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# Inhibitory effect of a taraxasterol derivative of *Manilkara zapota* (L.) on Ehrlich ascites carcinoma

**M.Ismail Hossain, Ziasmin Khatun, M.Rowshanul Habib, M.Rezaul Karim***
**Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi-6205, (BANGLADESH)**
**E-mail: rezaplazad@yahoo.com**

## ABSTRACT

This study has described the first time isolation and identification of a taraxasterol derivative from the leaves of *Manilkara zapota* (L.) and its effect on Ehrlich Ascites Carcinoma (EAC). A phytochemical study on the leaves of *Manilkara zapota* using chromatographic techniques led to the first time isolation of 21α-Hydroxy-taraxasterol as compound-1. Its structure was confirmed by spectroscopic analyses (Mass and NMR). *In vivo*, the effect of compound-1 on Ehrlich Ascites Carcinoma was assessed by evaluating the viable tumour cell count, survival time, body weight gain due to tumour burden and heamatological (hemoglobin content, RBC and WBC count) parameters of EAC cell bearing mice. At 5 mg/kg body weight dose, compound-1 showed significant decrease in ($p<0.05$) viable cell count, weight gain and elevated the life span of EAC cell bearing mice. Altered hematological profile such as RBC, hemoglobin, WBC and differential count were reverted to normal level in compound-1 treated mice. Results of this study conclude that *in vivo* the compound-1 (i.e., 21α-Hydroxy-taraxast) has inhibitory activity against Ehrlich Ascites Carcinoma with improving in cancer induced complications.


## KEYWORDS

*Manilkara zapota*;
Leaves;
Ehrlich ascites carcinoma;
Taraxasterol derivative.

## INTRODUCTION

Plants, vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of drug discovery and development[1]. In recent years, there has been a global trend towards the use of natural phytochemicals present in natural products such as fruits, vegetables, and their extracts as a medicine[2]. So there is a growing interest in the phytochemical and biological evaluation of various plants used in traditional system of medicine. *Manilkara zapota* (L.) P. Royen (Family: Sapotaceae) is a small to medium evergreen tree and it is widely cultivated medicinal and food plant in Indian subcontinent including Bangladesh. In Bangladesh, the leaves of *Manilkara zapota* (L.) has been used as a traditional medicine for the remedies of cough, cold, dysentery and diarrhea[3]. In an earlier screening, the leave of this plant was shown to possess antimicrobial and antioxidant activities[4-6]. Seeds of *Manilkara zapota* (L.) have been used as aperients, diuretic tonic and febrifuge whereas stem bark is astringent and febrifuge[7,8]. In addition, our published

work has also showed the potent antitumour activity of stem bark of *Manilkara zapota* (L.)[9]. Previous phytochemical investigation on the leaves of this plant has resulted in the isolation of lupeol acetate, oleanolic acid, apigenin-7-O-α-L-rhamnoside, myricetin-3-O-α-L-rhamnoside and caffeic acid[10]. As a part of our research focused on bioactive compounds from *Manilkara zapota* plant, here we have reported the first isolation and identification of a taraxasterol derivative from the leaves of this plant and evaluated *in vivo* the effect of this derivative on Ehrlich ascites carcinoma (EAC).

## MATERIALS AND METHODS

### Chemicals

All the chemicals used in present study were obtained from Sigma-aldrich and Carl-Roth, Germany.

### Plant material

The leaves of *Manilkara zapota* (L.) (Family: Sapotaceae) were collected from Rajshahi district of Bangladesh in 2014 and identified by Professor A.T.M Naderuzzaman, Department of Botany, University of Rajshahi. A voucher specimen has been deposited under the accession number DACB-23801 at the Bangladesh National Herbarium

### Preparation of plant extracts and isolation procedure

The dried powdered leaves (950 g) of *Manilkara zapota* (L.) was extracted with ethyl acetate (2.0 L) at room temperature. The extracts were concentrated by rotary vacuum evaporator to give 12 g (1.26%) ethyl acetate extract of leaves of *Manilkara zapota* (L.). Then 6 g of ethyl acetate extract was chromatographed over silica gel (60-120 mesh) with a gradient of n-hexane-ethyl acetate to give sixty eight (68) fractions. Among these fractions, fraction 08-11 afforded 28 mg amorphous powder (designated as compound-1). The purity of the isolated compound was checked on TLC plates.

### General methods

High Resolution TOF 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 and NMR spectra were recorded on Bruker 400 MHz FT spectrometer (DPX-400, Switzarland). All the spectra were taken in Analytical Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR) Laboratories, Dhaka-1205, Bangladesh.

### Animals

Male Swiss albino mice (20-25 gm) were procured from the Animal Research Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR,B). They were used throughout the study and housed in iron cages in a controlled environment (temperature 25±2°C and 12h dark/light cycle) with standard laboratory diet and water *ad libitum*. 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 collected from the 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

The previously described method[11] was applied to determine the effect of compound-1 on EAC cell growth inhibition. $1.5 \times 10^5$ EAC cells were inoculated (i.p.) into 3 groups of mice (8 in each) on day 0. The group 1 was treated with vehicle (2% Dimethylsulfoxide; DMSO) and it was considered as untreated control. Mice in group 2 received compound-1 at 5 mg/kg dose whereas mice of group 3 were treated with bleomycin (0.3 mg/kg). 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 untreated control.

### Studies on survival time and hematological parameters

Swiss Albino mice were divided into four groups (n

= 8). All the animals were injected with EAC cells ($2x10^5$ cells/mouse) intraperitoneally except for the normal control group. This was taken as day 0. Group 1 served as the normal control and group 2 served as the untreated tumour control. These two groups received 2% DMSO. Group 3 and 4 were treated with compound-1 and bleomycin at 5 and 0.3 mg/kg body weight, respectively. All these treatment were given 24 h after the tumour inoculation, once daily for 12 days. On 14th day after EAC cell inoculation hematological parameters (Hemoglobin, total RBC and WBC) were measured from freely flowing tail vein blood of each mice of each group[12]. Then the animals of group 2, 3 and 4 were kept to check the survival time of EAC-tumor bearing mice. The survival time was expressed as mean survival time (MST) in days and percent increase of life span (%ILS) was calculated[11] using the formula below:

**% ILS = (MST of treated group / MST of control group – 1) x 100**

Where, MST = (" Survival time in days of each mouse in a group)/Total number of mice

## Statistical analysis

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

## RESULTS AND DISCUSSION

## Characterization and identification of compound-1

Isolated and purified compound-1 was characterized by its mass and NMR spectral data. Molecular formula for compound-1 was deduced as $C_{30}H_{50}O_2$ through analysis of its mass spectrum which showed the molecular ion ($M^+$) peak at m/z 442.38 (Calcd for $C_{30}H_{50}O_2$). The $^1$H-NMR spectrum of compound-1 showed singlets of six tertiary methyl with the integration of three protons each at δ 0.97, 0.77, 0.83, 1.01, 0.89 and 0.78 whereas one doublet of one tertiary methyl with the integration of three protons was also observed at 1.24 (3H, d, *J* = 7.0 Hz, H-29) (TABLE 1). A double-doublet of one proton integration at δ 3.26 (*J* = 11.2 & 4.9 Hz) was due to H-3α. A secondary carbinolic methine proton resonating at δ 5.47 (dd, *J* = 4.9 & 8.9 Hz) was appeared which corresponds to H-21 (TABLE 1). $^1$H-NMR spectrum of compound-1 also showed two olefinic proton signals

**TABLE 1 : $^1$H- and $^{13}$C-NMR spectral data of compound-1 (i.e., 21α-Hydroxy-taraxasterol)**

| Compound-1 | | |
|---|---|---|
| **Carbon Number** | **$\delta_C$** | **$\delta_H$** |
| 1 | 38.4 | 1.55 (2H, m, H-1) |
| 2 | 28.4 | 1.61 (2H, m, H-2) |
| 3 | 89.1 | 3.26 (1H, dd, *J*ax.ax =11.2 Hz, *J*ax.eq = 4.9 Hz, H-3) |
| 4 | 39.1 | - |
| 5 | 56.5 | 0.63 (1H, bd, *J* = 9.0 Hz, H-5) |
| 6 | 17.9 | 1.38 (2H, m, H-6) |
| 7 | 34.1 | 1.40 (2H, m, H-7) |
| 8 | 41.6 | - |
| 9 | 51.2 | 1.29 (1H, m, H-9) |
| 10 | 37.0 | - |
| 11 | 21.7 | 1.54 (2H, m, H-11) |
| 12 | 26.3 | 1.63 (2H, m, H-12) |
| 13 | 39.1 | 1.57 (1H, m, H-13) |
| 14 | 41.7 | - |
| 15 | 29.2 | 1.69 (2H, m, H-15) |
| 16 | 37.9 | 1.31 (2H, m, H-16) |
| 17 | 34.0 | - |
| 18 | 49.6 | 1.18 (1H, d, *J* = 7.0 Hz, H-18) |
| 19 | 38.1 | 1.97 (1H, dq, *J* = 7.0 & 7.1 Hz, H-19) |
| 20 | 156.6 | - |
| 21 | 81.4 | 5.47 (1H, dd, *J* = 4.9 & 8.9 Hz, H-21) |
| 22 | 49.1 | 1.95 (2H, dd, *J* = 9.1 & 13.8 Hz, H-22) |
| 23 | 30.2 | 0.97 (3H, s, H-23) |
| 24 | 15.8 | 0.77 (3H, s, H-24) |
| 25 | 17.9 | 0.83 (3H, s, H-25) |
| 26 | 16.6 | 1.01 (3H, s, H-26) |
| 27 | 19.0 | 0.89 (3H, s, H-27) |
| 28 | 21.7 | 0.78 (3H, s, H-28) |
| 29 | 33.5 | 1.24 (3H, d, *J* = 7.0 Hz, H-29) |
| 30 | 113.2 | 4.29 (1H, d, *J* = 1.4 Hz, H-30); 4.42 (1H, s, H-30) |
| -O-H (C-3) | - | 2.04 (1H, s) |
| -O-H (C-21) | - | 2.00 (1H, s) |

**Proton resonance integral, multiplicity, and coupling constant (*J* = Hz) are in parentheses**

at 4.29 (1H, d, *J* = 1.4 Hz, H-30) and 4.42 (1H, s, H-30).

The $^{13}$C-NMR spectrum of compound-1 showed the presence of 30 signals, which were resolved through DEPT experiments as seven methyl, ten methylene, seven methine and six quaternary carbons (TABLE 1). The $^{13}$C-NMR of compound-1 also showed two olefinic carbons at δ 113.2 (C-30) and δ 156.6 (C-20). Based on the foregoing observations and a comparison of the data with the literature[13], the structure of compound-1 was confirmed as 21α-Hydroxy-taraxasterol (Figure 1). Isolation of 21α-Hydroxy-taraxasterol is reported for the first time from *Manilkara zapota*.

## Effect of compound-1 on cell growth and survival time

In the study, the average number of viable EAC cells per mouse and MST of untreated control group were found to be (1.214 ± 0.13) x $10^7$ cells/mL and 21.2 ± 2.7 days, respectively. Treatment with compound-1 (5 mg/kg) significantly ($p<0.05$) reduced the viable EAC cells and improved survival period as compared to that of untreated control (Figure 2 and 3). The percentage (%) increase in the life span of EAC cell bearing mice treated with compound-1 and bleomycin was found to be 40.56 and 95.28% at 5 and 0.03 mg/kg doses, respectively (Figure 3). Moreover, treatment with compound-1 (5 mg/kg) and bleomycin (0.03 mg/kg) significantly inhibited the body weight gain when compared to the untreated control (Figure 4). The reliable criterion

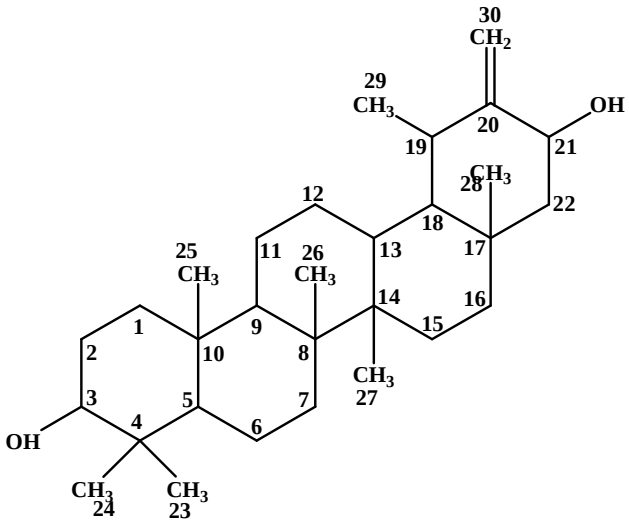


**Figure 1 : Chemical structure of 21α-Hydroxy-taraxasterol (compound-1)**

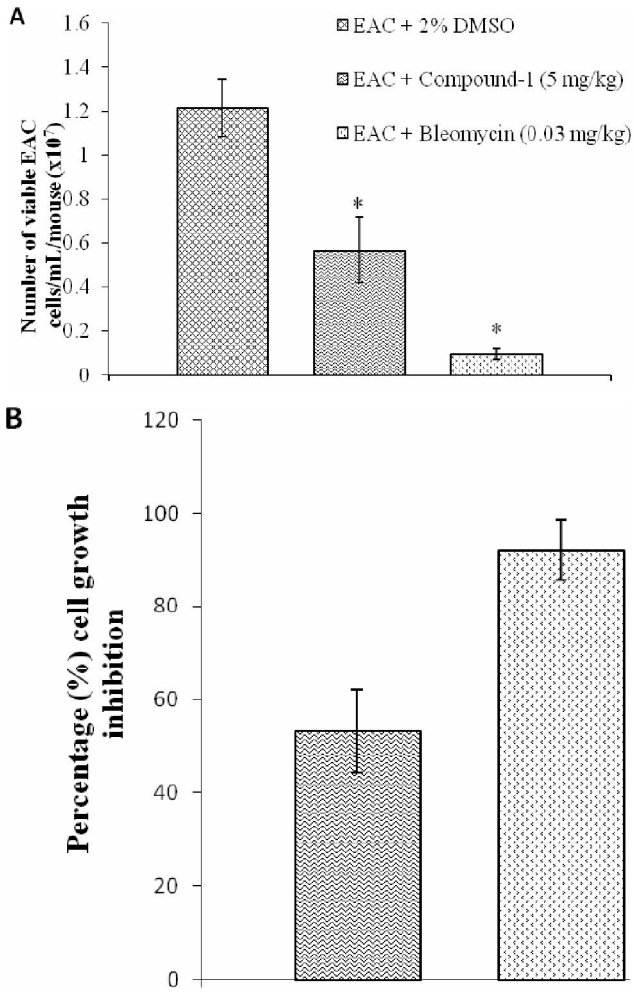


**Figure 2 : Effect of compound-1 and bleomycin on EAC cell growth. A: Viable EAC cells on day 6 after tumor cell inoculation, B: % of cell growth inhibition; Data are expressed as mean ± S.E.M (n = 8); *P<0.05 :Significance difference with respect to EAC control**

for judging the value of any anticancer drug is the prolongation of life span of the animals[14] and compound-1 was able to meet this criterion.

However, our previous study[15] showed that treatment with ethyl acetate extract of leaves of *Manilkara zapota* decreased the viable tumor cell count and body weight gain due to tumor burden as well as increased the average life span of animals when compared to the untreated EAC control. So as a constituent, compound-1 is one of contributors in the antineoplastic activity of ethyl acetate extract of *Manilkara zapota* leaves.

## Effect of compound-1 on heamatological parameters of EAC cell bearing mice

In this study, inoculation with EAC cells for 14 days

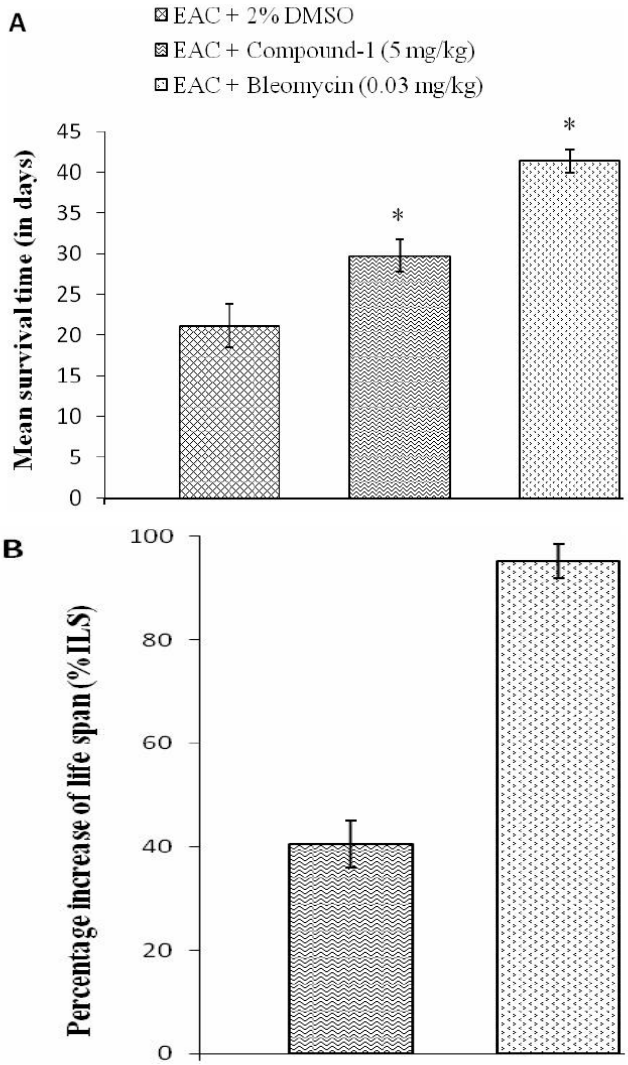


**Figure 3 : Effect of compound-1 and bleomycin on survival time of EAC cell bearing mice. A: Mean survival time (MST), B: Percentage increase in life span (%ILS); Data are expressed as mean ± S.E.M (n = 8); *P<0.05: Significance difference with respect to EAC control**

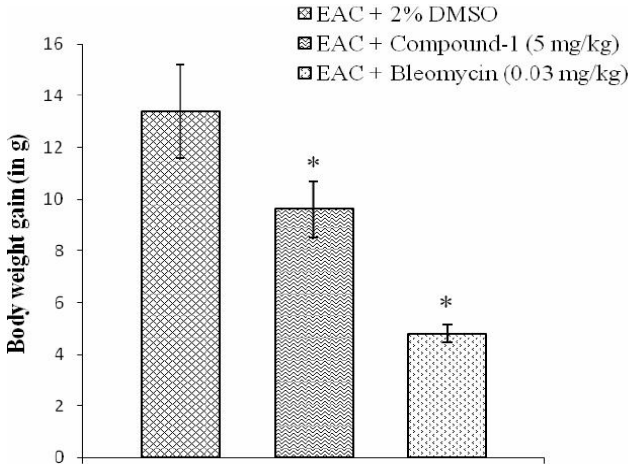


**Figure 4 : Effect of compound-1 and bleomycin on body weight gain of EAC cell bearing mice after 15 days. Data are expressed as mean ± S.E.M (n = 8); *P<0.05: Significance difference with respect to EAC control**

significantly (P<0.05) altered hematological parameters of untreated tumor control when compared to normal mice (TABLE 2). The total WBC count was found to increase with a reduction in the hemoglobin content, and total RBC count. At the same time interval, the intraperitoneal administration of compound-1 (5 mg/kg) and bleomycin (0.03 mg/kg) restored the altered WBC, RBC and hemoglobin content near to normal (TABLE 2). In cancer chemotherapy, myelosuppression and anemia are observed as the major problems[16,17]. In tumour bearing mice, the anemia is mainly encountered due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions[18]. This study showed that, compound-1 possesses protective action on the heamopoietic system.

**TABLE 2 : Effect of compound-1 and bleomycin on hematological parameters of EAC cell bearing mice**

| Treatment | Hb (g/dL) | RBC ($x10^9$cells/mL) | WBC ($x10^6$cells/mL) |
|---|---|---|---|
| Normal + 2% (v/v) DMSO | 14.1 ± 1.5 | 5.7 ± 0.6 | 7.8 ± 2.8 |
| EAC + 2% (v/v) DMSO | 7.2 ± 0.8* | 2.3 ± 0.2* | 25.5 ± 3.1* |
| EAC + Compound-1 (5 mg/kg) | 9.8 ± 1.0a | 3.1 ± 0.2 a | 13.6 ± 1.6a |
| EAC + Bleomycin (0.03 mg/kg) | 13.7 ± 0.9a | 3.1 ± 0.4a | 9.5 ± 0.7a |

**Data are expressed as mean ± S.E.M. for eight animals in each group. *P<0.05: against normal group and aP<0.05: against EAC control group**

## CONCLUSION

The results of this study showed an inhibitory effect of compound-1 (i.e., 21α-Hydroxy-taraxasterol) against EAC in Swiss albino mice. So this compound has the merit as lead compound to conduct research on structural modification and structure–activity relationship

for the development of new and more active agents.

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