15 version. P<0.05 were considered to be statistically significant.

Results

Toxicity study

Intraperitoneal administration of graded doses of EAEAR and DMEAR to Swiss albino mice produced a LD_{50} of 723±0.62 and 563±0.95 mg/kg body weight, respectively.

Effect of EAEAR and DMEAR on EAC Cell Growth

Effect of EAEAR and DMEAR on EAC cell growth on day 5 after tumor transplantation is shown in Table 1. Treatment with DMEAR resulted in pronounced cell growth inhibition at doses 20 mg/kg (56.47%; P<0.001) and 40 mg/kg (75.10%; P<0.001) whereas EAEAR showed 37.69% (P<0.01) and 57.69% (P<0.001) inhibition at 20 and 40 mg/kg doses, respectively.

Effect of EAEAR and DMEAR on Survival Time

The effect of EAEAR and DMEAR on the survival and average body weight gain of tumour bearing mice is shown in Table 2. In the EAC control group, the mean survival time (MST) was 19.0±2.16 days and it is increased dose dependently at 20 mg/kg and 40 mg/kg in the EAEAR and DMEAR treated groups (Table 2). The standard drug *bleomycin* (0.3 mg/kg)-treated group had a MST of 37±0.95 (P<0.001). The increase in the life span of tumour bearing mice treated with EAEAR (20 and 40 mg/kg), DMEAR (20 and 40 mg/kg) and *bleomycin* (0.3 mg/kg) was found to be 21.05%, 36.84%, 47.36%, 68.84% and 89.47%, respectively. The average weight gain of tumour control group was 18.7±0.83 g whereas it was significantly (P<0.001) reduced by treatment of EAEAR, DMEAR and *bleomycin* (Table 2).

Effect of EAEAR and DMEAR on Hematological Parameters

As shown in Table 3, hematological parameters of EAC cell bearing mice on the day 14 were showed significant (P<0.001) changes when compared to normal mice. The total WBC count was found to increase with a reduction in the hemoglobin content and total RBC count. Treatment with DMEAR at the dose of 40 mg/kg, significantly restored the altered hemoglobin content RBC and WBC count more or less to normal levels (Table 3). EAEAR (40 mg/kg) also significantly increased hemoglobin content and RBC and reduced total WBC when compared with EAC control. The differential count of WBC showed that the percentage of neutrophils increased while that of lymphocytes decreased significantly (P<0.001) in the EAC control group when compared to normal mice. DMEAR at 20 and 40 mg/kg doses reverted these altered parameters significantly (P<0.05 and P<0.001, respectively) more or less to the normal values. At 0.3 mg/kg, standard drug *bleomycin* significantly (P<0.001) restored all hematological parameters to normal level.

Effect of EAEAR and DMEAR on ALP, SGPT and SGOT

Only mice of EAC alone group showed significant (P<0.001) increase in the activities of ALP and SGOT when compared with the respective normal values (Table 4). Significant (P<0.001) depletion in the activities of ALP and SGOT was found by treatment with DMEAR at 20 and 40 mg/kg doses. EAEAR also significantly (P<0.001) reduced the activities of ALP and SGOT at 40 mg/kg (Table 4). However SGPT was not significantly altered by tumour growth in only tumour bearing mice but DMEAR at 40 mg/kg dose, increased SGPT (P<0.01) when compared with only EAC cell bearing mice.

Discussion

Cancer is a pathological state involving uncontrolled proliferation of tumor cells. The present study was carried out to investigate the antitumor potential of EAEAR and DMEAR against EAC bearing mice. EAC (Ehrlich Ascites Carcinoma) is a very rapidly growing carcinoma with very aggressive behavior (Segura et al. 2000). It is able to grow in almost all strains of mice. The Ehrlich Ascitic tumor implantation induces *per se* a local inflammatory reaction, with increasing

Table 1. Effect of *Amoora rohituka* stem extracts on EAC cell growth.

Group	No. of mice	Treatment	Viable EAC cells on day 6 after inoculation (x 10^7)	% of cell growth inhibition
1	6	EAC + 2% (v/v) DMSO	3.51 ± 0.18	-
2	6	EAC + EAEAR (20 mg/kg)	2.19 ± 0.35^b	37.69%
3	6	EAC + EAEAR (40 mg/kg)	1.48 ± 0.37^a	57.89%
4	6	EAC + DMEAR (20 mg/kg)	1.53 ± 0.43^b	56.47%
5	6	EAC + DMEAR (40 mg/kg)	0.87 ± 0.43^a	75.10%
6	6	*Bleomycin* (0.3 mg/kg)	0.29 ± 0.09^a	91.74%

^{a}Significantly different from group 1; P<0.001
^{b}Significantly different from group 1; P<0.01

Table 2. Effect of *Amoora rohituka* stem extracts on mean survival time and average body weight gain of EAC tumour bearing mice.

Group	No. of mice	Treatment	Mean survival time (MST) in days	% Increase life span (ILS)	Average increase in body weight (gm)
1	6	EAC + 2% (v/v) DMSO	19 ± 2.16	-	18.7 ± 0.83
2	6	EAC + EAEAR (20 mg/kg)	23 ± 1.70	21.05	16.1 ± 0.80[b]
3	6	EAC + EAEAR (40 mg/kg)	26 ± 1.41[c]	36.84	13.6 ± 0.71[a]
4	6	EAC + DMEAR (20 mg/kg)	28 ± 1.41[b]	47.36	10.4 ± 0.58[a]
5	6	EAC + DMEAR (40 mg/kg)	32 ± 1.87[a]	68.84	8.6 ± 0.98[a]
6	6	*Bleomycin* (0.3 mg/kg)	37 ± 0.95[a]	89.47	6.8 ± 0.65[a]

[a]Significantly different from group 1; P<0.001
[b]Significantly different from group 1; P<0.01
[c]Significantly different from group 1; P<0.05

Table 3. Effect of *Amoora rohituka* stem extracts on hematological parameters.

Group	No. of mice	Treatment	Hb (g/dl)	RBC (x10^{9}cells/ml)	WBC (x10^{6}cells/ml)	Differential Count (%)		
						Lymphocytes	Neutrophils	Monocytes
1	6	Normal + 0.9% NaCl	12.1±1.5	5.4±0.5	7.8±2.8	73.6±4.9	24.8±4.0	1.5±1.0
2	6	EAC + 2% (v/v) DMSO	6.6±0.9[*]	2.1±0.4[*]	25.5±3.1[*]	34.3±5.0[*]	64.0±5.5[*]	1.6±1.0
3	6	EAC + EAEAR (20 mg/kg)	7.8±1.0	2.6±0.2	17.6±6.6	32.8±5.6	66.1±5.9	1.0±0.6
4	6	EAC + EAEAR (40 mg/kg)	8.7±1.0[c]	3.1±0.4[c]	15.1±4.0[b]	34.6±5.2	63.7±4.9	1.3±0.8
5	6	EAC + DMEAR (20 mg/kg)	8.4±0.9	2.8±0.2	14.8±3.2[b]	45.8±4.2[c]	52.6±3.9[c]	1.5±0.5
6	6	EAC + DMEAR (40 mg/kg)	9.7±0.9[b]	4.8±0.3[a]	14.8±3.2[a]	61.2±6.7[a]	35.6±5.6[a]	1.3±1.0
7	6	Bleomycin (0.3 mg/kg)	11.7±0.7[a]	5.2±0.9[a]	10.5±2.8[a]	70.3±1.2[a]	28.2±0.9[a]	1.5±0.7[a]

[*]Significantly different from group 1; P<0.001
[a]Significantly different from group 2; P<0.001
[b]Significantly different from group 2; P<0.01
[c]Significantly different from group 2; P<0.05

Table 4. Effect of *Amoora rohituka* stem extracts on ALP, SGPT and SGOT.

Group	No. of mice	Treatment	ALP (U/L)	SGPT (U/L)	SGOT (U/L)
1	6	Normal + 2% (v/v) DMSO	122.5 ± 3.7	68.9 ± 2.6	39.6 ± 1.4
2	6	EAC + 2% (v/v) DMSO	234.6 ± 5.1[*]	66.6 ± 1.9	84.1 ± 2.3[*]
3	6	EAC + EAEAR (20 mg/kg)	225.0 ± 7.7[c]	68.3 ± 2.3	80.3 ± 1.2
4	6	EAC + EAEAR (40 mg/kg)	184.1 ± 5.0[a]	66.9 ±1.9	75.3 ± 0.9[a]
5	6	EAC + DMEAR (20 mg/kg)	144.3 ± 3.1[a]	65.2 ± 2.0	58.6 ± 0.9[a]
6	6	EAC + DMEAR (40 mg/kg)	126.5 ± 4.8[a]	75.6 ± 2.4	51.4 ± 0.7[a]
7	6	EAC + Bleomycin (0.3 mg/kg)	127.6 ± 4.1[a]	68.0 ± 2.4[b]	37.3 ± 1.6[a]

[*]Significantly different from group 1; P<0.001
[a]Significantly different from group 2; P<0.001
[b]Significantly different from group 2; P<0.01
[c]Significantly different from group 2; P<0.05

vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascitic fluid formation and accumulation (Fecchio et al. 1990). The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells (Shimizu et al. 2004). EAEAR and DMEAR significantly reduced the number of viable EAC tumor cells in peritoneum (Table 1) which indicate either a direct cytotoxic effect of EAEAR and DMEAR on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition.

The reliable criteria for judging the value of any anticancer drug is prolongation of life span (Bala et al. 2010). Treatment with EAEAR and DMEAR showed enhancement of mean survival time (MST) and decrease in body weight gain due to tumour burden (Table 2).

In cancer chemotherapy the major problems are myelosuppression and anemia (Jules Hirsch 2006). The anemia encountered in tumour 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 (Hogland 1982). Treatment with EAEAR and DMEAR reverted the hemoglobin content, RBC and WBC cell count near to normal values (Table 3). This indicates that both extracts possess protective action on the heamopoietic system.

Significant elevation in the levels of SGOT and ALP reflects that some extent cellular damages were associated after 14 days of inoculation with EAC (Saha et al. 2011). Treatment with the EAEAR and DMEAR restored the elevated biochemical parameters more or less to normal range (Table 4), indicating the protection of the tumor cell induced cellular damages by EAEAR and DMEAR. However, no such inference could be drawn from the SGPT assay, as it was not notably affected by the tumour growth. In our previous study, treatment of 'normal' mice with the extracts did not cause any noticeable abnormality in their glycolytic functions as indicated by their serum transaminase values (Karim & Habib 2009). In our studies, DMEAR have potential antitumour activity than EAEAR and it is comparable to that of *bleomycin*, which is commonly used as an active antitumour agent in vast series of preclinical and clinical studies (Tannock & Richard 1998). Preliminary phytochemical screening of EAEAR and DMEAR showed the presence of triterpenes, phenolic compounds, flavonoids and glycosides. Triterpenes and phanolic compounds have antitumour effects (Kim et al. 2004, Jin et al. 2001). Flavonoids have been

shown to possess antimutagenic and antimalignant effects (Kintzios 2006). Flavonoids show chemopreventive role in cancer through their effects on signal transduction in cell proliferation (Weber et al. 1996) and angiogenesis (Fotis et al. 1997). The antitumour properties of the extracts may be caused by the presence of these compounds. Further investigations are in progress in our laboratory to identify the active principles involved in this antitumour activity.

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