

Full Paper

Antitumor Activity of the Methanol Extract of *Hypericum hookerianum* Stem Against Ehrlich Ascites Carcinoma in Swiss Albino MiceSantoshkumar H. Dongre¹, Shrishailappa Badami^{1,*}, Senthilkumar Natesan¹, and Raghu Chandrashekhar H.¹¹Department of Pharmaceutical Chemistry, J.S.S. College of Pharmacy, Rocklands, Ootacamund – 643 001, Tamil Nadu, India

Received September 13, 2006; Accepted November 24, 2006

Abstract. A large number of plants belonging to the *Hypericum* family are known to possess strong antitumor properties. The methanol extract of *H. hookerianum* WIGHT and ARNOTT stem (MEHH) exhibited potent in vitro cytotoxic activity against various cancerous cell lines. In the present study, the high performance liquid chromatography (HPLC) standardized MEHH was tested for in vivo antitumor properties against Ehrlich ascites carcinoma (EAC) tumor bearing mice at 100, 200, and 400 mg/kg body weight doses given orally once daily for 14 days. The results indicate that administration of the extract not only increased the survival of animals with ascites tumor, decreased the body weight induced by the tumor burden, and reduced packed cell volume and viable tissue cell count, but also altered many hematological parameters changed during tumor progression, indicating the potent antitumor nature of the extract. Among the three doses tested, the 200 mg/kg body weight dose was found to be the most potent.

Keywords: *H. hookerianum*, methanol extract, Ehrlich ascites carcinoma (EAC), anticancer

Introduction

The plants of the genus *Hypericum* are widely used in folk medicine (1) and for the treatment of tumors (2). *H. perforatum* (3), *H. mysorence*, *H. patulum* (4), *H. polyanthemum* (5), *H. drummondii* (6), and so on, are reported to possess strong cytotoxic and anticancer properties. Several constituents including hypericin isolated from *H. perforatum* have been studied in detail for their potent anticancer properties (7). In an earlier study carried out in our laboratory, the methanol extracts of the aerial parts, leaves, and stem of *H. hookerianum* exhibited in vitro cytotoxicity against various cancerous cell lines (8). Among the extracts prepared from different parts of the plant, the stem extract was found to be more potent with CTC₅₀ (cytotoxic concentration to kill 50% cells) values of 2.02 µg/ml for RD, 10.25 µg/ml for HEp-2, and 100.6 µg/ml for Vero cell lines. The methanol extracts of *H. hookerianum* leaf and stem were also reported to possess wound healing (9) and antibacterial (10) properties. Except these studies, so far, no other investigations on the

biological activities *H. hookerianum* have been carried out and no phytoconstituents have been reported.

Over the past few years, cancer has remained a major cause of death and the number of individuals living with cancer is continuing to expand. Hence, a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets (11). Due to the enormous propensity of plants, which synthesize a variety of structurally diverse bioactive compounds, the plant kingdom is a potential source of chemical constituents with antitumor and cytotoxic activities (12–14). Therefore, based on the potent cytotoxic properties (8) and the genus possessing a large number of anticancer plants (3–6), we studied the MEHH for its in vivo anticancer activity against the EAC tumor model. In order to use a standardized extract for the present work, HPLC studies were carried out.

Materials and Methods*Plant material and extraction*

The plant material *Hypericum hookerianum* (Family: Hypericaceae) was collected in Ootacamund in the month of July 2004 and was authenticated by Dr. S. Rajan, Medicinal Plants Survey and Collection Unit, Government Arts College, Ootacamund. A speci-

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Published online in J-STAGE

doi: 10.1254/jphs.FP0061088

men voucher is preserved for future reference (Voucher No 2312). The stem of *H. hookerianum* was shade dried, powdered, and extracted (190 g) with methanol (1.2 l) in a Soxhlet extractor for 18–20 h. The extract was concentrated to dryness under reduced pressure and controlled temperature (40°C–50°C). The crude methanol extract was a dark brown solid weighing 40 g (yield, 21.04%). The extract was preserved in a refrigerator at 4°C until further use.

Chemicals

5-Fluorouracil (5-FU) was obtained from Ranbaxy, Ltd., Gurgaon, India. Hyperoside was obtained from Dyang Chemicals Co., Ltd., Hangzhou, China. Trypan blue was obtained from Hi-Media Laboratories, Ltd., Mumbai, India. HPLC grade solvents were obtained from E-Merck, Ltd., Mumbai, India. All the chemicals used were of analytical grade.

Tumor cells

Ehrlich ascites carcinoma (EAC) cells were supplied by Amala Cancer Research Centre, Trissur, Kerala, India. The cells were maintained in vivo in Swiss albino mice by intraperitoneal transplantation. EAC cells aspirated from the peritoneal cavity of mice were washed with saline and given intraperitoneally to develop ascitic tumor.

Preparation of suspensions and solutions

For the short term cytotoxicity assay against the EAC cell line, MEHH was dissolved in dimethyl sulphoxide (DMSO) and the volume made up to 10 ml with DMEM to obtain a 1000 µg/ml stock solution, which was stored at –20°C until further use. Serial two-fold dilutions were made using maintenance medium from the stock solution. The MEHH and standard 5-FU were suspended in distilled water using sodium carboxy methyl cellulose (CMC, 0.3%) and administered orally to the animals with the help of an intragastric catheter to study in vivo antitumor activity.

Animals

Healthy Swiss Albino mice weighing 25 ± 2.0 g were obtained from the animal house, J.S.S. College of Pharmacy, Ootacamund, India. The mice were grouped and housed in polyacrylic cages and maintained under standard conditions ($25 \pm 2^\circ\text{C}$) with 12 ± 1 h dark/light cycle. The animals were fed with rat pellet feed supplied by Hindustan Lever Ltd., Bangalore, India and water ad libitum. All the procedures were reviewed and approved by CPCSEA, Chennai, India (No. JSSCP/IAEC/PH.D/Ph.Chem/03/2005-2006).

HPLC standardization

Hyperoside is a flavonoid constituent present in most of the plants belonging to the genus *Hypericum*. Preliminary HPLC studies of the MEHH and its comparison with standard hyperoside indicated the presence of this compound in the extract. Hence, using hyperoside as a standard, HPLC standardization of the MEHH was carried out. The MEHH was dissolved in methanol (1 mg/ml) and filtered through Whatman filter paper and the filtrate was used for HPLC analysis. Chromatographic separation was performed on a Shimadzu® liquid chromatographic system equipped with a LC-10AT-vp solvent delivery system, SPD M-10 AVP photo diode array detector, and Rheodyne 7725i injector with 5 µl loop. A Phenomenex GEMINI C₁₈ column (25 cm × 4.6 mm i.d., 5 µm) was used for the separation. A mixture (75:25 v/v) of phosphate buffer (25 mM %) and acetonitrile was used as the mobile phase. It was delivered at a flow rate of 1.0 ml per min with detection at 360 nm. The retention time of hyperoside was found to be 5.80 min. The injection volume of the MEHH was 50 µl. Analysis was performed at ambient temperature. Based on the peak area of standard and sample solution, the amount of hyperoside (%) was calculated.

Short-term cytotoxic activity

Short-term cytotoxic activity of the MEHH was assayed by determining the percentage viability of EAC cells using the trypan blue dye exclusion technique (15). EAC cells were cultured in the peritoneal cavity of healthy albino mice weighing between 25 to 30 g by injecting a suspension of EAC cells (1×10^6 cells/ml) intraperitoneally. The cells were aspirated aseptically from the peritoneal cavity of the mice on day 15 and washed with Hank's balanced salt solution (HBSS) and centrifuged for 15 min at 1,500 rpm in a cooling centrifuge. The pellet was re-suspended with HBSS and the process was repeated three times. Finally, the cells were suspended in a known quantity of HBSS and the cell count was adjusted to 2×10^6 cells/ml. Then, 0.1 ml of this diluted cell suspension was distributed into Eppendorf tubes and exposed to 0.1 ml each of the different concentrations of the MEHH and incubated at 37°C for 3 h. After 3 h, the trypan blue dye exclusion test was performed to determine the percentage viability and the CTC₅₀ value was calculated.

Antitumor activity

Swiss Albino mice were divided into six groups (n = 12). All the animals were injected with EAC cells (2×10^6 cells/mouse) intraperitoneally except for the normal group. This was taken as day zero. Group I

served as the normal control and group II served as the tumor control. These two groups received sodium CMC suspension (0.3%). Group III, which served as the positive control, was treated with the suspension of 5-FU at 20 mg/kg body weight. Groups IV, V, and VI were treated with the MEHH at 100, 200, and 400 mg/kg body weight doses, respectively. All these treatments were given 24 h after the tumor inoculation, once daily for 14 days. After the last dose and 24-h fasting, six mice from each group were sacrificed. The blood was collected from the animals by retro-orbital puncture under slight anesthesia (diethyl ether) condition; and the hematological parameters such as red blood cells (RBC), white blood cells (WBC), differential count, and hemoglobin content were determined. The ascitic fluid was collected from the peritoneal cavity of the animals and divided into two parts. One part was centrifuged in a graduated centrifuge tube at 1,000 rpm for 10 min and the packed cell volume was measured. The cells in the other part of the ascitic fluid were separated by centrifugation and stained with trypan blue (0.4% in normal saline). The number of viable cells was counted. The rest of the animals were kept to check average life span and change in body weight for 6 weeks. Percent increase in life span (ILS) was calculated by the following formula: $\% \text{ ILS} = \{(\text{Life span of treated group} / \text{Life span of control group}) - 1\} \times 100$.

Statistical analysis

The significance of the *in vivo* data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison tests. $P < 0.05$ was considered as statistically significant.

Results

HPLC standardization

Typical chromatograms of hyperoside and the MEHH are shown in Fig. 1, a and b, respectively. The amount of hyperoside present in the MEHH was found to be $0.36 \pm 0.01\%$ w/w.

Short term cytotoxicity

The CTC_{50} value of MEHH against EAC was found to be $387.50 \pm 4.50 \mu\text{g/ml}$.

Effect of MEHH on antitumor parameters

The animals of the tumor control group inoculated with EAC survived for a period 16.66 ± 0.95 days. The treatment with the MEHH at 100, 200, and 400 mg/kg body weight increased the average life span of animals by 19.16 ± 2.28 , 23.83 ± 2.33 , and 22.00 ± 1.73 days, respectively (Fig. 2). The increases in life span at 200

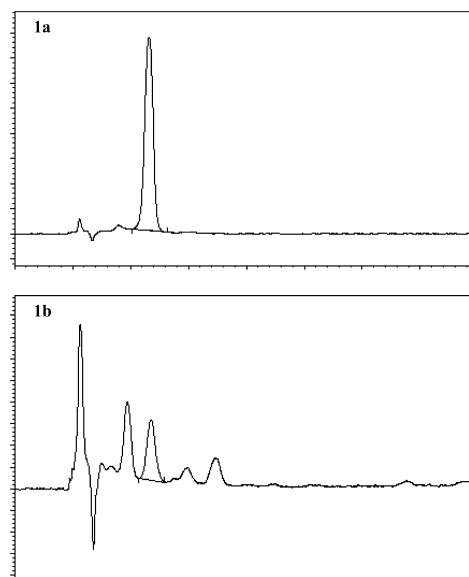


Fig. 1. HPLC of a) standard hyperoside and b) the MEHH.

and 400 mg/kg body weight were found to be significant. The MEHH at the 200 mg/kg body weight dose was found to be more potent in inhibiting the proliferation of EAC with the percentage increase in life span of 43.03%. The percent increase in body weight of the EAC tumor control group was found to be $35.26 \pm 0.58\%$. The MEHH treatment at 200 and 400 mg/kg doses significantly inhibited the percent increase in body weight when compared to the tumor control ($P < 0.001$). The packed cell volume (ml), viable tumor cell count ($\times 10^7$ cells/ml), and total WBC ($\times 10^3/\text{mm}^3$) were found to decrease significantly in animals treated with the MEHH at almost all the doses tested when compared to the EAC tumor control, indicating the antitumor nature of the extract.

Similarly, RBC count, hemoglobin content, and lymphocytes count, which were decreased after EAC inoculation, were found to be significantly restored to the normal levels in the animals treated with the MEHH at all the three doses (Table 1). The neutrophils count, which was increased in EAC tumor control animals, was found to be decreased towards the normal by the MEHH significantly ($P < 0.001$) at all the doses. All these results suggest the potent antitumor properties of the MEHH. However, the standard 5-FU treatment at 20 mg/kg body weight produced better results than the extract treatment in all these parameters.

Discussion

Cancer is a disease of misguided cells that have high potential of excess proliferation without apparent

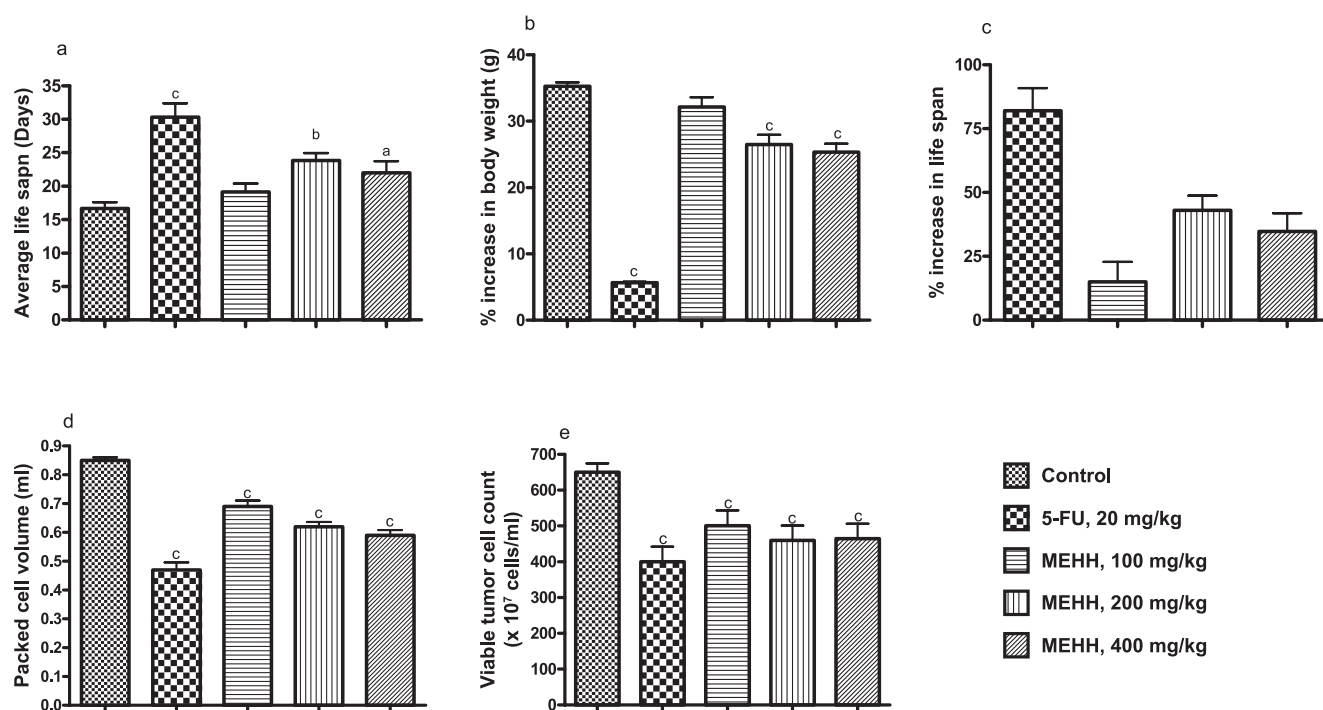


Fig. 2. Effect of MEHH on EAC tumor-bearing mice. a: average life span, b: % increase in body weight on day 15, c: % increase in life span, d: packed cell volume on day 15, and e: viable tumour cell count on day 15. Data are expressed as the mean $\pm$ S.E.M. (n = 6); ^a $P < 0.05$, ^b $P < 0.01$, and ^c $P < 0.001$: between tumor control and treated group.

Table 1. Effect of the MEHH stem on hematological parameters of EAC-bearing mice on day 15 of the experiment

Parameters	Treatment (Dose, mg/kg body weight)					
	Normal	EAC control (2×10^6 cells/ml per mice)	EAC + MEHH (100)	EAC + MEHH (200)	EAC + MEHH (400)	EAC + Standard 5-FU (20)
Total WBC ($\times 10^3/\text{mm}^3$)	7.61 $\pm$ 0.46	12.45 $\pm$ 0.62 ^a	10.43 $\pm$ 0.74	9.96 $\pm$ 0.71 ^b	9.78 $\pm$ 0.24 ^b	9.36 $\pm$ 0.26 ^c
RBC ($\times 10^6/\text{mm}^3$)	11.22 $\pm$ 0.47	5.70 $\pm$ 1.16 ^a	10.26 $\pm$ 0.39 ^d	9.30 $\pm$ 0.39 ^c	9.41 $\pm$ 0.48 ^c	9.08 $\pm$ 0.74 ^c
Hgb (g/dl)	15.56 $\pm$ 0.44	7.22 $\pm$ 0.71 ^a	11.02 $\pm$ 0.41 ^d	11.41 $\pm$ 0.87 ^d	11.67 $\pm$ 0.52 ^d	12.68 $\pm$ 0.53 ^d
WBC Differential count (%)						
Lymphocytes	66.25 $\pm$ 0.79	35.75 $\pm$ 0.70 ^a	61.00 $\pm$ 1.96 ^d	65.25 $\pm$ 1.37 ^d	60.00 $\pm$ 1.15 ^d	66.75 $\pm$ 0.70 ^d
Neutrophils	28.00 $\pm$ 0.51	64.83 $\pm$ 0.90 ^a	38.50 $\pm$ 1.58 ^d	34.16 $\pm$ 1.44 ^d	35.35 $\pm$ 1.72 ^d	33.83 $\pm$ 2.18 ^d
Eosinophils	4.75 $\pm$ 0.47	5.75 $\pm$ 0.62	5.57 $\pm$ 0.62	4.75 $\pm$ 0.47	4.50 $\pm$ 0.64	5.50 $\pm$ 0.64

Values are expressed as the mean $\pm$ S.E.M. for six animals in each group. ^a $P < 0.001$: between normal and EAC tumor group values; ^b $P < 0.05$, ^c $P < 0.01$, and ^d $P < 0.001$: between tumor control and treated groups.

relation to the physiological demand of the process. It is the second largest cause of death in the world. Of all the available anticancer drugs during 1940–2002, 40% were natural products *per se* or natural product derived, with another 8% being natural product mimics (16). The greatest recent impact of plant derived drugs is observed in the area of antitumor research, where compounds such as taxol, vinblastine, vincristine, and

camptothecin have dramatically improved the effectiveness of chemotherapy against some of the dreaded cancers (17). Hence, there is a great potential for the development of anticancer drugs from the essentially untapped reservoir of the plant kingdom. A large number of plants possessing anticancer properties have been documented (12, 18–21). Plants belonging to the genus *Hypericum* and several of their constituents have shown

potent anticancer properties in many models based on the studies conducted throughout the world (2–8). Earlier studies carried out in our laboratories have shown potent cytotoxic properties of *H. hookerianum* (8). Based on these observations, in the present study, the MEHH was evaluated for its *in vivo* antitumor properties.

Ehrlich tumor is a rapidly growing carcinoma with very aggressive behavior (22). It is able to grow in almost all strains of mice. The Ehrlich ascitic tumor implantation induces *per se* a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, and a progressive ascitic fluid formation (23). The ascitic fluid is essential for tumor growth, since it constitutes a direct nutritional source for tumor cells (20). The packed cell volume and the number of viable EAC tumor cells in peritoneum were significantly lower in mice treated with MEHH when compared to the tumor control group. These results could indicate either a direct cytotoxic effect of MEHH on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition. The direct strong cytotoxic effect of MEHH was already demonstrated in the *in vitro* experiments against RD and HEP-2 cell lines (8) and in the present studies, against EAC cells.

The reliable criterion for judging the value of any anticancer drug is the prolongation of life span of the animals and the disappearance of leukemic cells from blood (21). The MEHH treatment decreased the packed cell volume and viable tumor cell count. It also inhibited the increase in body weight due to tumor burden and increased the average life span of animals when compared to the EAC control. Hence, it may be concluded that MEHH, by a direct cytotoxic effect or by decreasing the nutritional fluid volume and arresting the tumor growth, increased the life span of EAC-bearing mice. The percentage increase in life span at the 200 mg/kg body weight dose of the MEHH was found to be the highest among the three doses tested, indicating its potent anticancer nature. No toxic symptoms were observed for all three doses during the period of study.

A significant decrease in hemoglobin and the number of erythrocytes and a significant increase in total WBC in the tumor-bearing mice are known. Anemia is found frequently in cancer patients (24). Similar results were observed in the present study in animals of the EAC tumor control group. A significant decrease in the total WBC and neutrophils count was observed by the MEHH treatment when compared to the tumor control. A significant increase in the RBC, hemoglobin, and lymphocytes towards the normal values by the extract treatment was observed (Table 1). The reversal of

hematological parameters indicates that the MEHH may possess protective action on the hemopoietic system. Similar results were obtained by other workers (21).

The preliminary phytochemical studies indicated the presence of flavonoids, saponins, triterpenoids, glycosides, and tannins in the MEHH. Many such compounds are known to possess potent antitumor properties (13). The potent antitumor properties of the MEHH may be due to the presence of any of these phytoconstituents. In conclusion, the present study demonstrates the potent antitumor properties of the MEHH and the plant merits further investigation in isolating its active constituents.

Acknowledgments

The authors thank Department of Biotechnology (DBT), Ministry of Science & Technology, New Delhi for funding the work. One of the authors (SHD) thanks the DBT for awarding him Junior and Senior Research fellowships.

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