

Antitumor activity of *Manilkara zapota* (L.) fruits against Ehrlich ascites carcinoma in mice

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As a part of searching for potential anticancer agents from natural sources, this investigation was carried out to evaluate the antitumor effect of ethyl acetate extract of *Manilkara zapota* (L.) fruits (EEFM) against Ehrlich ascites carcinoma (EAC) in Swiss albino mice. The antitumor activity of EEFM has been evaluated against Ehrlich ascites carcinoma (EAC) at the doses of 50 and 100 mg/kg body weight. Treatment with EEFM (at 100 mg/kg body) showed a significant increase in the survival time and decrease in the viable tumor cell count and weight gain in the EAC tumor hosts. Improvement in the altered hematological parameters following the EEFM treatment, like hemoglobin content, RBC and WBC count of the tumor bearing mice, have also been observed. During tumor progression, altered biochemical (SALP and SGOT) parameters were also significantly restored in EEFM-treated mice at 100 mg/kg. In brine shrimp lethality bioassay, the cytotoxicity against *Artemia salina* in terms of LD₅₀ was found to be 3.06 µg/ml for EEFM. So, the results of this study conclude that *in vivo* the EEFM was effective in inhibiting the growth of EAC.

Key words: *Manilkara zapota*, antitumor, fruits, Ehrlich ascites carcinoma

INTRODUCTION

Cancer is one of the most frequent neoplastic diseases in human population. Due to lack of effective drugs, it is a fatal disease rating the top three causes of death. Many of the chemotherapeutic agents sold for the treatment of cancer are highly expensive and toxic for normal tissues. Narrow inhibition of these agents limits their applica-

tions (Gaidhani et al., 2013). Therefore, the quest for effective anti-cancer drug is an active research field. Medicinal plants have a long history of use in the treatment of cancer. Over 60% of currently used anti-cancer agents are derived in one or another way from natural sources, including plants, marine organisms and microorganisms (Newman et al., 2003). The medicinal importance of plants lies in some chemical substances that produce a definite physiological action on the human body (Cragg, Newman, 2000). Compounds

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derived from plants such as alkaloids, steroids, flavonoids, tannins, phenolics compounds and terpenoids have antitumour activity (Edeoga et al., 2005; Roslin, Anupam, 2011). Due to the ability of plants to synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of anti-neoplastic and cytotoxic agents.

Manilkara zapota (L.) P. Royen, which belongs to the family Sapotaceae, is an evergreen, glabrous tree that is cultivated throughout the Indian subcontinent including Bangladesh (Ghani, 2003). The seeds of *Manilkara zapota* are an aperient, a diuretic tonic and a febrifuge. Stem bark is an astringent and a febrifuge (Patricia et al., 2008). The leaves and bark are used to treat cough, cold, dysentery and diarrhea (Chanda, Nagani, 2010). Antimicrobial and antioxidant activities are also reported from the leaves of *Manilkara zapota* (Nair, Chanda, 2008; Kaneria et al., 2009). We had also showed the antineoplastic activity of leaves and stem bark of *Manilkara zapota* in our previous study (Osman et al., 2011; Rashid et al., 2014). Previous phytochemical investigations on the leaves of this plant have resulted in the isolation of luteol acetate, oleanolic acid, apigenin-7-O- α -L-rhamnoside, myricetin-3-O- α -L-rhamnoside and caffeic acid (Fayek et al., 2012). Moreover, the major constituents isolated from fruits of *Manilkara zapota* are polyphenols (methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+)-catechin, (-)-epicatechin, (+)-gallic acid, and gallic acid (Ma et al., 2003). But no studies to date have been conducted to demonstrate the antitumour activity of *Manilkara zapota* fruit. This study was carried out to evaluate the *in vivo* antitumour activity of the ethyl acetate extract of *Manilkara zapota* (EEFM) fruits against Ehrlich ascites carcinoma (EAC)

MATERIALS AND METHODS

Plant materials

Fruits of *Manilkara zapota* (Family: Sapotaceae) were collected in August 2013 from the Rajshahi district of Bangladesh. The plant

material was taxonomically identified by Dr. A. H. M. Mahabubur Rahman, Associate Professor, Department of Botany, University of Rajshahi, and a voucher specimen was deposited under the accession number DACB-23801 at the Bangladesh National Herbarium.

Extraction and phytochemical screening

The collected fruits were cleaned and cut into small pieces. The small pieces were shade-dried and then pulverized into a coarse powder by a grinding machine (FFC-15, China). The powdered fruits (450 g) were extracted with ethyl acetate at room temperature. These extracts were then filtered through filter papers and filtrates were evaporated under reduced pressure at 40 °C using a rotary evaporator to get 5.5 g ethyl acetate extract of *Manilkara zapota* (designated as EEFM) fruits. For phytochemical screening, this extract was run on a pre-coated silica gel plate using a mixture of n-hexane and ethyl acetate in different proportions as a mobile phase and a vanillin-H₂SO₄ reagent was used as a spray reagent. R_f values were also calculated for every spots.

Brine shrimp lethality bioassay

This experiment was carried out using the method described by Meyer (Meyer et al., 1982). In brief, *Artemia salina* Leach (brine shrimp eggs) was allowed to hatch and mature as nauplii (Larvae) in seawater for 48 h at 25 °C. Five doses (10, 20, 40, 80 and 100 μ g/mL) of each sample were used for the lethality test against brine shrimp nauplii. 10, 20, 40, 80 and 100 μ L of each sample (EEFM and Ampicillin trihydrate) were transferred from their stock solution (concentration: 5 mg/mL) to the seawater (5 mL), containing 10 nauplii. After the incubation for 24 h at 25 °C, the number of survivors was counted. LC₅₀ (50% lethal concentration, μ g/mL) was determined from triplicate experiments. Ampicillin trihydrate was used as a positive control.

Animals

Male Swiss albino mice (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 with eight animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C; humidity $55 \pm 5\%$) with 12 hrs dark/light cycle. The mice were allowed free access to the standard dry pellet diet (Collected from ICDDR,B) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before the beginning of the experiment. 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.

Tumour cells

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

Acute toxicity study

The acute toxicity study was conducted by the method of Lorke (1983) to determine the LD_{50} value of EEFM in mice. This method was carried out by a single intraperitoneal injection in thirty animals (5 in each group) at different doses (100, 200, 400, 800, 1 600 and 3 200 mg/kg body weight). LD_{50} was evaluated by recording mortality after 24 hours.

Studies on cell growth inhibition

In vivo cell growth inhibition was carried out following the method as described by Sur, Ganguly (1994). For this study the mice were divided into three groups (6 mice in each group), and for therapeutic evaluation, the mice of all groups were inoculated with 1.5×10^5 cells/mouse on the day zero. After 24 hours of the EAC cell inoculation, treatment was started and continued for 5 days. The mice in group 1 were given 2% v/v dimethylsulfoxide (DMSO) at 5 mL/kg/mouse/day and considered as an untreated EAC control. EEFM (50 and 100 mg/

kg/mouse/day) was administered intraperitoneally (i. p.) in groups 2 and 3. The mice were sacrificed on the 6th day after the transplantation of tumour cells, and EAC cells were collected by a repeated intraperitoneal wash with normal saline (0.9% NaCl). Viable EAC cells were counted with a haemocytometer using trypan blue, and a total number of viable cells per mouse of the treated group was compared with those of control.

Studies on survival time

Animals were divided into three groups, consisting of 6 mice in each and inoculated with 1.5×10^5 cells/mouse on the day zero. The control group (group 1) was treated with only 2% DMSO solution at 5 mL/kg/mouse/day. After 24 hours of the inoculation, treatment (i. p.) with EEFM was started on group 2 and 3 at doses of 50 and 100 mg/kg/mouse/day, respectively, and continued for 10 days. The average body weight of each group was noted on 15th day after the EAC cell inoculation. The survival time was recorded and expressed as the mean survival time (MST) in days and percent increase of the life span (%ILS) was calculated (Senthilkumar et al., 2008) as follows:

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

Mean survival time (MST) = $(\Sigma \text{ survival time in days of each mouse in a group}) / \text{total number of mice}$.

Haematological and biochemical studies

In order to detect the effect of EEFM on the hematological parameters of EAC cell bearing mice, a comparison was made among four groups (n = 6) of mice on the 12th day after the inoculation (Raj Kapoor et al., 2003). All the groups were injected with EAC cells (0.1 mL of 1.5×10^5 cells/mouse) intraperitoneally except the normal group (i. e. group 1) at the day zero. After 24 hours of the inoculation, normal saline (5 mL/kg/mouse/day) and 2% DMSO (5 mL/kg/mouse/day) were administered intraperitoneally to the normal group

(group 1) and the EAC control (group 2), respectively, for 10 days. EEFM at 50 and 100 mg/kg/mouse/day was administered in groups 3 and 4, respectively. On 12th day after the EAC cell inoculation, hematological parameters (Hemoglobin, RBC and WBC) were measured from freely flowing tail vein blood of each mice of each group (Mukherjee et al., 1988). Then every mouse was sacrificed and blood was collected from each individual mouse by heart puncture in the sterilized eppendorf tubes. The whole blood was placed immediately on ice and subsequently centrifuged at 4 000 rpm for 10 minutes at 4 °C. The supernatant was then taken as a serum and the serum was analyzed for serum alkaline phosphatase (SALP), serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate trans aminase (SGOT) in a bioanalyzer using commercial kits according to the manufacture protocol.

Statistical analysis

All values were expressed as mean \pm S.E.M (Standard Error of Mean). Statistical analysis was performed with the one way analysis of variance (ANOVA) followed by by Dunnett's 't' test using the SPSS statistical software of 14 version. $P < 0.05$ was considered to be statistically significant when compared with the control.

RESULTS AND DISCUSSION

The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites such as alkaloids, flavonoids, glycosides, phenols, saponins, sterols, and terpenoids (Satheesh et al., 2012). The preliminary phytochemical screening of EEFM showed the presence of terpenoids and flavonoids (Table 1).

In the brine shrimp lethality bioassay, EEFM showed potent cytotoxicity against brine shrimp nauplii (*Artemia salina*) with LC_{50} of 3.06 μ g/mL, whereas ampicillin trihydrate showed LC_{50} of 7.21 μ g/mL. In the acute toxicity study, EEFM did not show any toxic effect up to the dose of 2 g/kg b.wt. Based on this observation, 50 and 100 mg/kg b.wt were taken as low and high doses of EEFM for the experiment.

In the case of EAC cell growth inhibition study, the EEFM treatment significantly reduced the viable cell count compared to those of EAC control mice. The effect of EEFM on EAC cell growth is shown in Fig. 1. The average number of viable tumour cells per mouse of the untreated EAC control group was found to be $(3.924 \pm 0.30) \times 10^7$ cells/mL. On the other hand, the average number of viable tumour cells per mouse treated with EEFM at the dose of 50 and 100 mg/kg body weight were $(2.060 \pm 0.17) \times 10^7$ and $(1.363 \pm 0.29) \times 10^7$ cells/mL, respectively. EEFM showed 47.5% and 65.2% EAC cell growth inhibition at the dose of 50 and 100 mg/kg body weight, respectively (Fig. 1).

The reliable criteria for evaluating an anticancer drug are prolongation of the lifespan of the animal. In this study, the animals of the EAC control group survived for a period of 21.7 ± 2.55 days, whereas it was 30.6 ± 4.91 and 36.4 ± 3.88 ($P < 0.05$) (Fig. 2) for the group treated with EEFM at 50 and 100 mg/kg, respectively. The increase in the life span of EAC cell bearing mice treated with EEFM (50 and 100 mg/kg) was found to be 41.0 and 67.7% (Fig. 2). Moreover, the average weight gain of the EEFM-treated group was significantly decreased in respect to the untreated EAC control (Fig. 3). The previous study in our laboratory (Habib et al., 2010) showed that bleomycin (0.3 mg/kg) significantly reduced viable EAC cells (92.37% cell growth

Table 1. Types of compounds present in EEFM

Sample	Solvent system	R_f value	Colour with vanillin- H_2SO_4	Possible compound
EEFM	n-Hexane : ethyl acetate (9 : 1)	0.38	Brown	Heterocyclic
		0.57	Yellow	Flavonoid
		0.82	Violet	Terpenoid
		0.94	Violet	Terpenoid

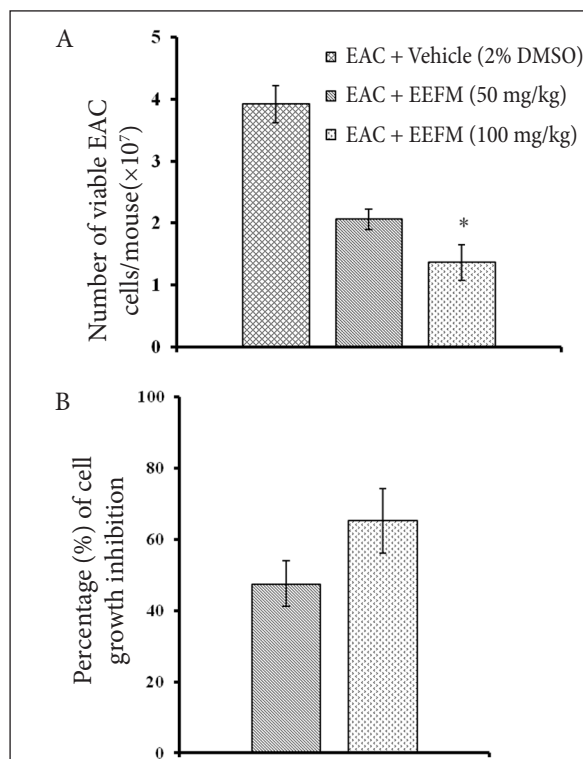


Fig. 1. Effect of EEFM and bleomycin on the EAC cell growth. **A:** Viable EAC cells on day 6 after tumor cell inoculation, **B:** % of cell growth inhibition; Data are expressed as mean \pm S.E.M (n = 6); *P < 0.05: Significance difference with respect to the EAC control

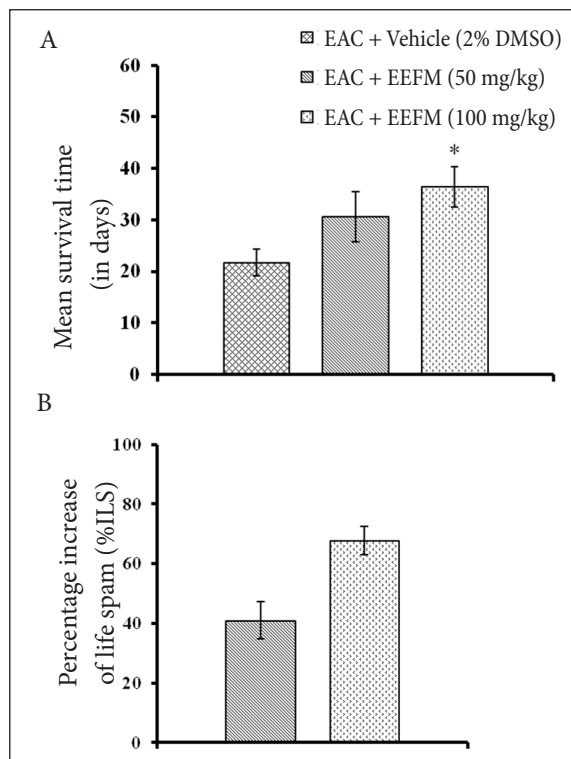


Fig. 2. Effect of EEFM on the survival time of EAC cell bearing mice. **A:** Mean survival time (MST), **B:** Percentage increase in life span (%ILS); Data are expressed as mean \pm S.E.M (n = 6); *P < 0.05: Significance difference with respect to the EAC control

inhibition) and increased the mean survival time (ILS% = 81.4) of EAC cell bearing mice. So the results of this study clearly demonstrate the inhibitory activity of EEFM against EAC.

The anemia encountered in tumour bearing mice is mainly due to reduction in RBC and hemoglobin and this may occur either due to iron deficiency or hemolytic or myelopathic conditions (Hogland, 1982). In addition, elevation in the activity of several serum enzymes was also clinically manifested in malignant conditions (Scott et al., 1986; Bacci et al., 1993; Sagman et al., 1991). This study showed that the haematological parameters of the EAC control group were changed significantly when compared to those of normal mice on day 12. The WBC count was found to be increased with a reduction in the haemoglobin and RBC count (Table 2). Treatment with EEFM at all doses restored the altered haemoglobin level, RBC

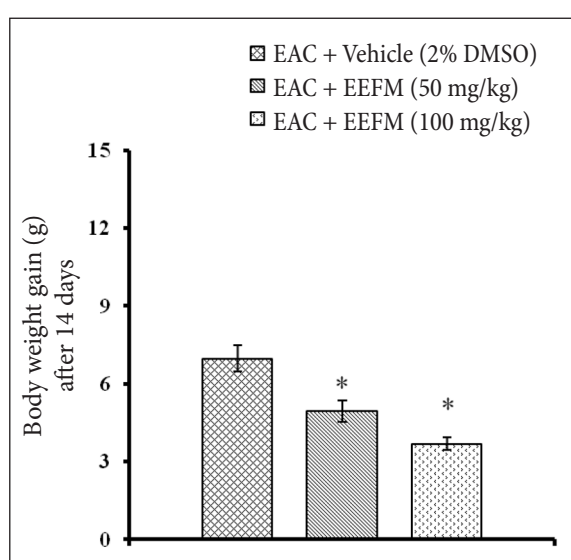


Fig. 3. Effect of EEFM on the body weight gain of EAC cell bearing mice after 14 days. Data are expressed as mean \pm S.E.M (n = 8); *P < 0.05: Significance difference with respect to the EAC control

Table 2. Effect of EEFM on hematological and biochemical parameters of EAC cell bearing mice

Parameters	Normal	EAC + Vehicle (2% DMSO)	EAC + EEFM (50 mg/kg)	EAC + EEFM (100 mg/kg)
Hgb (g/dL)	12.48 ± 0.22	8.50 ± 0.20 [*]	8.40 ± 0.11	9.33 ± 0.17 ^t
RBC (×10 ⁹ cells/mL)	5.67 ± 0.10	3.53 ± 0.15 [*]	3.56 ± 0.41	4.40 ± 0.13 ^t
WBC (×10 ⁶ cells/mL)	6.75 ± 0.23	12.0 ± 1.57 [*]	8.81 ± 1.15	4.66 ± 0.33 ^t
SALP (U/L)	124 ± 1.90	159 ± 1.54 [*]	164 ± 2.28	147 ± 1.08 ^t
SGPT (U/L)	45.0 ± 1.52	41 ± 1.10	53.0 ± 0.89	34 ± 0.52
SGOT (U/L)	43.7 ± 2.84	103 ± 3.68 [*]	102 ± 4.77	84 ± 2.46 ^t

Data are expressed as mean ± S.E.M. for six animals in each group. ^{*}P < 0.05: against the normal group, and ^tP < 0.05: against the EAC control group.

count, WBC count. Moreover, twelve (12) days of the inoculation with EAC brought the significant (P < 0.05) elevation in the activities of SALP and SGOT when compared with the respective normal values (Table 2). Significant (P < 0.05) depletion in the activities of SALP and SGOT was found by the treatment with EEFM (at 100 mg/kg). Therefore, this study showed that EEFM possesses a protective action on the complications induced by the malignant condition.

The preliminary phytochemical screening indicated the presence of terpenoids, flavonoids and heterocyclic compounds in EEFM. These compounds are known to possess potent antitumor properties (Kintzios, 2006; Viswanatha et al., 2010). In addition, flavonoids could also induce mechanisms that may kill cancer cells and inhibit tumor invasion (Lotito, Frei, 2006). On the other hand, it has been reported that the major constituents isolated from fruits of *Manilkara zapota* are polyphenols (methyl chlorogenate, dihydromyricetin, quercitrin, myricitrin, (+)-catechin, (-)-epicatechin, (+)-gallocatechin, and gallic acid (Ma et al., 2003). The inhibitory properties of EEFM against EAC may be due to the presence of these compounds.

CONCLUSIONS

In the light of the above observation, it can be concluded that fruits of *Manilkara zapota* have the potential anticancer activity and our study demonstrates that EEFM merit further investi-

gation to elucidate the mechanism of their inhibition against EAC as well as identify the main active phytochemicals responsible for the inhibition of EAC.

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***Manilkara zapota* (L.) VAISIŲ
PRIEŠVĖŽINIS AKTYVUMAS PRIEŠ PELIŲ
EHRLICHIO PILVO VANDENĖS KARCINOMĄ**

Santrauka

Ieškant potencialių antivėžinių medžiagų iš natūralių šaltinių, buvo tiriamas *Manilkara zapota* (L.)

vaisių etilacetato ekstrakto (EEFM) poveikis baltųjų pelių Ehrlichio pilvo vandenės karcinomai (EAC). Priešvėžinis EEFM aktyvumas prieš EAC buvo tikrinamas naudojant 50 ir 100 mg/kg dozes. Gydymas 100 mg/kg EEFM rodo patikimą atsparumo padidėjimą ir gyvybingų vėžinių ląstelių skaičiaus ir svorio sumažėjimą, taip pat buvo nustatyti geresni hematologiniai rodikliai: hemoglobino, RKK, BKK kiekis. Progresuojant vėžiui, pakitę biocheminiai rodikliai (SALP ir SGOT), paveikus peles 100 mg/kg EEFM, taip pat buvo patikimai atkurti. Tiriant jūrų krevečių letalumą, LD₅₀ citotoksiškumas *Artemia salina* nustatytas naudojant 3,06 µg/ml EEFM. Šio tyrimo rezultatai rodo, kad *in vivo* EEFM yra efektyvus EAC augimo inhibitorius.

Raktažodžiai: *Manilkara zapota*, vaisiai, priešvėžinis aktyvumas, Ehrlichio pilvo vandenės karcinoma