Indian J Physiol Pharmacol 2018; 62(2) : 259–266

Original Article

Evaluation of Antitumor Activity of *Crotalaria Burhia* Buch.-Ham. Roots Against Ehrlich's Ascites Carcinoma Treated Mice

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Abstract

The study was aimed to evaluate antitumor activity of methanolic extract of *Crotalaria burhia* Buch.-Ham. roots (MECB) in Swiss Albino mice against Ehrlich's Ascites Carcinoma (EAC). The mice were divided into seven groups (n=20) in which Group I was treated with Sterile Physiological Saline (SPS) (Normal mice), Group II served as EAC control and Group III, IV, V or VI were treated with MECB at 100, 150, 300 or 400 mg/kg body weight i.p., respectively. Group VII were treated with standard drug 5-Fluorouracil (20 mg/kg i.p). The MECB was administered for 9 consecutive days. After 24 hours of last dose and 18 h of fasting, ten mice from each group were sacrificed and remaining mice from each group were kept to evaluate mean survival time. Antitumor activity of MECB was assessed by determining tumor volume, tumor weight, viable cells and nonviable cells count, hematological parameters, biochemical parameters and liver antioxidant status of EAC bearing host. The MECB was shown potent dose dependent antitumor activity. MECB at different doses level showed significant (P<0.05) reduction in tumor volume, tumor weight, and viable cells count with increased the life span of EAC mice. Interestingly, no mortality was observed during nine consecutive days of treatment. In further, the imbalanced hematological parameters of tumor bearing mice was significantly (P<0.05) regained after treatment of MECB. Moreover, altered biochemical parameters such as SGOT, SGPT, ALT, albumin and total protein in EAC mice were regained after treatment of MECB. Fascinatingly, MECB was showed significant antioxidant activity and which was characterized by reducing

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(Received on January 1, 2018)

level of lipid peroxidation with increasing the level of catalase and reduced-glutathione. Results of present study revealed that the MECB had significant dose dependent antitumor and antioxidant activity that is comparable to 5-FU. In further, *C. burhia* roots can be a good source as antitumor and antioxidant in future.

Introduction

Plants are a potential and important source for discovery and development of newer pharmacological agents for chemotherapy (1). Numerous plants have been studied for their anticancer activity using various experimental models and this was resulted in availability of nearly 30 effective anticancer molecules (2). Since the ancient times herbs are recognized as a source of remedies. India is a richest source of medicinal plants and known as botanical garden of the world. Different types of plants or plant extracts are used in various systems of medicine (Ayurveda, Unani, and Siddha) for treatment of various diseases. Only a few of them are scientifically evaluated and still many more medicinal plants are left behind to be evaluated. Secondary metabolites of medicinal plants such as flavonoids, terpenes, alkaloids have received considerable attention in recent scenario due to their various pharmacological properties (3, 4).

C. burhia Buch.-Ham. also known as *Khip*, is an under shrub and fibrous plant, commonly found in the arid parts of India, West Pakistan and Afghanistan. In ancient Indian medicinal system of ayurveda, *khip* has been recognized as a potential medicinal plant. The leaves, roots and branches of *C. burhia* is use as a cooling and antitumor medicine, while fresh plant juice is useful for eczema, gout, hydrophobia, pain and swelling. Roots extract with sugar is use to cure chronic kidney pain and roots decoction is use for treatment of typhoid (5, 6).

Phytochemical screening revealed the presence of pyrrolizidine alkaloids as main compounds in *C. burhia*. In addition, flavonoid (quercetin) and steroid (β-sitosterol) were isolated from this plant. Anticancer, anti-inflammatory, analgesic, antimicrobial and antibacterial activities of *C. burhia* have been reported in various literatures (7, 8).

In the present investigation the roots of *C. burhia* was selected for assessment of their antitumor activity, because as per traditional medicine system of India and other Asian countries *C. burhia* roots are commonly use for treatment of tumors, pain, swelling and fever. However, till date there is no report of either ethnopharmacological or pharmacological study to evaluate the antitumor activity. Therefore, the present study was carried out to evaluate antitumor activity of *C. burhia* and establish the scientific basis to supports their traditionally claimed uses.

Materials & Methodology

Materials

Plant material

C. burhia Buch.-Ham. roots were collected from Rajasthan University campus Jaipur, Rajasthan (India), during the months of Oct-Nov 2010. The plant was identified by Mr. P.J. Parmar, Joint Director in Botanical Survey of India (BSI), Jodhpur (Rajasthan, India). Authenticated voucher specimen (JNU/JPR/PC/SK-1) has been deposited at Botanical Survey of India, Jodhpur, India.

Chemical

All the required chemicals were purchased from Merck Ltd., Mumbai, India and standard for biochemical and antioxidant were purchased from Sisco Research Laboratory Mumbai, India. All other chemical and reagent were used of analytical grade.

Animal care and handling

The animal care and handling was done according to guidelines set by the WHO (World Health Organization), Geneva, Switzerland, INSA (Indian

National Science Academy, New Delhi, India) and the "Guide for the care and use of Laboratory Animals" (9). Female Swiss Albino mice weighing 20-22 g was selected from departmental animal house. The mice were divided into groups and housed in polyacrylic cages under standard laboratory conditions (temperature 25±2°C; dark-light cycle [14-10 h]). Mice were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. All the described procedures were reviewed and approved by Institutional Animal Ethical Committee of Gyan Vihar School of Pharmacy, Jaipur, India.

Tumor model

EAC cells were procured from the Division of Radiobiology and Toxicology, Manipal Life Science Centre, Manipal University, Manipal, India. The EAC cells were maintained in *Swiss Albino* mice (*in vivo*) by intraperitoneal innoculation of 2×10^6 cells/mice after every 10 days in an aseptic environment. Ascitic fluid was drawn out from EAC tumor bearing mice at the log phase (days 7-8 of tumor bearing) of the tumor cells. Tumor was produced by intraperitoneal injection of 0.1 ml of tumor cell suspension containing 2×10^6 tumor cells.

Methodology

Preparation of methanolic extract

The air and shade dried powdered roots of *C. burhia* was exhaustively extracted with methanol by Soxhlet continuous extraction method. The final extract (MECB) was concentrated under reduced pressure, on a rotary evaporator at 40-45°C and percentage yield of crude extract was 2.1% w/w.

Preparation of drug and treatment mode

The MECB solution was prepared according to method described by Jagetia and Rao (2006) with minor modifications (10). Briefly, MECB and 5-Flurouracil (5-FU) were suspended in Sterile Physiological Saline (SPS) containing 0.5% Carboxy Methyl Cellulose (CMC). All animal were treated via intraperitoneal (i.p.) route of administration.

Acute toxicity study

The acute toxicity study was performed according to method described by Litchfield and Wilcoxon (11). The oral LD_{50} value of MECB in Swiss Albino mice was determined and found to be 2 g/kg body weight.

Treatment schedule

The mice were divided into the following groups (n=20) (12).

All mice in each group except group-I received EAC cell (2×10^6 cell/mouse i.p). This was taken as day '0'.

Group I (Normal Control): mice treated with 0.5 ml/kg SPS i.p, once daily, consecutively for 9 days.

Group II (EAC Control): EAC inoculated mice, served as EAC control (2×10^6 cell/mouse i.p).

Group III (MECB 100): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 100 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group IV (MECB 150): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 150 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group V (MECB 300): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 300 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group VI (MECB 400): 24 hrs after transplantation of EAC, mice were treated with MECB at the dose of 400 mg/kg body weight i.p, once daily and consecutively for 9 days.

Group VII (Standard 5-FU): 24 hrs after transplantation of EAC, mice were treated with 5-flurouracil at the dose of 20 mg/kg body weight, i.p, consecutively for 9 days.

After 24 hrs of last dose and 18 hrs of fasting, 10

mice from each group were sacrificed to determine antitumor and antioxidant activity along with biochemical and hematological parameters. The remaining mice were kept under observation to evaluate life span of the tumor host.

Tumor volume

The ascitic fluid was aspirated aseptically from peritoneal cavity. The volume was measured by taking it in a sterile graduated centrifuge tube (13).

Tumor weight

Tumor weight was measured by evaluating mice weight before and after the collection of ascitic fluid from peritoneal cavity.

Percentage increase in life span

The effect of MECB on percentage increases in life span was calculated on the basis of mortality of experimental mice (14).

$$MST = \frac{\text{First death} + \text{last death in the group}}{2}$$

(MST: mean survival time and time is denoted in days)

The increase in life span (% ILS) was calculated by following formula:

$$ILS(\%) = \frac{\text{MST of treated mice} - \text{MST of control}\ X100}{\text{MST of control}}$$

Tumor cell count

The ascitic fluid was collected by WBC pipette and diluted 100 times. A drop of collected cells suspension was placed on the Neubauer's counting chamber and the numbers of cells in 64 small squares were counted.

Viable and nonviable tumor cell count

Viabile and non viabile EAC cells were counted by trypan blue dye assay. The cells were stained with trypan blue dye (0.4% in normal saline). The cells that did not take up the dye were noted as viable and those are took the dye were noted as nonviable. These viable and nonviable cells were counted by following formula.

$$\text{Cell count} = \frac{\text{Number of cell} \times \text{Dilution factor}}{\text{Area} \times \text{Thickness of film}}$$

Hematological and biochemical parameters

At end of the experimental period, the next day after an overnight fasting blood samples were collected from tail vein and used for estimation of hematological (hemoglobin (Hb) content, red blood cell (RBCs) count, white blood cell (WBCs) count & lymphocyte count) and biochemical parameters (serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, alkaline phosphatase and total protein) by standard procedures. Further, the mice were sacrificed and liver were taken out for evaluation of antioxidant activity.

Antioxidant activity

Ten percent of liver tissue homogenate was prepared in Tris-HCl buffer (0.1M, pH 7.4) for estimation of lipid peroxidation (15), catalase (16) and reduced glutathione (17) by following standard procedures.

Statistical analysis

All the data are expressed as Mean±S.E.M. (n=10 mice per group). The data were analyzed by one-way ANOVA between the treated group and the EAC control followed by dunnett's post hoc test. The $P<0.05$ and $P<0.01$ were considered significant and highly significant, respectively.

Results

Intraperitoneal administration of methanolic extract of *C. burhia* (MECB) was shown dose depended antitumor activity in EAC bearing mice. No mortality was observed for nine consecutive days. Antitumor activity of MECB against EAC was assessed by evaluating tumor volume, tumor weight, viable and

non-viable cells count, MST and %ILS (Table I). Administration of MECB at different doses level caused significant (P<0.05) reduction in tumor volume, tumor weight and viable cells count, and increased MST, %ILS and non-viable cells count when compared to EAC control group (Table I). High dose of MECB (400 mg/kg) was found with higher antitumor activity than 300 mg/kg and followed by 150 mg/kg. Lower dose of MECB (100 mg/kg) was shown negligible antitumor activity (Table I).

Moreover, MST of EAC control group was noted 20 days, while it was significantly (P<0.05) increased by 1 day (MST was 21 days; increased 10% ILS), 9.5 days (MST was 29.5 days; increased 47.5% ILS), 17 days (MST was 37 days; increased 85% ILS), and 19.5 days (MST was 39.5 days; increased 97.5% ILS) with treatment of MECB at the dose of 100 mg/kg, 150 mg/kg, 300 mg/kg and 400 mg/kg, respectively, when compared to EAC control group. MECB at the low dose (100 mg/kg) had negligible impact on MST and %ILS.

On 10th day, after the completion of study period, hematological parameters of EAC bearing mice were found significantly altered when compared to normal control group (Table II). The total WBCs count was increased and RBCs count was decreased in EAC bearing mice when compared normal group. In differential count, WBCs and the percentage of lymphocytes were found elevated in EAC bearing mice when compared to normal mice. Intraperitoneal administration of MECB at different doses were shown significant (P<0.05) effect to regain altered hematological parameters towards saline control in dose depended manners (Table II).

Effect of MECB on biochemical parameters of tumor bearing mice is shown in Table III. The biochemical parameters such as SGOT, SGPT and ALT level were increased, whereas albumin level and total protein content were significantly decreased in tumor bearing mice when compared to saline treated mice. In MECB treated mice, the biochemical parameters showed significant restoration toward saline control (Table III).

TABLE I: Effect of the MECB on tumor volume, tumor weight, mean survival time (MST), percentage increase life span (%ILS), viable cells and nonviable tumor cells count in EAC bearing mice.

Parameters	*EAC control*	*100 mg/kg*	*150 mg/kg*	*300 mg/kg*	*400 mg/kg*	*5-FU*
Tumor volume (ml)	3.27±0.1	3.1±0.15^{NS}	1.53±0.11*	1.13±0.11*	0.68±0.17*	0.46±0.03*
Tumor weight (g)	3.5±0.13	2.93±0.25*	1.17±0.11*	1.18±0.16*	0.71±0.16*	0.54±0.04*
MST (day)	20	21	29.5	37	39.5	42
%ILS	0	10	47.5	85	97.5	107.5
Viable cell	8.21×10^7±0.33	7.11×10^7±0.3	3.62×10^7±0.29*	2.44×10^7±0.22**	1.4×10^7±0.1**	0.84×10^7±0.1**
Nonviable cell	0.35×10^7±0.06	0.67×10^7±0.2	1.13×10^7±0.11**	2.13×10^7±0.19*	3.09×10^7±0.11*	3.6×10^7±0.15**
Total cell	8.56×107	7.78×10^7	4.75×10^7	4.57×10^7	4.49×10^7	4.44×10^7
Viable %	95.91	91.38	79.38	53.39	31.18	19.14
Non viable %	4.08	8.61	20.61	46.6	68.81	81.08

Values are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when compared with EAC control

TABLE II: Effect of the MECB on hematological parameters in EAC bearing mice.

Parameters	*WBC (cell×10^6/µl)*	*RBC (cell×10^6/µl)*	*Lymphocytes (%)*	*Hemoglobin (g/dl)*
Normal	6.2±0.54	6.6±0.47	75.5±0.3	12.46±0.42
EAC	15.9±0.27**	3.9±0.23*	86.3±0.36**	4.97±0.03**
100 mg/kg	14.7±0.3^a	4.06±1.2^b	85.7±0.5^b	5.39±0.05^a
150 mg/kg	12.4±0.28^b	4.5±0.22^a	78.9±0.3^b	6.22±0.16^b
300 mg/kg	10.2±0.8^a	5.1±0.23^a	76.6±0.4^a	7.62±0.17^a
400 mg/kg	8.63±0.2^a	5.54±0.31^b	75.93±0.1^b	8.71±0.3^b
5-FU	7.7±.0.17^a	6.1±0.17^a	74.1±0.4^b	10.6±0.17^b

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when with control (Normal).
^{a}P<0.05, ^{b}P<0.01, Values are significantly different when with EAC control.

TABLE III: Effect of the MECB on biochemical parameter in EAC bearing mice.

Treatment	*Total protein (gm/dl)*	*Albumin (gm/dl)*	*SGOT (IU/L)*	*SGPT (IU/L)*	*ALP (IU/L)*
Normal	5.65±0.29	1.6±0.09	34.08±1.16	28.17±1.89	77.44±3.47
EAC control	2.9±0.18*	1.17±0.1**	64.97±1.72**	59.14±1.82**	122±2.55*
100 mg/kg	3.14±0.2[a]	1.2±0.1[b]	61.17±1.32[a]	54.37±0.82[b]	116.1±1.9[a]
150 mg/kg	3.54±0.27[b]	1.25±0.08[a]	56.75±2.42[b]	50.14±0.59[b]	106.57±1.98[a]
300 mg/kg	4.4±0.5[a]	1.34±0.02[a]	49.52±1.04[a]	40.32±1.83[a]	94.65±2.81[b]
400 mg/kg	4.98±3.8[a]	1.43±2.61[b]	43.97±1.63[a]	34.17±2.25[a]	87±2.75[a]

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different when compared with control (Normal).
[a]P<0.05, [b]P<0.01, Values are significantly different when compared with EAC control.

TABLE IV: Effect of MECB on lipid peroxidation and antioxidant parameters in EAC bearing mice.

Treatment	*Lipid peroxidation (nMol/mg protein)*	*Catalase (Unit/mg/protein/min)*	*Reduced-glutathione (mMol/gm)*
Normal	162.28±0.54	4.36±0.22	28.87±0.56
EAC control	438.48±0.3**	1.3±0.19**	7.46±0.2**
100 mg/kg	389.68±0.23[a]	1.96±0.25[b]	9.16±0.2[b]
150 mg/kg	325.19±0.2[a]	2.52±0.07[a]	12.92±0.2[a]
300 mg/kg)	234.96±0.18[a]	3.2±0.5[b]	22.63±0.21[a]
400 mg/kg	197.3±0.4[a]	3.97±0.09[b]	24.73±0.1[a]

Value are expressed as Mean±S.E.M. (n=10).
*P<0.05, **P<0.01, Values are significantly different compared with control (Normal).
[a]P<0.05, [b]P<0.01, Values are significantly different when compared with EAC control.

As shown in Table IV, significant elevation in lipid peroxidation were observed in tumor bearing mice when compared to normal mice (P<0.05). MECB treatment at different tested dose level caused significant reduction in lipid peroxidation. In addition, the level of reduced-glutathione and catalase were significantly decreased in EAC mice when compared to EAC treated mice. Administration of MECB at different doses level was able to restore the level of reduced-glutathione and catalase toward normal in tumor bearing mice (Table IV). Finally, results clearly indicate that the MECB has remarkable capacity to inhibit the growth of EAC tumor in a dose dependent manner.

DISCUSSION

Preliminary phytochemical investigation was indicated that the MECB is rich in alkaloids, flavanoids and steroids. Previously alkaloids, flavanoids and steroids are reported with antitumor activity. Flavanoid have been shown to possess antimutagenic and antimalignant effect (18). In present investigation, MECB was shown potent dose depended antitumor and antioxidant activity in EAC bearing mice.

Prolongation of life span and reduction in WBCs count are the reliable criteria for judging antitumor activity of any medicinal agents. In this study, reduction in tumor volume and increased mean survival time of EAC bearing mice imply the delaying impact of MECB on EAC cell division (14). In present study, MECB has shown antitumor activity in dose depended manner, which is amusingly supported by increased life span and reduced viable cells count further supported by reduced tumor volume and tumor weight.

A regular and rapid raised in ascites tumor volume were noted in EAC bearing mice. An ascitic fluid is direct nutritional source for tumor cell and rapid raise in ascitic fluid with growth of tumor would be a means to meet nutritional requirement of EAC cell.

In the present study, reduction in tumor volume, tumor weight, and viable tumor cells count, and prolongation of life span and increased number of nonviable cells were noted in tumor bearing mice after treatment of

MECB when compared to EAC control mice. So this event is suggesting that the MECB had promising effect to prolong life span of EAC bearing mice by diminishing nutritional fluid volume with arresting tumor growth. In further, MECB decreased the numbers of viable cells count and increased number of nonviable cells in tumor bearing mice. These exploit indicates that the MECB has direct association with tumor cell as tumor cell directly absorbed drug from peritoneal cavity and this antitumor agent produces lysis action on cell wall by direct cytotoxic mechanism (19).

Myelosuppression and anemia are major complication during cancer chemotherapy. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin content. This may occur either due to iron deficiency or due to hemolytic or myelopathic condition (20). Treatment with MECB brought back the hemoglobin content, RBCs and WBCs count more or less towards normal levels in EAC bearing mice. This indicates that the MECB possess non toxic or protective effect on the hemopoietic system of tumor bearing mice (21).

Superoxide dismutase and catalase enzyme in serum are markers for early indication or detection of neoplasia and also helps to judge progression and regression of disease (22). Hepatotoxicity may occur due to cytotoxic agent itself or due to its toxic metabolites. In certain circumstances they can be carcinogenic (i.e. they may themselves cause cancer). Rapid cell destruction with extensive purine catabolism can be characterize with precipitation of ureates in renal tubules and which is responsible for kidney damage. Elevated level of biochemical parameters such as SGOT, SGPT and ALP is reported, whereas the levels of total protein and albumin were decreased in tumor bearing mice (22). In present investigation the MECB showed promising effect to restore these biochemical parameters toward normal level that means the MECB did not produce toxicity on liver or kidney.

The Ehrlich's tumor growth induces an inhibition of superoxide dismutase and catalase enzymes (23) which are fundamental in the elimination of free radicals as superoxide and hydrogen peroxide (24). In Ehrlich's tumor-bearing mice the antioxidant acts by modulating lipid peroxidation and augmenting antioxidant defense system (25).

Oxidative stress which is one of the key factor in cancer pathology and that is evident in the present study by reduced levels of glutathione in tumor bearing mice. Elevated lipid peroxidation is producing degeneration effect on tissues as the consequences of excessive oxidation is also reported in the present study lipid peroxide aggravate the damage by propagating process of lipid peroxidations (26). Malondialdehyd (MDA), the end product of lipid peroxidation was reported too higher in carcinomatous tissue than in non disease tissue (27), and their levels can be correlated with tumor progression (28). Moreover, MDA is reported as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes (26, 29). Glutathione is a potent inhibitor of neoplastic process, which plays an important role as an endogenous antioxidant system (30). The potent reduction in lipid peroxidation and elevation in glutathione (GSH) and catalase (CAT) level were observed in MECB treated mice.

Conclusion

In conclusion, the present study illustrates that the MECB reduces tumor volume and tumor weight, increases the mean survival time and life span of EAC bearing mice. Decreasing lipid peroxidation and thereby augmented the endogenous live antioxidant enzymes in liver. Improvement in hematological pofile and all other biochemical parameters suggests that the methanolic extract of *C. burhia* roots exhibits potent antitumor and antioxidant activity. Further pharmacological and phytochemical investigations are required to elucidate exact mechanism and active chemical constituents which are responsible for their antitumor and antioxidant activity. Thus our present study suggests that the MECB possess potent dose depended antitumor and antioxidant activity against EAC tumor. In future, *C. burhia* can be a good source for cancer treatment.

Acknowledgements

Authors would like to thanks Department of Pharmacology, Gyanvihar University, Jaipur, India for providing research facilities.

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