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Evaluation of antitumor activity of *Manilkara zapota* leaves against Ehrlich ascites carcinoma in mice

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Abstract

This study has been designed to explore *in vivo* the antitumuor activity of the ethyl acetate extract of leaves of *Manilkara zapota* (EALM) against Ehrlich ascites carcinoma (EAC). The antitumour activity of EALM against EAC was evaluated at doses of 50, 100 and 200 mg kg^{-1} day^{-1}. Intraperitoneal treatment with EALM (at 100 and 200 mg kg^{-1} day^{-1}) showed a significant increase in survival time and decrease in viable EAC cell count and weight gain in the EAC tumour hosts. Improvement in the altered hematological parameters following EALM treatment, like hemoglobin content, red blood corpuscle and white blood corpuscle count of the EAC cell bearing mice were also observed. Overall, the findings of the study indicate antitumour activity of EALM. In addition, erythrodiol, which was for the first time isolated from EALM, also showed 70.8% reduction in viable tumour cell count, in comparison with the untreated control.

Key words: antitumour activity, Ehrlich ascites carcinoma, erythrodiol, *Manilkara zapota*, mice.
Abbreviations: EAC, Ehrlich ascites carcinoma; EALM, ethyl acetate extract of leaves of *Manilkara zapota*; DMSO, dimethylsulphoxide; ILS, increase in life span; MST, mean survival time; RBC, red blood cells; WBC, white blood cells.

Introduction

Natural phytochemicals derived from medicinal plants have gained significant recognition in the potential management of several human clinical conditions, including cancer. Plant extracts as prophylactic agents offer great potential to inhibit the carcinogenic process. In traditional medication systems, various plants have been used in the treatment of cancer (Indap et al. 2006; Guilford, Pezzuto, 2008).

Manilkara zapota (L.) P. Royen is a plant in the Sapotaceae family. It grows widely throughout the Indian subcontinent, including Bangladesh, but it is a plant native to Mexico and Central America. In traditional medicine of Bangladesh, the leaves of *M. zapota* are used to treat cough, cold, dysentery and diarrhea (Ghani 2003). The leaves of this plant have been reported to possess antimicrobial and antioxidant activities (Nair, Chanda 2008; Kaneria et al. 2009; Osman et al. 2011a). Seeds of *M. zapota* have been used as aperients, diuretic tonic and febrifuge, and stem bark is astringent and febrifuge (Patricia et al. 2008; Chanda, Nagani 2010). In addition, we have also reported the potent antitumour activity of stem bark of *M. zapota* (Osman et al. 2011b). Previous phytochemical investigation on the leaves of *M. zapota* reported the isolation and identification of lupeol acetate, oleanolic acid, apigenin-7-O-α-L-rhamnoside, myricetin-3-O-α-L-rhamnoside and caffeic acid (Fayek et al. 2012). However, no investigation has been made on the antitumour properties of leaves of *M. zapota*.

Since Ehrlich ascites carcinoma (EAC) is a rapidly growing experimental tumour with very aggressive behavior and resembles human tumours (Segura et al. 2000), here we used EAC to evaluate the *in vivo* antitumor activity of isolated erythrodiol and ethyl acetate extract of the leaves of *M. zapota* (EALM) in mice.

Materials and methods

Plant material

Leaves of *M. zapota* were collected in January, 2011 from Rajshahi district of Bangladesh. The plant material was 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-23801 in the Bangladesh National Herbarium.

Extraction and isolation

A sample of shed-dried powdered leaves (950 g) of *M. zapota* was extracted with ethyl acetate (2.0 L) at room temperature. The solvent was completely removed by rotary vacuum evaporator from the crude extract to yield 12 g ethyl acetate extract of leaves of *M. zapota* (EALM). Then 6 g of EALM was applied on silica gel (60 to 120 mesh) chromatography using n-hexane with a gradient of ethyl acetate up to 100% and followed by chloroform. Sixty eight fractions were collected. Among these fractions, fraction 14 to 19 gave 35 mg colourless crystals (designated

as compound-1). The purity of the isolated compound was checked on thin layer chromatography plates.

General methods

High resolution time-of-flight mass spectra of compound-1 were obtained using a Waters LCT Premier mass spectrometer (UK) coupled with a Waters Aquity HPLC system, with data acquisition achieved using MassLynx software, version 4.0. NMR spectra were recorded on a Bruker 400 MHz FT spectrometer (DPX-400, Switzerland). All spectra were produced in the Analytical Research Division, Bangladesh Council of Scientific and Industrial Research Laboratories, Dhaka-1205, Bangladesh.

Animals

Male Swiss albino mice (22 to 25 g) were obtained from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh. The animals were housed in an air-conditioned (25 ± 2 °C) room with controlled lighting (from 6:00 AM to 8:00 PM). The animals were fed on pelleted food and tap water was available ad libitum. The animals were acclimatized to the laboratory for at least 10 days before testing. The experiments were carried out after approval of the protocol by the Institutional Ethics Committee for Experimentations on Animal, Human, Microbes and Living Natural Sources (225/320-IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

Ehrlich ascites carcinoma cells

Ehrlich ascites carcinoma (EAC) cells were obtained by the courtesy of Indian Institute for Chemical Biology, Kolkata, India and were maintained by weekly intraperitoneal inoculation of 10^5 cells per mouse in the laboratory.

Acute toxicity study

Acute toxicity studies were conducted for EALM in order to select a suitable dose for evaluation of the antitumor activity. An acute toxicity study relating to the determination of LD_{50} was performed by the method of Lorke (1983). This method was carried out by a single intraperitoneal injection in thirty animals (five in each group) at different doses (100, 200, 400, 800, 1600 and 3200 mg kg^{-1} day^{-1}). LD_{50} was evaluated by recording mortality after 24 h.

Cell growth inhibition

In vivo EAC cell growth inhibition was carried out by the method described by Osman et al. (2011b). EAC cells (1.5×10^5) were inoculated intraperitonealy into five groups of mice (eight in each) on day 0. Group 1 was treated with vehicle (2% dimethylsulfoxide; DMSO) and considered as the untreated control. Mice in group 2, 3 and 4 were treated by intraperitoneal injection with EALM at 50, 100 and 200 mg kg^{-1} day^{-1} doses and group 5 received bleomycin (0.3 mg kg^{-1} day^{-1}). Treatment was continued for 5 days and on day 6 after EAC cell inoculation, animals were sacrificed. EAC cells were collected by repeated washing with 0.9% saline and viable EAC cells per mouse of the treated groups were compared with the untreated control.

In another separate experiment, mice were divided into two groups (eight mice in each group). After inoculation with EAC cells, group 1 was treated with the vehicle (2% v/v DMSO) and designated as untreated control whereas group 2 received compound-1 at 5 mg kg^{-1} day^{-1}. Then the experiment was continued by the method as described above.

Studies on survival time and hematological parameters

Swiss albino mice weighing 22 ± 3 g were divided into six groups (n = 8). All the groups were injected with EAC cells (0.1 mL of 2×10^5 cells per mouse) intraperitoneally except the normal group. This was taken as day zero. On the first day, 5 mL kg^{-1} of normal saline was administered in group 1 (control). 2% DMSO was administered in group 2 (EAC control). EALM at different doses (50, 100 and 200 mg kg^{-1} day^{-1}) and the standard drug bleomycin (0.3 mg kg^{-1} day^{-1}) were administered by intraperitoneal injection in groups 3, 4, 5 and 6 respectively, for 10 days. After the last dose and 18-h fasting, hematological parameters hemoglobin (Hgb), red blood cells (RBC), white blood cells (WBC) and differential count of WBC were measured from freely flowing tail vein blood of each individual of each group under aseptic condition (Mukherjee 1988). Then animals of each group were kept to determine survival time and body weight gain of each EAC-cell bearing host. The survival time was expressed as mean survival time (MST) in days and percent increase of life span (%ILS) was calculated (Osman et al. 2011b) using the formula

$$\% \text{ ILS} = (\text{MST of treated group} / \text{MST of control group} - 1) \times 100,$$

where MST = (Σ survival time in days of each mouse in a group) / total number of mice.

Statistical analysis

All values were expressed as mean ± SE. Statistical analysis was performed with one way analysis of variance (ANOVA) followed by Dunnett's *t* test using SPSS statistical software 14 version. $P < 0.05$ was considered to be statistically significantly different when treatment effects were compared with the control values.

Results

Identification of compound-1

Isolated and purified compound-1 was characterized by its mass and NMR spectral data. The molecular formula for compound-1 was deduced as $C_{30}H_{50}O_2$ through EI-MS, which showed a molecular ion (M^+) peak at m/z 442.92 (calculated for $C_{30}H_{50}O_2$). Its ^{1}H-NMR spectrum exhibited one olefinic proton at δ 5.63 and a singlet of

one methylene with the integration of two protons at 3.44 (H-28). The ^{13}C-NMR spectrum of compound-1 showed the presence of 30 signals, which were resolved through DEPT (Distortion enhancement by polarization transfer) experiments as seven methyl, eleven methylene, five methine and seven quaternary carbons. The ^{13}C-NMR of compound-1 also showed two olefinic carbons at δ 122.8 (C-12) and δ 145.1 (C-13). Based on the foregoing observations and a comparison of the NMR data (Table 1) data with the literature (Mehmood et al. 2008), the structure of compound-1 was confirmed as erythrodiol or 12-oleanene-3,28-diol (Fig. 1). Isolation of erythrodiol (compound-1) is reported for the first time from *M. zapota*. Erythrodiol is a constituent of many higher plants and is widely distributed as aglycone of various saponins (Juan et al. 2008).

Table 2. ^{1}H- and ^{13}C-NMR spectral data of Compound 1 (erythrodiol). Proton resonance integral, multiplicity, and coupling constant (*J* = Hz) are in parentheses

Carbon	δ_C	δ_H
1	36.7	1.35 (2H, d, *J* = 11.5 Hz)
2	27.1	1.65 (2H, s)
3	79.0	3.19 (1H, dd, *J*ax.ax =11.2 Hz, *J*ax.eq = 4.5 Hz)
4	38.7	-
5	55.5	1.42 (1H, s)
6	18.8	1.37 (2H, s)
7	32.6	1.45 (2H, s)
8	39.8	-
9	48.7	1.48 (1H, s)
10	36.9	-
11	23.6	1.65 (2H, m)
12	122.8	5.63 (1H, t, *J* = 3.6 Hz)
13	145.1	-
14	41.3	-
15	25.9	1.26 (2H, m)
16	22.0	1.92 (2H, d, *J* = 11.5 Hz)
17	35.7	-
18	48.7	1.51 (1H, m)
19	49.3	1.53 (2H, m)
20	29.9	-
21	35.1	1.58, (2H, m)
22	31.0	1.59 (2H, m)
23	28.0	0.97 (3H, s)
24	15.4	0.78 (3H, s)
25	17.5	0.92 (3H, s)
26	18.8	0.94 (3H, s)
27	25.9	1.25 (3H, s)
28	67.1	3.44 (2H, s)
29	33.7	0.81 (3H, s)
30	23.6	0.90 (3H, s)
–OH (C-3)	-	2.04 (1H, s)
–OH (C-28)	-	2.00 (1H, s)

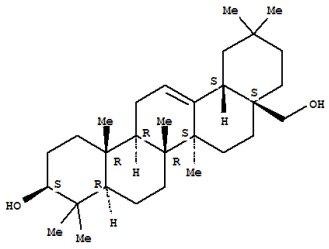

Fig. 1. Chemical structure of erythrodiol (compound-1).

Acute toxicity study

In acute toxicity study, intraperitoneal administration of graded doses of EALM to Swiss albino mice produced a LD_{50} of 2853.1 mg kg^{-1} day^{-1}.

Effect of EALM on cell growth and survival time

In this study of antitumor activity, the average number of viable EAC cells per mouse and MST of untreated control group were found to be (2.483 ± 0.57) × 10^7 cells mL^{-1} and 18.55 ± 1.34 days, respectively. EALM groups treated at doses of 100 and 200 mg kg^{-1} day^{-1} significantly (P < 0.05) reduced the counts of viable EAC cells and improved survival in a dose dependent manner, compared to those of the untreated control (Table 2). The percentage increase in the life span of EAC cell bearing mice treated with EALM was found to be 17.25, 67.11 and 73.85% at 50, 100 and 200 mg kg^{-1} day^{-1} doses, respectively (Table 2). Moreover, the EALM treatment at 100 and 200 mg kg^{-1} day^{-1} doses significantly inhibited body weight gain when compared to the untreated control (Table 2). However, the standard bleomycin produced better results in all these parameters at the dose of 0.3 mg kg^{-1} day^{-1}.

The isolated and purified compound-1 (i.e., erythrodiol) also significantly (P < 0.05) reduced the average number of viable EAC cells at 5 mg kg^{-1} day^{-1} dose (Fig. 2) and showed 70.82% cell growth inhibition.

Effect of EALM on heamatological parameters of EAC cell bearing mice

In this study, inoculation with EAC cells for 12 days significantly (P < 0.05) altered hematological parameters of untreated tumour control when compared to normal mice (Table 3). The total WBC count and the percentage of neutrophils were found to increase with a reduction in the hemoglobin content, total RBC count and percentage of lymphocytes. At the same time interval, the intraperitoneal administration of EALM (100 and 200 mg kg^{-1} day^{-1}) restored the altered WBC, RBC and hemoglobin content near to normal (Table 3). Treatment of EALM could not normalize the altered differential count.

Table 2. Effect of EALM on viable cell count, survival time, life span and body weight gain in EAC cell bearing mice. Data are expressed as mean ± SE for eight animals in each group. *, significant differences from EAC control ($P < 0.05$)

Treatment	Viable tumour cells on day 6 after inoculation ($\times 10^7$ cells mL^{-1})	Median survival time (in days)	Increase of life span (%)	Body weight gain (g) after 12 days
EAC control	2.48 ± 0.57	18.55 ± 1.34	-	14.7 ± 0.88
EAC + EALM (50 mg kg^{-1} day^{-1})	1.46 ± 0.51	21.75 ± 2.52	17.25	12.1 ± 0.93
EAC + EALM (100 mg kg^{-1} day^{-1})	0.97 ± 0.28*	31.00 ± 2.34*	67.11	8.4 ± 0.75*
EAC + EALM (200 mg kg^{-1} day^{-1})	0.90 ± 0.34*	32.25 ± 2.01*	73.85	7.9 ± 0.52*
EAC + bleomycin (0.3 mg kg^{-1} day^{-1})	0.23 ± 0.05*	36.84 ± 0.85*	98.59	4.1 ± 0.36*

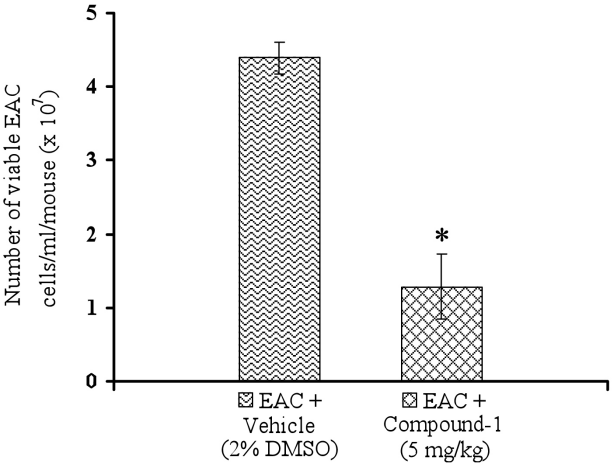

Fig. 2. Effect of compound-1 (erythrodiol) on viable EAC cells on day 6 after EAC cell inoculation. *, significant differences ($P < 0.05$) between EAC control and compound-1 treated group of mice.

Discussion

The results of this study showed an antitumour effect of EALM against EAC in Swiss albino mice. The EALM treatment decreased the viable tumour cell count and body weight gain due to tumour burden as well as increased the average life span of animals, when compared to the untreated EAC control values (Table 2). A reliable criterion for judging the value of any anticancer drug is the prolongation of life span of the animals and the disappearance of elevated WBC from blood (Hirsch 2006). EALM was able to meet this criterion.

Myelosuppression and anemia (reduced hemoglobin) have been frequently observed in ascites carcinoma. Anemia is a common complication in cancer and the situation becomes further aggravated during chemotherapy, since the majority of antineoplastic agents exert suppressive effects on erythropoiesis and thereby limiting the use of these drugs (Hogland 1982). Anemia encountered in ascites carcinoma is mainly due to iron deficiency, either by haemolytic or myelopathic conditions, which finally lead to reduced RBC number (Hogland 1982). In this study, EALM treatment and subsequent tumour inhibition resulted in appreciable improvements in hemoglobin content, RBC and WBC counts (Table 3). These observations indicate the haematopoietic protecting activity of EALM, without inducing myelotoxicity.

On thin layer chromatography screening, EALM gave positive test for glycosides, terpenoids and flavonoids, which was consistent with literature (Fayek et al. 2012). In addition, the isolated and purified erythrodiol also significantly reduced the average number of viable EAC cells and showed cell growth inhibition. In vitro antiproliferative and apoptotic activities of erythrodiol have already been reported (Ukiya et al. 2002; Juan et al. 2008). Thus, erythrodiol with other phytoconstituents contribute an important role in inhibition of EAC by EALM.

In conclusion, the overall findings of this study demonstrate that EALM and isolated erythrodiol of *M. zapota* have noteworthy antutumour effect and that

Table 3. Effect of EALM on hematological parameters of EAC cell bearing mice. *, significant differences from control group; ^{t}, significant differences fromEAC control group ($P < 0.05$)

Parameter	Control	EAC + vehicle	EAC + EALM (50)	EAC + EALM (100)	EAC + EALM (200)	EAC + bleomycin (0.3)
Hgb (g dL^{-1})	13.25 ± 0.22	5.77 ± 0.15*	7.22 ± 0.47	7.94 ± 0.49^t	7.80 ± 0.41^t	13.67 ± 0.25^t
RBC ($\times 10^9$ cells mL^{-1})	6.20 ± 0.10	1.53 ± 0.03*	2.19 ± 0.31	3.70 ± 0.48^t	3.62 ± 0.25^t	5.90 ± 0.09^t
WBC ($\times 10^6$ cells mL^{-1})	7.29 ± 0.53	21.25 ± 3.81*	15.8 ± 1.59	10.6 ± 0.92^t	11.6 ± 2.97	8.49 ± 0.59^t
Lymphocytes (%)	75.2 ± 1.36	40.8 ± 1.40*	38.5 ± 2.65	45.6 ± 3.15	47.2 ± 2.15	69.4 ± 0.90^t
Neutrophils (%)	20.3 ± 1.38	55.1 ± 2.10*	56.4 ± 3.55	52.3 ± 2.80	50.7 ± 3.15	25.8 ± 0.93^t
Monocytes (%)	2.75 ± 0.40	2.00 ± 0.22	3.62 ± 0.27	1.87 ± 0.15	1.65 ± 0.15	3.78 ± 0.27

they might be a source of herbal drugs in the respective therapeutic area. Further investigations are in progress in the laboratory to identify more active principles from EALM involved in this antitumour activity and investigate their mechanism of action.

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