Antiproliferative and hepatoprotective activity of metabolites from *Corynebacterium xerosis* against Ehrlich Ascites Carcinoma cells

Farhadul Islam, Soby Ghosh, Jahan Ara Khanam*

Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi 6205, Bangladesh

PEER REVIEW

Peer reviewer
Dr. Zahangir Alam Saud, Professor, Department of Biochemistry and Molecular Biology, University of Rajshahi, Bangladesh.
E−mail: zasaud@yahoo.com

Comments
This is an excellent and well designed research work in which the researchers have explore the anticancer and hepatoprotective activity of metabolites from *C. xerosis* against EAC cells in Swiss albino mice. Cell growth inhibition, survival time, tumor weight and hematological parameters were studied to assess its anticancer activity. Biochemical and histopathology of mice organ were studied for its hepatoprotective potential. It was found from the study that the metabolites have promising anticancer and hepatoprotective activity in mice model.
Details on Page S291

ABSTRACT

Objective: To find out the effective anticancer drugs from bacterial products, petroleum ether extract of *Corynebacterium xerosis*.
Methods: Antiproliferative activity of the metabolite has been measured by monitoring the parameters like tumor weight measurement, tumor cell growth inhibition in mice and survival time of tumor bearing mice, *etc*. Hepatoprotective effect of the metabolites was determined by observing biochemical, hematological parameters.
Results: It has been found that the petroleum ether extract bacterial metabolite significantly decrease cell growth (78.58%; *P*<0.01), tumor weight (36.04 %; *P*<0.01) and increase the life span of tumor bearing mice (69.23%; *P*<0.01) at dose 100 mg/kg (*i.p.*) in comparison to those of untreated Ehrlich ascites carcinoma (EAC) bearing mice. The metabolite also alters the depleted hematological parameters like red blood cell, white blood cell, hemoglobin (Hb%), *etc*. towards normal in tumor bearing mice. Metabolite show no adverse effect on liver functions regarding blood glucose, serum alkaline phosphatases, glutamic pyruvic transaminase, glutamic oxaloacetic transaminase activity and serum billirubin, *etc*. in normal mice. Histopathological observation of these mice organ does not show any toxic effect on cellular structure. But in the case of EAC bearing untreated mice these hematological and biochemical parameters deteriorate extremely with time whereas petroleum ether extract bacterial metabolite receiving EAC bearing mice nullified the toxicity induced by EAC cells.
Conclusion: Study results reveal that metabolite possesses significant antiproliferative and hepatoprotective effect against EAC cells.

1. Introduction

Cancer is a non−communicable disease and continues to represent the second largest cause of mortality in the world. The prevention and control of cancer in developed and developing countries deserve urgent attention because it is expected to be double in the next 20 to 25 years[1]. Research in the field of cancer eradication is going on all over the world and a number of clinically used drugs/methods have already been developed but most of them were ineffective against devastating cancer due to divergences of cancer. During the past decade, cancer drugs discovery or development is based on the single−target, single−compound paradigm. These conventional treatments modalities are self limited due to their major side effects and it prompted to development of many new approaches for the treatment of cancer and this concept contrasts with a more recent drug design approach, where a single drug can target with high efficacy multiple steps in cancer progression. Such second−generation drugs are often much more efficacious with little side effects. One such example involves the use of live, attenuated bacteria or their purified products or secondary metabolites. The mode

 *Corresponding author: Jahan Ara Khanam, Professor, Department of Biochemistry and Molecular Biology, Rajshahi University, Rajshahi 6205, Bangladesh.
   Tel: +88−0721−750180
   Fax : +880−721−750064
   E−mail: jahanara_khanam@yahoo.com

of action of some strains is through the production of cytotoxic factors, enzymes, antibiotics and others secondary metabolites that can be used or adapted in a proper manner to specifically target cancer cells. Interestingly, bacteria naturally produce several chemokine/adhesion receptor inhibitors, so this appears as a new appealing approach as an anticancer therapy such as superantigen produced by *Staphylococcus aureus* showed anticancer activity against different types of cancer by interacting with cell surface receptors[2,3]. Bacterial enzymes arginine deaminase of *Mycoplasma arginini* act as candidate therapeutic agents for cancer treatment and show potential antitumor activity against different cancer *in vivo* and *in vitro* as well as inhibit the growth of HIV and hepatitis C virus by depletion of arginine[4,5]. Exotoxin A–immunotoxins kill cancer cells by binding specifically to overexpress cell–surface receptors, where they arrest protein synthesis and induce apoptosis and showed positive result against leukemia and bladder cancer in clinical trial[6–8]. Microbial secondary metabolites, such as manumycin A (fernesyl trasferase) and gliotoxin showed both *in vitro* cytotoxic activities against several cell lines and *in vivo* against human cancer xenografted models[9,10]. Prodiginines are bacterial secondary metabolites has been shown anticancer activity in several cancer derived cell lines and also *in vivo* in mice with a xenografted liver cancer by inducing apoptosis[11]. Epothilones were discovered as cytotoxic metabolites in the myxobacterium *Sorangium cellulosum*. They show anti–tumor activity in cancer cell lines inducing its polymerization and stabilization which causes cell cycle arrest at the G_2/M transition and apoptosis[12].

Microbial–based therapy of cancer is one of the emerging cancer treatment modalities. Over the past few years, important advances have been made to study and develop bacterial products with cancer killing ability. Attention has also recently been directed to the use of bacterial products that can enter preferentially to cancer cells and disrupt their growth or kill them by multiple mechanisms. We report experimental tumor mouse model as it have great importance in developing potential drugs using Ehrlich ascites carcinoma (EAC) because EAC cell is one of the commonest tumors which is referred to as an undifferentiated carcinoma and is originally hyperdiploid, has high transplantable capability, no–regression, rapid proliferation, shorter life span, 100% malignancy and resembles to human tumors which are the most sensitive to chemotherapy[13]. Anticancer activity of secondary metabolites extracted from cultured bacteria against EAC cells was investigated to find out effective host friendly anticancer drugs.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and reagents used throughout the investigation were of reagent grade and from BDH, England, E'MERK, Germany and Sigma Aldrich, USA.

2.2. Test animals

Adult male Swiss albino mice, six to eight weeks old [(25± 5) g body weight] were collected from animal resource branch of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR'B) and used throughout the studies. Animals were housed in polypropylene cages containing sterile paddy husk as bedding material under hygienic conditions with a maximum of ten animals in a cage. They were maintained under controlled conditions (12:12 h light–dark), temperature (22±5) °C. The mice were fed with standard mice food–pellets (collected from ICDDR'B) and water was given in *ad libitum*.

2.3. Cell lines

EAC cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as ascites tumour in swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6 cells/mouse.

2.4. Ethical clearance

Protocol used in this study for the use of mice as animal model for cancer research was approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) for Experimentations on Animal, Human, Microbes and Living Natural Sources (225/320–IAMEBBC/IBSc), Institute of Biological Sciences, University of Rajshahi, Bangladesh.

2.5. Identification of bacteria and extraction of metabolites

The isolated cellulytic soil bacteria were identified by their colony morphology, staining characters, pigment production, motility and other relevant biochemical test as per standard methods[14,15]. The isolated pure bacteria were cultured in broth media at 37 °C for 72 h and then centrifuge the media at 8 000 r/min for 10 min. The supernatant was collected and the secondary metabolites produced by the bacteria were extracted with petroleum ether. The solvent was evaporated by rotary evaporator and the semisolid metabolites designated as petroleum ether bacterial metabolites (PEBM), preserved in decicator for further use.

2.6. Determination of median lethal dose (LD_{50})

The bacterial metabolites (PEBM) was dissolved in distilled water and were injected intraperitoneally to eight groups of mice ($n=4$) at different doses of [100 mg/kg (*i.p.*), 400 mg/kg (*i.p.*), 800 mg/kg (*i.p.*), 1 200 mg/kg (*i.p.*), 1 600 mg/kg (*i.p.*), 1 800 mg/kg (*i.p.*), 2 000 mg/kg (*i.p.*) 2 100 mg/kg (*i.p.*)]. The LD_{50} value was then estimated by the procedure as described in

the literature[16].

2.7. Brine shrimp lethality bioassay

Cytotoxicity of the metabolites was screened against *Artemia salina* in a 1–day *in vivo* assay according to published protocol[17]. For the experiment 3 mg of the metabolite was dissolved in 0.6 mL (600 µL) of distilled water to get a concentration of 5 µg/µL and by serial dilution technique, solutions of varying concentrations such as 5, 10, 20, 40, 80 and 100.0 µg/mL were obtained. After 24 h. of incubation, the percentage of mortality of the nauplii was calculated for each concentration and the LC_{50} value was determined using Probit analysis as described in the literature.

2.8. Determination of cell growth inhibition (in vivo)

To determine the cell growth inhibition[18] of the metabolites, five groups of Swiss albino mice (*n*=6) were used. For therapeutic evaluation $136×10^4$ EAC cells in every mouse were inoculated on day 0. Treatments were started after 24 h of tumor inoculation and continued for 5 d. Group one to three received the metabolite at the doses of 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*), and 100 mg/kg (*i.p.*). Group four received bleomycin at the dose of 0.3 mg/kg/day[19] (*i.p.*) and group five was used as control. Mice in each group were sacrificed on day six and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypen blue and then counted by a haemocytometer. Total number of viable cells in every animal of the treated groups was compared with those of control (EAC treated only) group.

2.9. Bioassay of EAC cells

In this experiment[20] three groups of mice (*n*=6) were inoculated with $115×10^5$ EAC cells. Group 1 and 2 was treated with the bacterial metabolites and bleomycin at the dose of 100 and 0.3 mg/kg (*i.p.*) respectively for five consecutive days and group 3 served as control. On day 7, tumor cells from the mice were harvested in cold (0.9%) saline, pooled, centrifuged and re–inoculated ($2×10^5$ cells/mice) into 3 fresh groups of mice (*n*=6) as before. No further treatment was done on these mice. On day 5, they were sacrificed and viable tumor cells count/mice were performed.

2.10. Determination of average tumor weight and survival time

For this determination, a brief description of the method[21] used is given bellow; five groups of Swiss albino mice (6 in each group) were used. For therapeutic evaluation $136×10^4$ EAC cells per mouse were inoculated in to each group of mice on day 0. Treatment was started after 24 h of tumor cell inoculation and continued for 10 d. Tumor growth

were monitored by recording daily weight change and host survival was recorded and expressed as mean survival time in days and percent increase of life span was calculated by using the following formula:

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time (days) of each mouse in a group}}{\text{Total number of mice}}$$

$$\text{Percent increase of life span (ILS) \%} = \left\{ \frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right\} × 100$$

2.11. Monitoring of the hematological profile

To assess the effects of PEBM on the hematological parameters like WBC, RBC, Hb content, *etc.* were determined by the standard methods[22] using cell dilution fluids and hemocytometer. For this purpose, four groups (*n*=24) of mice were taken. Group 1, normal mice (without any treatment), group 2 EAC bearing control mice (only EAC treated), group 3 and group 4, normal mice treated with PEBM and EAC bearing mice treated with PEBM at 100 mg/kg/d (*i.p.*) respectively. Blood was collected from six mice of each group on day 5, 10, 15 and 25 by tail puncture in anticoagulant containing tube.

2.12. Measurement of biochemical parameters

The parameters such as serum GPT (glutamic pyruvic transaminase), GOT (glutamic oxaloacetic transaminase), ALP (alkaline phosphatases), serum glucose, cholesterol, urea, billirubin, triglyceride, *etc.* were determined for both normal and EAC bearing mice. For this experiment, on day 5, 10, 15 and 25, six mice from each group were sacrificed. Blood was collected from heart in plastic centrifuge tubes. These were then allowed to clot at room temperature for half an hour and centrifuged at 4 000 r/min for 15 min using a WIFUNG centrifuge LABOR–50M. The clear straw colored serum was then collected from the upper part of the tubes in vials with a micro–pipette. All the parameters were determined according to the procedures[23] established earlier.

2.13. Effect of metabolites on normal peritoneal cells

Effects of PEBM on normal peritoneal cells were determined[20] by counting total peritoneal cells and number of macrophages. Normal mice (6 in each group) were treated with PEBM (*i.p.*) at the dose of 25 mg/kg, 50 mg/kg and 100 mg/kg for three consecutive days. The untreated group was used as control. After 24 h of last treatment animals were sacrificed after injecting 5 mL of normal saline (0.98%) into peritoneal cavity of each mouse. Intraperitoneal exuded cells and number of macrophages were counted with 1% neutral red by haemocytometer.

2.14. Histopathology

The major body organs like brain, liver, kidney, heart, lung

and spleen, were collected from the experimental animals on 15th day and processed by standard methods[23] to prepare slides of tissues by hematoxylin and eosin staining. The slides were viewed under Motic Advanced system microscope (B, series) with the help of Motic J. 1 software in a Macintosh computer. PEBM induced hepatotoxicity as well as toxicity on other organs was observed.

2.15. Statistical analysis

The experimental results have been expressed as the mean ±SEM (Standard Error of Mean). Data have been calculated by one way ANOVA followed by Dunnett 't' test using statistical package for social science (SPSS) software of 10 version.

3. Results

3.1. Median lethal dose (LD$_{50}$)

The LD$_{50}$ value of the metabolites from *Corynebacterium xerosis* was found to be 1050 mg/kg (*i.p.*) for intraperitoneal treatment in male Swiss albino mice. The experimental animal showed toxicity at this dose regarding body weight or general appearance.

3.2. Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was done to assess the *in vitro* cytotoxic effect of the metabolites. Medium lethal concentration (LC$_{50}$) of brine shrimp lethality was found to be 22.49 μg/mL and percent of mortality of nauplii were increases with the concentration of PEBM (Figure 1).
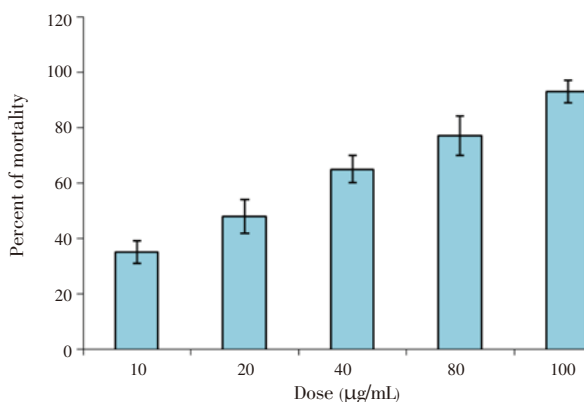
Figure 1. Effects of PEBM on the mortality of brine shrimp nauplii.

3.3. Cell growth inhibition (in vivo)

Maximum cell growth inhibition was found with the treatment of metabolites at the doses 100 mg/kg (*i.p.*) and 50 mg/kg (*i.p.*), as evident from 78.56% and 66.5% reduction of tumor cells respectively whereas treatment with *bleomycin* at dose 0.3 mg/kg showed 89.57% cell growth inhibition (Figure 2)
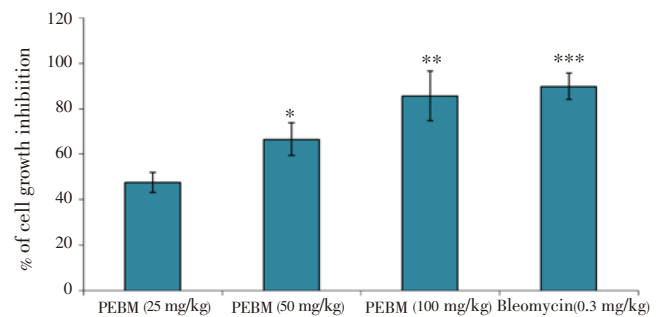
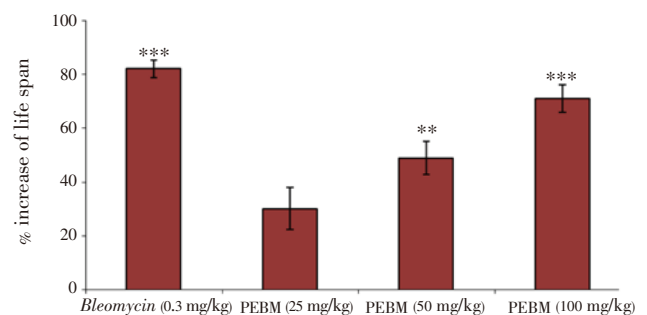**Figure 2.** Effects of PEBM on EAC cell growth inhibition.
Results are shown as mean±SEM, where significant values are *P<0.05, **P<0.01 and ***P<0.001 when compared with control (EAC bearing only).

3.4. Bioassay of EAC cells

Transplantability of EAC cell treated with PEBM was found to be decreased remarkably as 72.4% reduction in EAC cell growth was observed when 5 d treated (at the dose 100 mg/kg *i.p.*) EAC cells were re−inoculated into fresh mice and sacrificed on day 5.

3.5. Tumor weight and survival time

An effective chemo−preventive anti−cancerous drug shows a significant increase on survival time of EAC cell bearing mice. The effect of metabolites on survival time at different doses was summarized in Figure 3. It has been observed that tumor bearing mice treated with the PEBM at doses 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*), and 100 mg/kg (*i.p.*) resulted in, increase of life span significantly, when compared to that of control mice. Thus the survival time was found to be increased when the dose of the metabolites was increased. *Bleomycin* increased life span by 82.00% when compared to control. It was observed that % of increase of life span of mice at dose of 100 mg/kg (*i.p.*) of the test metabolite is quite comparable (69.23% *P*<0.01) to that of standard anticancer agent *bleomycin* (0.3 mg/kg).
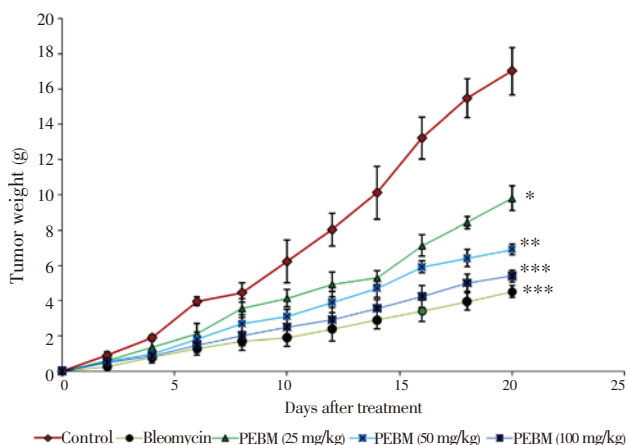

Figure 3. Effects of PEBM on survival time of tumor bearing mice
Results are shown as mean±SEM, where significant values are *P<0.05, **P<0.01 and ***P<0.001 when compared with control (EAC bearing only).

Effect of bacterial metabolites at the doses of 25 mg/kg, 50 mg/kg, 100 mg/kg and the antitumor drug *bleomycin* (0.3 mg/kg) on average tumor weight is shown in Figure 4. Treatment of the animals with PEBM, previously inoculated with EAC cells, resulted in the inhibition of tumor growth. In the case

of control (EAC bearing) group, the body weight was increased by 81.22% in 20 d when compared to the normal. Mice treated with bacterial extract at the doses of 25 mg/kg (*i.p.*), 50 mg/kg (*i.p.*) and 100 mg/kg (*i.p.*) the body weight increased only by 47.34%, 36.85%, 28.44%, respectively on 20 d.

Figure 4. Effects of PEBM on tumor weight of EAC bearing mice.
Results are shown as mean±SEM, where significant values are $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ when compared with control (EAC bearing only).

3.6. Effect of PEBM on hematological parameters

The hematological parameters like RBC, WBC, Hb, *etc* of both treated and non–treated mice were examined. For normal mice receiving PEBM at 100 mg/kg/day, all the examined parameters were found to be slightly changed during the treatment period from normal values. After 25 d of the initial treatment they were restored to almost normal values. In the case of parallel treatment of EAC bearing mice, these parameters were found to be significantly deteriorated as compared to those of the normal mice due to tumorogenesis. But these deteriorated parameters reverse towards normal values when treated with PEBM at dose 100 mg/kg (*i.p.*). All the experimental data are presented in Table 1.

Table 1
Effects of PEBM on hematological parameters in normal and experimental mice.

Name of Exp.	Days	RBC Cells/mL	WBC Cells/mL	% of Hb (g/dL)
Normal mice	0	$(6.80\pm0.67)\times10^9$	$(9.45\pm1.61)\times10^6$	11.00 ± 1.20
Normal+PEBM	5	$(4.69\pm0.27)\times10^{9a}$	$(6.97\pm0.85)\times10^{6c}$	5.80 ± 0.32^c
	10	$(5.14\pm0.75)\times10^{9b}$	$(7.12\pm1.00)\times10^{6b}$	6.88 ± 0.91^c
	15	$(5.71\pm0.22)\times10^9$	$(10.30\pm2.70)\times10^6$	5.50 ± 2.10
	25	$(5.85\pm0.41)\times10^9$	$(9.75\pm1.20)\times10^{6b}$	12.00 ± 1.10
EAC control	5	$(4.20\pm0.31)\times10^9$	$(13.00\pm0.34)\times10^6$	9.10 ± 0.11
	10	$(3.80\pm1.40)\times10^9$	$(15.00\pm1.30)\times10^6$	7.70 ± 0.36
	15	$(3.20\pm0.14)\times10^9$	$(17.00\pm0.31)\times10^6$	5.60 ± 0.40
	25	$(2.32\pm0.20)\times10^{9*}$	$(25.70\pm0.40)\times10^{6*}$	$4.00\pm1.00^{**}$
EAC+ PEBM	5	$(3.80\pm1.10)\times10^9$	$(15.80\pm3.10)\times10^6$	6.70 ± 2.10
	10	$(4.10\pm1.40)\times10^9$	$(14.00\pm1.30)\times10^6$	7.20 ± 1.30
	15	$(4.30\pm1.20)\times10^9$	$(11.00\pm3.40)\times10^6$	7.40 ± 1.10
	25	$(5.70\pm1.20)\times10^9$	$(10.90\pm2.70)\times10^6$	8.10 ± 2.30

Results are shown as mean±SEM, where significant values are $^aP<0.05$, $^bP<0.01$ and $^cP<0.001$ when PEBM treated mice compared with normal mice and $^*P<0.05$ and $^{**}P<0.01$ when (EAC+PEBM) treated mice compared with EAC bearing controlmice (EAC bearing only).
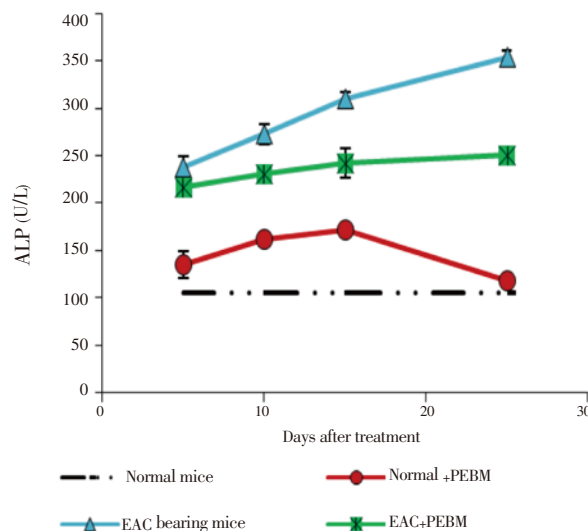
3.7. Effect of PEBM on enzymes

Effects of the metabolites on the enzyme activities like GPT, GOT and ALP have been presented in Figure 5–7. With normal mice, activities of these enzymes were found to be moderately increased during the treatment period (14 consecutive days at dose 100 mg/kg/day *i.p.*), after which these values were gradually return to the more or less normal level. For EAC bearing untreated mice, all such values increased almost linearly with time while EAC bearing mice treated with PEBM however, limit the increment of GPT, GOT and ALP values remarkably. After treatment, the GPT values returned to normal levels with time (Figure 5–7) while the ALP values reduced effectively (Figure 5 and 6). In the case of GOT, the test metabolite partially reduced the rate of its increment (Figure 7).
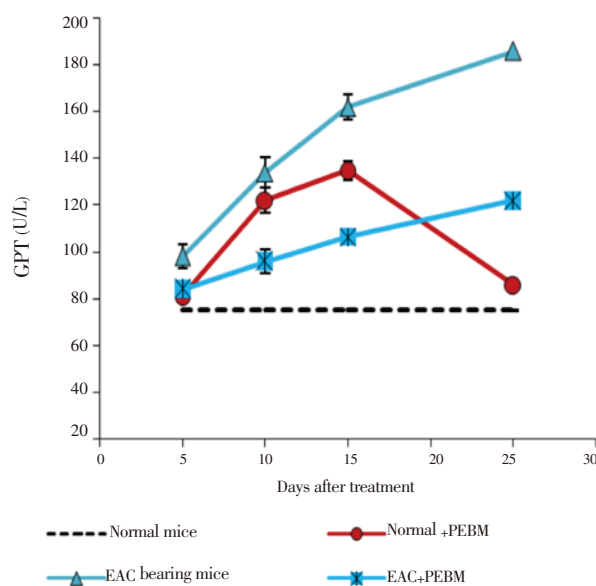
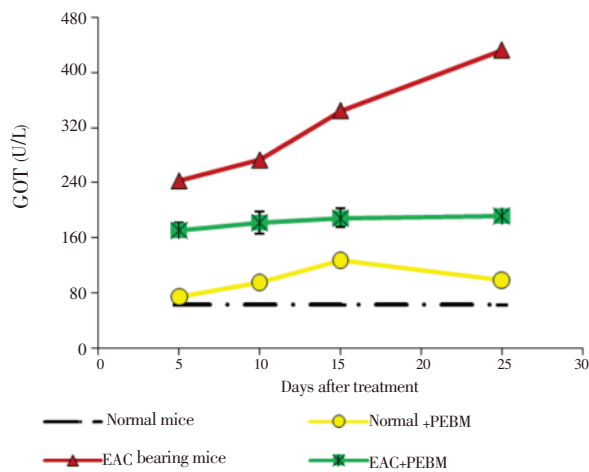**Figure 5.** Effects of PEBM on SGPT of experimental mice.
Results are shown as mean±SEM.

Figure 6. Effects of PEBM on SALP of experimental mice.
Results are shown as mean±SEM.

Figure 7. Effects of PEBM on SGOT of experimental mice.
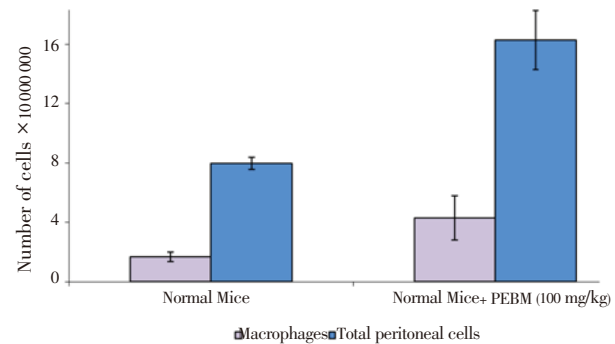Results are shown as mean±SEM.

3.8. Effect of PEBM on biochemical parameters

Table 2 shows the effects of PEBM on serum biomolecules such as glucose, bilirubin, cholesterol, urea, triglyceride and creatinine content of both normal and EAC bearing mice. All these parameters except glucose were increase in both normal and EAC bearing mice but they regain to its normal range in PEBM treated normal mice after the treatment. This values return back to more or less normal level in EAC bearing mice treated with PEBM. The glucose content was found to be increased to some extent during the treatment period in normal mice, after the treatment period, it slowly reversed back towards normal level. For EAC bearing mice, the glucose content was found to be reduced abruptly from normal level due to hypoglycemic effects of EAC cells. The supplementation of PEBM increased the glucose value close to normal level.

3.9. Effect of PEBM on peritoneal cells

The average number of peritoneal exudates cells of normal

mice treated with the metabolite were found to be $(20.0\pm 1.7)\times 10^6$ of which the macrophage counts were $(5.06\pm 0.15)\times 10^6$. Treatment with PEBM at dose 100 mg/kg/day for three consecutive days significantly enhanced the number of macrophages. Results are shown in Figure 8.

Figure 8. Effects of PEBM on enhancement of peritoneal cells.
Results are shown as mean±SEM, where significant values are [**]$P<0.01$ and [***] $P<0.001$ when compared with control (normal mice).
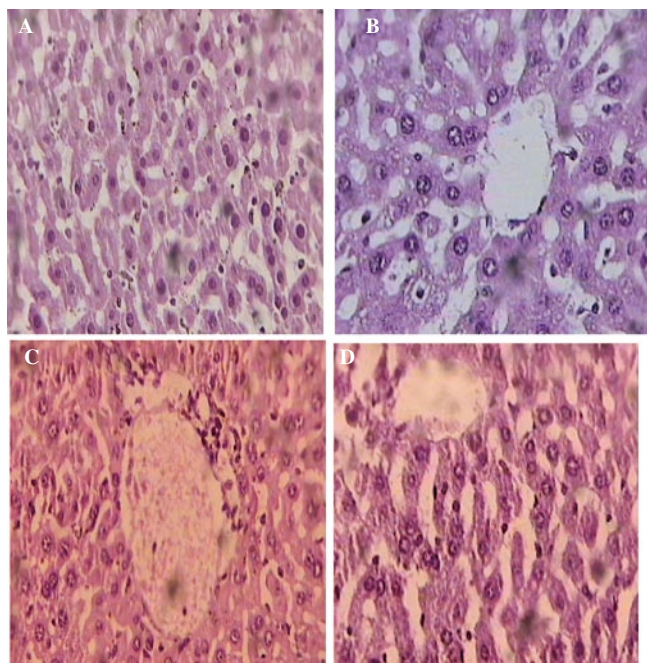
3.10. Histopathology

Histology of major organs like liver, kidney, heart, lung, spleen and brain were performed to observe any changes in tissues level (infiltration, inflammation, congestion, degradation and regeneration, *etc*) of the mice receiving the metabolite at dose 100.0 mg/kg/day for 14 consecutive days with respect to control group (normal mice). In mice of the treated group (normal mice receiving PEBM), no abnormalities in the histopathology of kidney, spleen, lung and brain were detected in comparisons with control (receiving saline only) group under microscope. Liver tissues showed very little infiltration with no central vein dilation, fatty generation or nodule formation, necrosis *etc* in normal mice whereas major organs of EAC bearing mice showed significant cellular degeneration/regeneration due to carcinogenesis. The hepatotoxicity induced by EAC cells were nullified by the protective effects of PEBM supplementation to a great extent (Figure 9).

Table 2
Effect of PEBM on biochemical parameters in experimental mice.

Name of Exp.	Days	Serum glucose mg/dL blood	Serum cholesterol mg/dL blood	Serum billirubin mg/dL blood	Serum urea mg/dL blood	Serum triglyceride mg/dL blood	Serum creatinine mg/dL
Normal mice	0	152.3±1.3	142.7±3.5	1.32±0.10	37.2±4.2	110.0±2.5	0.26±0.21
	5	102.0±10.5[b]	155.5±7.3	1.07±0.20	26.0±2.7	140.5±5.0	0.29±0.20
Normal	10	138.7±8.3	221.0±15.7	1.59±0.10	38.5±5.1	162.0±6.0	0.35±0.10
+PEBM	15	170.0±12.6[a]	235.0±5.0[a]	1.77±0.30[c]	48.8±7.5	172.8±5.3[c]	0.45±0.15
	25	156.0±14.0	160.7±6.4	1.45±0.25	39.6±4.4	120.0±5.7	0.28±0.10
EAC control	5	87.0±2.3[*]	152.0±3.7	1.52±0.50	64.1±1.8[*]	188.6±6.2	0.40±0.20
	10	73.0±3.7[**]	156.7±2.6	1.95±0.63	77.0±0.8[*]	192.0±3.1	1.10±0.31
	15	65.0±2.4[**]	165.0±3.7	2.40±0.45	84.1±1.2[**]	205.0±5.2[**]	2.70±1.10
	25	54.3±2.9[**]	183.0±3.8[**]	2.45±1.10	93.0±3.1[***]	231.0±3.1[***]	3.20±1.20[**]
EAC+ PEBM	5	96.5±4.5	142.0±3.3	1.50±0.25	47.3±4.3	158.0±2.2[*]	0.81±0.40
	10	92.2±10.4	155.0±4.6	1.75±0.40	50.5±2.5	172.1±8.2	0.92±0.20
	15	90.5±6.5	165.4±2.5	1.85±0.43	55.2±2.5	178.0±1.2	1.00±0.50
	25	88.5±8.5	175.0±5.1	1.92±0.54	62.2±5.3	198.2±1.5	1.30±1.00

Results are shown as mean±SEM, where significant values are [a]$P<0.05$, [b]$P<0.01$ and [c]$P<0.001$ when PEBM treated mice compared with normal mice and [*]$P<0.05$ and [**]$P<0.01$ when (EAC+PEBM) treated mice compared with EAC bearing controlmice (EAC bearing only).

Figure 9. Histopathological examinations of experimental mice.
A: Liver tissues from control mice with no abnormality; B: Liver tissue from PEBM treated normal mice shown very little infiltration with no central vein dilation, fatty generation or nodule formation; C: Liver tissues from untreated EAC bearing mice with necrosis, central vein dilation; D: Liver tissues from EAC bearing PEBM treated mice with very little necrosis and no central vein dilation.

4. Discussion

The potency and efficacy of the metabolites as potential anticancer agent has been judged by measuring reduction of average tumor weight, cell growth inhibition and enhancement of life span of the EAC cell bearing mice as well as hematological parameters. The efficiency of the metabolites has been compared with data obtained by running parallel experiments with a known effective clinically used anticancer drug, *bleomycin* at the dose of 0.3 mg/kg (*i.p.*). The average tumor weight reduction ability of the metabolite has been examined. For tumor bearing mice, body weight has been found to be increased gradually with time. The treatment of such mice with PEBM has reduced the growth rate significantly. The metabolites also inhibits the cell growth rate effectively, more than 78.56% ($P<0.001$) inhibition has been achieved at dose 100 mg/kg (*i.p.*) which is quite comparable to that of bleomycin(0.3 mg/kg).

The metabolites also increased the life span of tumor bearing mice very effectively. The potency has been found to be increased with the further enhancement of dose. In the present study, the dose up to 100 mg/kg (*i.p.*) has been employed only. Further enhancement of the life span of tumor bearing mice is therefore expected with still higher doses. The enhancement of life span has been assigned as a very important parameter for judging its suitability of a compound as potential chemopreventive anticancer agent[24].

High LD_{50} value (1050 mg/kg) indicates that PEBM has minor or no toxicity to the host which is also rectified by the hematological and histopathology studies as well.

Hematological parameters for normal mice, EAC cell bearing mice and EAC bearing mice treated with the metabolites have been studied. Although the treatment of the metabolites in normal mice showed little toxic effect (during the treatment period), but they were found to be recovered back towards normal level after the treatment period. The effect is more pronounced in the case of EAC bearing mice. Literature reveals that progression of tumor was accompanied by the following hematological changes compared to normal gradual decrease in hemoglobin content, RBC count and gradual increase in leukocytes[19] which was also observed in our control mice. The RBC count was almost reversed back to normal range on the treatment of PEBM in EAC bearing mice. It could also improve the WBC level efficiently. The hemoglobin level was in the near normal range in the PEBM treated EAC bearing group. Recovery of the hematological parameters like hemoglobin content, RBC and WBC cells count in the experimental mice indicates the protective action of PEBM on the hemopoietic system and this certainly ratify that PEBM posses pronounced anticancer activity with a little or no host toxic effect.

It has been found that the metabolites have significantly increased the peritoneal cells in normal mice. Treatment with the metabolites at dose 100 mg/kg (*i.p.*) increases the number of macrophages to great extent. This is considered to be a very important indicator for acquiring self destroying ability of the animals/ living beings towards cancer cells[20] by cell mediated immunity. Enhancement of macrophages might produce some candidate cytokines such as tumor necrosis factors, interleukins, *etc.* inside the peritoneal cavity, which in turn may be responsible for killing of tumor cells[16]. The bioassay of EAC cells shows a significant reduction of viability of cancer cells *in vivo* after the treatment with PEBM.

Hepatoprotective effects of PEBM have been performed by studying biochemical parameters as well as histopathological analysis of experimental animal. It is well known that there are significant elevations in the levels of serum GPT, GOT and ALP in liver diseases and disorders in hepatocellular damage caused by a number of agents. An increase in these enzyme levels is also observed in patients with cardiac damage due to myocardial infarction and with liver disorders[19].

Biochemical measurements of these parameters in normal mice treated with PEBM showed some extent of increase due to mild hepatotoxicity during treatment period but they become normal after completion of treatment schedule. The slight host toxic effects observed in mice during treatment time are mostly reversible. This means that, the treatments of the extract do not cause any acute or permanent damage to the liver. But in case of tumor bearing mice, these parameters were found to be increase more drastically with time due to the acute and permanent toxicities induced by EAC cells on host. After treatment with PEBM in the EAC bearing mice these values remain near the normal range in the treated group. From this it follows that the damage generated by EAC was prevented by PEBM supplementation.

Treatment of normal mice with PEBM slightly changed

biomolecules level which also rectified more or less to normal after treatment. This indicates that after short term treatment the extract did not cause any extreme abnormality at the dose used in this study.

The development of hypoglycaemia and hyperlipidaemia in experimental animals with carcinoma has been previously reported[21]. In this experiment, the reduced glucose level and elevated cholesterol, triglycerides and serum urea were returned to more or less normal levels in PEBM–treated mice, thereby indicating a potent antitumour efficacy of PEBM.

The histopathology of major organs of normal mice also revealed the relatively less toxic nature of the metabolite as compared to control group when viewed under microscope. The histopathology of kidney tissues from PEBM treated mice did not show any cellular regeneration/regenerations, glomerular infiltration, and there is no sign of tubular necrosis, casts and also glomerular congestion. Tissues from brain as well as other organs did not show changes in cellular architecture. The histology of liver showed very little infiltration (inflammation) with no central vein dilation, fatty generation or nodule formation in normal mice having PEBM. This mild hepatotoxicity causes some biochemical parameters deteriorate during treatment period which become normal after closing treatment. All these slight host toxic effects observed in mice during treatment time are mostly reversible and so treatment with PEBM do not cause any acute or permanent damage to the host.

Histology of EAC bearing mice showed major abnormities in almost all studied organs and it is interesting that the hepatic damage induced by EAC cells were nullified by PEBM supplementation.

The aim of this study was to determine the antiproliferative and hepatoprotective effects of the metabolite obtained from our isolated bacteria to find out less host toxic potential anticancer agents. The results presented above showed that the metabolites at dose 100.0 mg/kg (*i.p.*) can inhibit cell growth of tumor bearing mice satisfactorily, reduce tumor growth rate markedly and increase life span dramatically. All these parameters are considered as very important and promising aspects in justifying the potency of a compound in cancer chemopreventive therapy[24]. As the metabolites enhances macrophages and other peritoneal cells remarkably, so it is speculated that the metabolite kill or destroy tumor cells by boosting cell mediated tumor immunity of the host. One of the major problems usually encountered in cancer chemotherapy is myelosuppression followed by anemia[25] due to the reduction of RBC and hemoglobin contents in host. This is probably owing to the deficiency of iron in hemolytic or myelopathic condition. The treatment with PEBM under investigation can also reverse all the depleted hematological parameters back towards almost normal level which indicate that it have protective effect on hemopoetic system. The host toxic effect of PEBM is not high and almost in all cases the toxic effects of EAC cells on biomolecules as well as on cellular structure have been found to be nullified by such

treatment. In most cases antagonistic effects have been found instead of additive or synergistic effects. Further elevation of glucose levels of EAC bearing mice by the treatment of the metabolite indicates their partial recovery from tumor growth.

As the major organs of the treated mice do not show any histopathological abnormalities, these findings in conjunction with those obtained from the measurement of hematology and serum biomolecules definitely give positive support to conclude that PEBM is an effective antineoplastic agent with comparatively less toxic effects in our experimental model. However, further chronic toxicological studies and its anti–tumor activity and its mechanism should be carried out against other tumor cell lines which may bring promising results in cancer chemotherapy.

Conflict of interest statement

We declare that we have no conflict of interest.

Comments

Background

Toxicity of chemotherapy to host is main problem for its efficacy and outcome. Untoward side effects of available chemopreventive agents limit their application and usage. Therefore, host friendly and effective chemo–agents discovery and development is needed for cancer treatment with better outcome.

Research frontiers

The present research work explores the anticancer and hepatoprotective activities of secondary metabolites extracted from a bacterial species namely *Corynebacterium xerosis* against EAC cells in animal model. Its anticancer activity was determined by observing different parameters like tumor weight, survival time, cell growth inhibition and the hepatoprotective effects of the metabolites was measured by studying biochemical and histological studies.

Related reports

Many microbial metabolites/products show important pharmacological properties and used as antibiotic, anticancer agents, enzyme inhibitors *etc*. Bacterial products like exotoxin A, manumycin, gliotoxin showed *in vitro* cytotoxic activity against different cell lines. Some metabolites induce apoptosis of different cancer cell both *in vitro* and *in vivo*.

Innovations and breakthroughs

To find out an effective, cheap and host friendly anticancer agents from nature, anticancer and hepatoprotective activity of the metabolites carried out. The authors first time reported the hepatoprotective and antiproliferative potential of metabolites extracted from the *Corynebacterium xerosis*

against EAC cell *in vivo*.

Applications

The data obtained from the study is very much promising and lead to further studies in this field. This study may open new biotechnological approaches to find effective cancer chemo–agents from microbes.

Peer review

This is an excellent and well designed research work in which the researchers have explore the anticancer and hepatoprotective activity of metabolites from *Corynebacterium xerosis* against EAC cells in Swiss albino mice. Cell growth inhibition, survival time, tumor weight and hematological parameters were studied to assess its anticancer activity. Biochemical and histopathology of mice organ were studied for its hepatoprotective potential. It was found from the study that the metabolites have promising anticancer and hepatoprotective activity in mice model.

References

[1] Islam F, Khatun H, Khatun M, Ali SM, Khanam JA. Growth inhibition and apoptosis of Ehrlich ascites carcinoma cells by the methanol extract of Eucalyptus camaldulensis. *Pharm Biol* 2014; **52**(3): 1–10.

[2] Kreitman RJ, Tallman MS, Robak T, Coutre S, Wilson WH, Stetler–Stevenson M, et al. Phase I trial of anti–CD22 recombinant immunotoxin moxetumomab pasudotox (CAT–8015 or HA22) in patients with hairy cell leukemia. *J Clin Oncol* 2012; **30**(15): 1822–1828.

[3] Ben–Baruch A. Site–specific metastasis formation: chemokines as regulators of tumor cell adhesion, motility and invasion. *Cell Adh Migr* 2009; **3**(4): 328–333.

[4] Lind DS. Arginine and cancer. *J Nutr* 2004; **134**(10): 2837S–2841S.

[5] Feun L, You M, Wu CJ, Kuo MT, Wangpaichitr M, Spector S, et al. Arginine deprivation as a targeted therapy for cancer. *Curr Pharm Des* 2008; **14**(11): 1049–1057.

[6] Arsénio M, Fialho A, Chakrabarty M. Pseudomonas exotoxin–A based immunotoxins for targeted cancer therapy. In: Fialho A, Chakrabarty A, editors. *Emerging cancer therapy: microbial approaches and biotechnological tools*. 1st ed. Hoboken, NJ, USA: John wiley & Sons, Inc.; 2010.

[7] Oloomi M, Bouzari S, Shariati E. *In vivo* characterization of fusion protein comprising of A1 Subunit of Shiga toxin and human GM–CSF: assessment of its immunogenicity and toxicity. *Iran Biomed J* 2010; **14**(4): 136–141.

[8] Westphal K, Leschner S, Jablonska J, Loessner H, Weiss S. Containment of tumor–colonizing bacteria by host neutrophils. *Cancer Res* 2008; **68**: 2952–2957.

[9] Xiong Q, Rikihisa Y. The prenylation inhibitor manumycin A reduces the viability of *Anaplasma phagocytophilum*. *J Med Microbiol* 2011; **60**(6): 744–749.

[10] Pan J, Xu G, Yeung SC. Cytochrome c release is upstream to activation of caspase–9, caspase–8 and caspase–3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel. *J Clin Endocrinol Metab* 2001; **86**(10): 4731–4740.

[11] Williamson NR, Chawrai S, Leeper FJ, Salmond GPC. Prodiginines and their potential utility as proapototic anticancer agents. In: Fialho A, Chakrabarty A, editors. *Emerging cancer therapy: microbial approaches and biotechnological tools*. 1st ed. Hoboken, NJ, USA: John wiley & Sons, Inc.; 2010.

[12] Trivedi M, Budihardjo I, Loureiro K, Reid TR, Ma JD. Epothilones: a novel class of microtubule–stabilizing drugs for the treatment of cancer. *Future Oncol* 2008; **4**(4): 483–500.

[13] Mehmet O, Isik D K, Ibrahim HK, Muhammed EG. Ehrlich ascites carcinoma. *Afr J Biotechnol* 2011; **10**(13): 2375–2378.

[14] Sonnenwirth AC, Jarett L. *Gradwohl's clinical laboratory methods and diagnosis* Vol. 2. 8th ed. New Delhi: B.I. Publication; 1980, p. 1698.

[15] Collee JG, Marr W. Culture of bacteria. In: Collee JG, Fraser AG, Marmion BP, Simmons A, editors. *Mackie and McCartney practical medical microbiology*. 14th ed. New York: Churchill Livingstone; 1996, p. 121–124.

[16] Khanam JA, Islam MF, Jesmin M, Ali MM. Antineoplastic activity of acetone semicarbazone(ASC) against ehrlich ascites carcinoma (EAC) bearing mice. *J Natn Sci Foundation Sri Lanka* 2010; **38**(4): 225–231.

[17] Kabir SR, Hossen A, Zubair A, Alom J, Islam F, Hossain A, et al. A new lectin from the tuberous rhizome of *Kaempferia rotunda*: isolation, characterization, antibacterial and antiproliferative activities. *Protein Pept Lett* 2011; **18**(11): 1140–1149.

[18] Kabir SR, Zubair MA, Nurujjaman M, Haque MA, Hasan I, Islam MF, et al. Purification and characterization of a Ca(2+)–dependent novel lectin from *Nymphaea nouchali* tuber with antiproliferative activities. *Biosci Rep* 2011; **31**(6): 465–475.

[19] Muhammad RH, Muhammad AA, Muhammad RK. Inhibition of Ehrlich's ascites carcinoma by ethyl acetate extract from the flower of *Calotropis gigantia* L. in mice. *J Appl Biomed* 2010; **8**: 47–54.

[20] Islam F, Khatun H, Ghosh S, Ali MM, Khanam JA. Bioassay of *Eucalyptus* extracts for anticancer activity against ehrlich ascites carcinoma (EAC) cells in Swiss Albino mice. *Asian Pac J Trop Biomed* 2012; **2**(5): 394–398.

[21] Rasida P, Islam F, Khanum J, Yeasmin T. Preventive effect of Ethanol Extract of Alpinia calcarata Rosc on Ehrlich's ascitic carcinoma cell induced malignant ascites in mice. *Asian Pac J Trop Med* 2012; **5**(2): 121–125.

[22] Kabir SR, Islam MF, Alom MJ, Zubair MA, Absar N. Purification, characterizations of a snake guard seeds lectin with antitumor activity against Ehrlich ascites carcinoma cells *in vivo* in mice. *Protein Pept Lett* 2012; **19**(3): 360–368.

[23] Ali MM, Jesmin M. Hepatotoxicity of Schiff bases derived from benzoin salicylaldehyde, aminophenol and 2,4 dinitrophenyl hydrazine. *J Natn Sci Foundation Sri Lanka* 2010; **38**(2): 145–149.

[24] Clarkson D, Burichenal JH. Preliminary screening of antineoplastic drugs. *Prog Clin Cancer* 1965; **1**: 625–629.

[25] Aravind SR, Joseph MM, Varghese S, Balaram P, Sreelekha TT. Antitumor and immunopotentiating activity of polysaccharide PST001 Isolated from the seed kernel of *Tamarindus indica*: an *in vivo* study in mice. *Sci World J* 2012; **2012**: 361–382.