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Original Article

ANTICANCER AND ANTIOXIDANT ACTIVITY OF METHANOL EXTRACT OF *HIPPOPHAE SALICIFOLIA* IN EAC INDUCED SWISS ALBINO MICE

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ABSTRACTS

Objective: Anticancer activity of methanol extract of *Hippophae salicifolia* (MEHS) bark was evaluated using the Ehrlich Ascites Carcinoma (EAC) cells on Swiss albino mice.

Methods: *In vitro* cytotoxicity assay has been evaluated by using the trypan blue and MTT assay method. The determination of *in vivo* anticancer activity was performed using EAC cells (2×10^6) induced mice groups (n = 12). After treatment with MEHS at the doses of 50 and 100 mg/kg b. w. respectively for 9 days, half of the mice of each group were sacrificed and the rest were kept for increase in life span determination. The anticancer potential of MEHS was assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight, hematological parameters and biochemical estimations. Furthermore, tissue antioxidant parameters were checked from liver homogenate.

Results: In MEHS treated groups (50 and 100 mg/kg b. w.) tumor volume, tumor weight, viable cell count was significantly decreased as compared to that of the EAC control group. Life span increased by 34% and 43% in a dose dependant manner as compared to EAC control group. The hematological, biochemical and liver tissue antioxidant parameter are significantly ($p < 0.05$) restored towards the normal level after treatment with MEHS.

Conclusion: From the above study it can be concluded that the MEHS has significant anticancer activity in the dose dependent manner.

Keywords: Trypan blue, MTT, EAC, Antioxidant, Tumor.

INTRODUCTION

Cancer has wreaked havoc in terms of mortality over years and the number of people affected with cancer is steadily increasing every day [1]. An estimate of 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer (within 5 years of diagnosis) in 2012 worldwide was observed. 57% (8 million) of new cancer cases, 65% (5.3 million) of the cancer deaths and 48% (15.6 million) of the 5-year prevalent cancer cases occurred in the less developed regions [2]. Naturally current pharmacological research focuses on discovering new drugs or compounds to fight cancer. Nature has been providing man with remedies to diseases since time immemorial. Natural sources like plants and marine products can synthesize a variety of structurally and functionally diverse bioactive compounds which can mitigate a varied number of ailments including cancer. Several plant products like taxol, vincristine etc have proven anticancer activities and are now available in the market for cancer treatment [3].

Hippophae salicifolia, commonly known as Sea-buckthorn, is a versatile plant with multipurpose uses. In India, two species of Sea-buckthorn, namely, *Hippophae salicifolia* D. Don and *Hippophae rhamnoides* L. are very common. *H. salicifolia* is a shrub-to-tree in nature and is restricted to the Himalayan region, whereas *H. rhamnoides* is bushy, growing at higher altitude in India, and widely distributed in Europe and Asia [4]. The bark is traditionally used for its anti diarrhoeal, antitumor and cosmetic purposes and also its ash has burn healing properties [5]. It has been reported that the plant has anti bacterial and anti fungal activities [6]. The hydro alcoholic extract of bark has also shown the antioxidant activity [7]. The present study was carried out to evaluate the anticancer effect of the methanol extract of *Hippophae salicifolia* bark against Ehrlich's Ascites Carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

Chemicals

Sodium chloride, Propylene glycol, Trypan blue, Methyl violet, Sodium sulphate, Methylene blue, 5-Fluorouracil, MTT (MERCK Limited, Mumbai, India). All other chemicals and reagents used were of highest analytical grade.

Plant collection and extraction

Hippophae salicifolia barks were collected from the Gangtok, Sikkim, India. The barks were cleaned and air dried for a week at 35–40°C and pulverized in electric grinder. Coarse bark (1.4 kg) was extracted by methanol (13.8% w/w, yield) by using soxhlet apparatus. The solvents were completely removed under reduced pressure in a rotary vacuum evaporator (Buchi R-210). The concentrated extracts were stored in vacuum desiccators for further use.

Animals

Male Swiss albino mice weighing 20–22 g were taken. They were obtained from the animal house, B. N. Ghosh & Co. Kolkata, India. The mice were grouped and housed in poly acrylic cages (38 × 23 × 10 cm) with not more than 6 animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 °C and dark/ light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animal Ethical Committee.

Transplantation of tumor cell

Ehrlich ascites tumor cells were maintained in our laboratory according to the standard protocol. EAC cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (day 7 – 8 of tumor bearing) of the tumor cells. Each animal received 0.1 ml of tumor cell suspension containing 2×10^6 EAC cells intra peritoneally [8].

Determination of *in vitro* cytotoxicity

In vitro cytotoxicity studies of MEHS were done by standard trypan blue (0.4%) exclusion method and MTT assay [8, 9]. The experiment was performed in triplicate.

Determination of acute toxicity

The acute oral toxicity of MEHS in Swiss albino mice was performed as per OECD guideline 425 (OECD, 2008) [10].

Treatment schedule

Swiss albino mice (60) were divided into five groups (n = 12). All the animals in each group, except Group-I (Normal control) received EAC cells (2×10^6 cells/mouse i. p.). This was taken as day '0'. Group-I served as normal saline (5 ml/kg i. p.) and group-II (EAC control) served as EAC control. 24 h after EAC transplantation, Group-III and IV received MEHS at a dose of 50 and 100 mg/kg i. p. for nine consecutive days, respectively. Group-V received reference drug 5-FU (20 mg/kg i. p.) for nine consecutive days [11].

Twenty four hours of the last dose and 18 hr of fasting, 6 animals of each group were sacrificed by cervical dislocation to measure antitumor, haematological and biochemical parameters (livers) and rest of the animals were kept with food and water *ad libitum* to check percentage increase life span of the tumor host. The anti cancer activity of MEHS was measured in EAC animals with respect to the following parameters.

Determination of tumor volume, packed cell volume and tumor weight

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. Tumor volume, packed cell volume and tumor weight was measured according to the previously described standard methods in our laboratory [12].

Determination of viable and non-viable tumor cell count

The viable and nonviable cells were counted in neubauer chamber like white blood cell counting procedure according to the previously used standard methods [12].

Determination of percentage increase life span (% ILS)

The effect of MEHS on percentage increased in life span was calculated on the basis of the mortality rates of the experimental mice according to the standard protocol [12].

Determination of hematological parameters

Collected blood was used for the estimation of haemoglobin (Hb) content, red blood cell count (RBC) and WBC according to the standard protocol [13].

Determination of biochemical parameters

Serum biochemical parameter like total proteins, serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP) and serum bilirubin was done by using commercially available kits manufactured by the Span Diagnostics Ltd., Surat, India.

Determination of tissue antioxidant parameters

The tissue antioxidant assay was performed with liver tissues and the evaluation was carried out by measuring the level thiobarbituric acid reactive substances (TBARS) in lipid peroxidation assay, the amounts of enzymatic Catalase, superoxide dismutase and non enzymatic antioxidant system such as reduced glutathione [14, 15].

Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.0, Graph Pad Software Inc., San Diego, CA) Software. The experimental results were expressed as mean ± standard error of mean (SEM). Statistical significance was analyzed by one-way ANOVA followed by Dunnett's post hoc test of significance. $p < 0.05$ was considered as statistically significant.

RESULTS

Determination of *in vitro* cytotoxicity

In the assay for *in vitro* cytotoxicity study, Trypan blue exclusion assay and MTT assay on MEHS showed the direct cytotoxic effect on the EAC cell line in a dose dependent manner with IC_{50} value of 43.2 µg/ml and 50.3 µg/ml respectively (fig. 1 and fig. 2).

Determination of acute toxicity

The extract was safe up to the dose of 800 mg/kg for mice. Dose was selected on the basis of $1/10^{th}$ of the LD_{50} dose (50 and 100 mg/kg).

Determination of direct tumor related parameters

Anticancer activity of MEHS at the dose 50 mg/kg and 100 mg/kg, against EAC tumor bearing mice was assessed by tumor volume, tumor weight, viable and non-viable cell count, mean survival time and % increase in life span. The tumor volume, tumor weight and viable cell count were found significantly increased and non- viable cell count was significantly reduced ($p < 0.05$) in EAC control animals when compared with normal control animals and treated group (table 1).

Hematological parameters

Hematological parameters of tumor bearing mice were found to be significantly altered as compared to normal control and MEHS at the dose 50 mg/kg and 100 mg/kg table. 2.

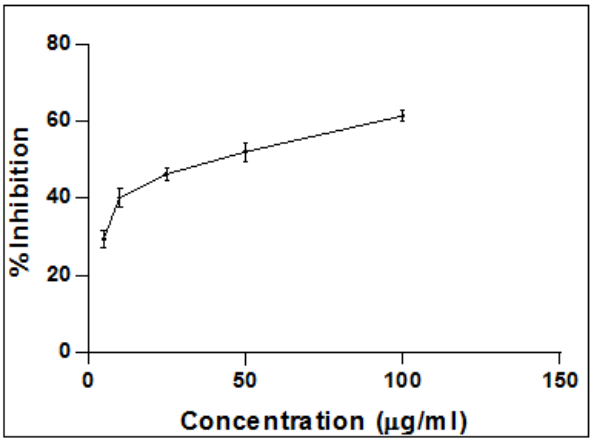

Fig. 1: Cytotoxic effect of MEHS on *in vitro* EAC cell line by Trypan blue. Values are Mean ± SEM; where n = 6

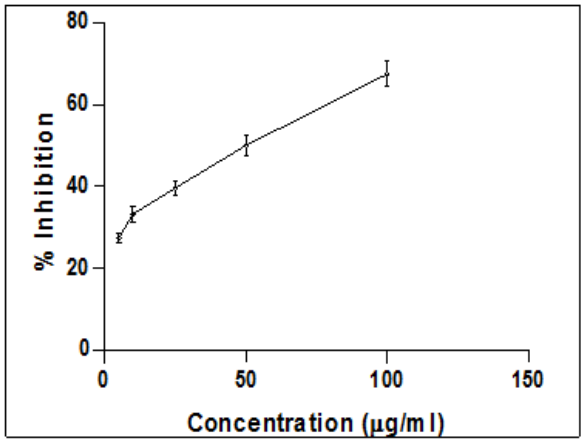

Fig. 2: Cytotoxic effect of MEHS on *in vitro* EAC cell line by MTT assay. Values are Mean ± SEM; where n = 6

Table 1: Effect of MEHS on tumor volume, tumor weight, total cell count, viable and nonviable cell count, mean survival time (MST) and percentage increase life-span (% ILS) in EAC bearing mice

Parameters	EAC Control (2×10^6cells/mouse)	EAC + MEHS (50 mg/kg)	EAC + MEHS (100 mg/kg)	EAC + 5-FU (20 mg/kg)
Tumor volume (ml)	2.36± 0.06	1.02± 0.13*	0.75± 0.10*	0.55± 0.05*
Tumor weight (g)	4.97± 0.36	1.67± 0.56*	1.12± 0.28*	0.51± 0.04*
Total cell	8.49×10^6±0.07	4.69×10^6± 0.27*	2.54×10^6± 0.09*	3.81×10^6± 0.09*
Viable cell	8.19×10^6±1.20	3.11×10^6± 0.27*	0.99×10^6± 0.08*	0.60×10^6± 0.08*
Nonviable cell	0.40×10^6±0.06	2.11×10^6± 0.15*	1.34×10^6± 0.05*	3.21×10^6± 0.09*
MST (days)	21	34*	43*	53*
% ILS	00	61.90	104.76	152.38

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* $p < 0.05$). Each point represents the mean±SEM. (n = 6 mice per groups)

Table 2: Effect of MEHS on hematological parameters in EAC bearing mice

Parameters	Normal saline (5 ml/kg)	EAC control (2×10^6 cells/mouse)	EAC + MEHS (50 mg/kg)	EAC + MEHS (100 mg/kg)	EAC + 5-FU (20 mg/kg)
RBC (cell × 10^6/mm^3)	5.29 ± 0.20	1.63 ±0.09	4.83 ±0.08*	3.53 ±0.25*	5.19 ±0.20*
WBC (cell × 10^3/mm^3)	4.98 ± 0.32	7.84 ±1.13	4.70 ±0.62*	4.25 ±0.65*	5.09 ±0.33*
Hb. (g/dL)	11.48 ±0.38	4.23 ±0.14	6.9 ±0.12*	8.4 ±0.21*	10.35 ±0.46*

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* $p < 0.05$). Each point represents the mean±SEM (n = 6 mice per groups)

Serum biochemical parameters

The serum biochemical parameters like amount of SGOT, SGPT and SALP in the EAC control group were significantly ($p < 0.05$) increased as compared to the normal control group and MEHS at the dose 50 mg/kg and 100 mg/kg. The total protein content was found to be significantly ($p < 0.05$) declined in the EAC control group when compared with the normal control group (table 3).

Table 3: Effect of MEHS on serum biochemical parameters in EAC bearing mice

Parameters	Normal saline (5 ml/kg)	EAC control (2×10^6 cells/mouse)	EAC + MEHS (50 mg/kg)	EAC + MEHS (100 mg/kg)	EAC + 5-FU (20 mg/kg)
SGOT (IU/l)	10.84 ± 0.62	19.96 ± 0.86	9.61 ± 0.79*	11.14±0.35*	8.53 ± 0.33*
SGPT (IU/l)	15.45 ± 0.29	2.35 ± 0.73	19.05± 0.60*	22.73±0.62*	18.30 ± 0.56*
SALP (IU/l)	76.87 ± 1.70	200.1 ± 3.89	117.20±2.12*	127.8±1.53*	104.20 ± 4.46*
Protein (g/dL)	9.36 ± 0.14	4.63 ± 0.22	7.22 ± 0.17*	6.62± 0.27*	8.51 ± 0.11*

Statistical significance (p) calculated by one way ANOVA between EAC control group and the treated groups followed by Dunnett's test (* $p < 0.05$). Each point represents the mean±SEM (n = 6 mice per groups)

Tissue antioxidant assay parameters

The level of lipid peroxide in liver tissue was significantly increased in EAC control mice when compared to normal control animals. After treatment with MEHS (50 and 100 mg/kg b. w.), MDA content of liver and were significantly reduced with compared to EAC control mice (fig. 3). Liver homogenates of EAC control group the SOD activity was reduced as compared to the normal controls. The dose dependent enhancement of SOD was observed in case of MEHS treatment group, as compared with EAC control group (fig. 3). The reduction in antioxidant enzyme catalase activity in EAC control mice was improved in the liver by the treatment with MEHS (fig. 3). The reduced GSH level in liver was depleted in EAC control group. Treatment with MEHS significantly elevated reduced GSH level in a dose dependent manner (fig. 3).

DISCUSSION

In the present study, EAC cell line was used to evaluate the anticancer activity of MEHS. EAC or Ehrlich Ascites Carcinoma cells are spontaneous murine mammary rapidly growing adenocarcinoma cells which are highly aggressive in nature and can affect almost all strains of mice. The Ehrlich Ascitic tumor implantation induces a local inflammatory reaction with increasing vascular permeability, which results in an intense edema formation, cellular migration and a progressive ascitic fluid formation and accumulation [16]. The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells [17]. MEHS treatment was able to reduce tumor volume, packed cell volume, viable cell count when compared to the tumor control group probably due to its potency in inhibiting the peritoneal ascitic fluid. These results could connote either a direct cytotoxic effect of MEHS on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition [11]. Increase in life span of the treated animals is a reliable criterion for judging the value of any anticancer drug [3]. The MEHS treated group showed an enhancement of life span compared to the tumor treated group which indicates that MEHS could certainly be a potential candidate for an anticancer drug. The major problems faced during cancer chemotherapy are myelosuppression and anemia [18, 19]. Anemia occurs due to reduction in RBC or destruction of hemoglobin. Treatment with MEHS significantly restored RBC and hemoglobin towards their respective normal levels as compared to the tumor control group. Also the WBC cell count which elevated in the tumor induced group was brought down to near normal levels following MEHS treatment. Enzymes in serum have been studied for many

years as possible early indicators of neoplasia and as aids in following the progression and regression of disease [20]. Hepatotoxicity may occur due to cytotoxic agent itself or due to its toxic metabolites. In certain circumstances, they can be carcinogenic [8].

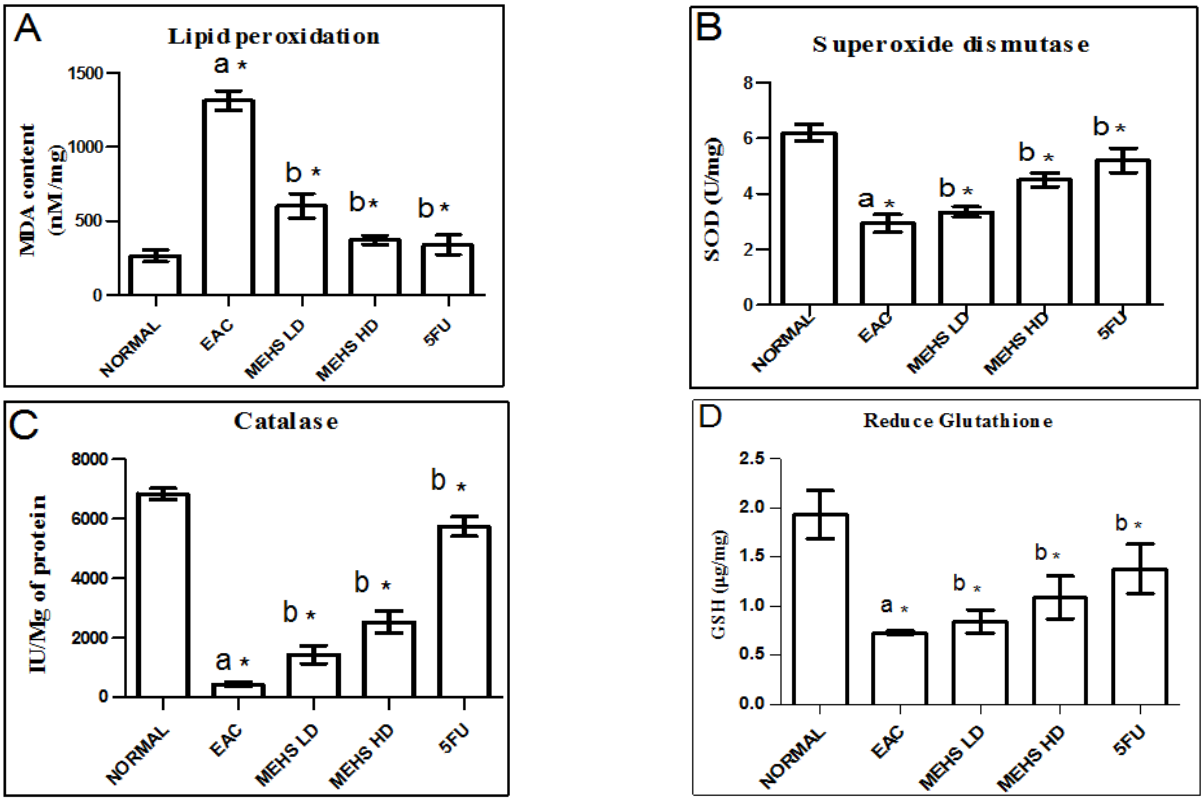

Fig. 3: Effect of MEHS on tissue antioxidant defense parameters like lipid peroxidation (A), superoxide dismutase (B), catalase (C), reduce glutathione (D) in EAC bearing mice. Values are represented as mean± SEM, where n=6. [a]normal control groupvsEAC control group, *$p < 0.05$; [b] EAC control group vs All treated groups, *$p < 0.05$

From the experiment, we found that EAC control group exhibited increased levels of liver enzymes such as SGOT, SGPT and ALP while the levels of total protein were decreased due to hepatocellular damages. The MEHS treated group showed restoration of these biochemical parameters to more or less normal levels. The misbalance between the reactive oxygen metabolites and the antioxidant defence systems leads to 'oxidative stress' which deregulates various cellular functions causing pathological conditions [21, 22]. The oxidative stress may lead to damage of the macromolecules such as lipids and can induce lipid peroxidation *in vivo* [23]. In EAC bearing mice, the level of lipid peroxide in the liver was significantly elevated, which was however reduced to near normal level in the MEHS treated group animals.

This reflects the ability of the extract to decrease free radical production and to subsequently reduce the oxidative stress. Glutathione (GSH), a potent inhibitor of neoplastic proliferation process, plays a crucial role as an endogenous antioxidant system. It was found particularly in high concentration in liver and is known to have a key function in the protective process [24]. The level of non enzymatic antioxidant reduced glutathione (GSH) was reduced in cancer bearing mice which may be due to its utilization by the excessive amount of free radicals generated in the disease state. Treatment with MEHS was found to increase the GSH content in the liver as compared to the tumor control group. The free radical scavenging antioxidant system of endogenous enzymatic antioxidants (SOD and CAT) are present in all oxygen-metabolizing cells, and their function is to provide a defence against the potentially damaging reactivates oxygen species like superoxide anion free radical and hydrogen peroxide [25]. Inhibition of SOD and CAT activity as the result of EAC induced tumor was reported earlier [12]. Treatment with MEHS significantly increased the SOD and CAT levels indicating antioxidant and free radical scavenging activity of the extract.

CONCLUSION

The result of the present investigation is quite encouraging and it explores the potent anticancer activity of MEHS probably because of its direct cytotoxic effect which is further potentiated by its antioxidant properties. Further investigation was going on to find out the molecular mechanism for which anticancer activity shown.

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CONFLICT OF INTERESTS

Declared None

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