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Antitumor Activity of Flavonoids against Ehrlich Ascites Carcinoma-induced Mice

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ABSTRACT

Flavonoids are phenolic compounds widely present in plants and foods of plant origin. Methylhesperidine and chrysin were evaluated for *in vivo* antitumor activity against Ehrlich ascites carcinoma (EAC)-bearing Swiss albino mice. The present study deals with the effect of methyl hesperidine and chrysin on the growth of transplantable murine tumor, life span of EAC-bearing hosts, hematological profile, and biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and serum creatinine levels. Test compounds were administered at the dose of 20 mg/kg body weight per day for 14 days after 24 h of tumor inoculation. After the last dose and 18 h fasting, the blood samples were collected from tail vein of all the mice. Both flavonoids produced a significant ($P < 0.05$) decrease in tumor volume, packed cell volume and viable tumor cell count, and they prolonged the life span of EAC-bearing mice. Compared to control mice, hematological profile is more or less normal levels in flavonoids-treated mice. Selected flavonoids significantly ($P < 0.05$) decreased the levels of serum creatinine levels. The results indicate that methylhesperidine and chrysin exhibited significant *in vivo* antitumor activity in EAC-bearing mice.

Keywords: EAC mice, tumor groth, mean survival time, anti tumor activity
Abbreviations: CAT, catalase; **DMSO**, dimethyl sulfoxide; **EAC**, Ehrlich ascites carcinoma; **ILS**, increased life span; **MST**, mean survival time; **RBC**, red blood cell; **SGOT**, serum glutamic oxaloacetic transaminase; **SGPT**, serum glutamic pyruvic transaminase; **SOD**, super oxide dismutase; **WBC**, white blood cell

INTRODUCTION

India is a rich source of many medicinal plant derived natural products such as flavonoids, alkaloids, terpenes (Stevenson and Lowe 2009; Hounsome *et al.* 2010), which have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxicity and cancer chemo-protective effects (Roja and Heble 1994). Flavonoids are nearly ubiquitous in plants. They are rich in seeds, citrus fruits, olive oil, tea, and red wine. Flavonoids, like methylhesperidine and chrysin, have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances, and pigments and light screens (Carroll *et al*. 1998). They inhibit many enzymes like kinases, lipoxygenases and cyclooxygenases, phospholipase C, cyclic nucleotide phosphodiesterase, reverse transcriptase, RNA and DNA polymerases (Middleton *et al*. 2000). Flavonoids have been reported to possess a number of biological activities and are well known for their antioxidant properties (Rajnarayana *et al*. 2001). There are a number of reports on different natural products derived from plants indicate that they exert multiple biological effects due to their anti oxidant and free radical scavenging abilities of the flavonoids. These natural compounds were reported to produce protective effects against tumors, heart disease and different diseases (DeFeudis *et al*. 2003). Based on the above facts the present work has been carried out to evaluate the *in vivo* antitumor activity of methylhesperidine and chrysin against Ehrlich ascites carcinoma (EAC) in Swiss albino mice.

MATERIALS AND METHODS

Flavonoids

The flavonoids methylhesperidine and chrysin were procured from Sigma-Aldrich Chemicals Ltd. (Germany). The stock solutions (100 mg/ml) of flavonoids were prepared using 1% DMSO and further diluted with water to obtain the required solutions (20 mg/kg) which were to be administered in the study. 5-fluorouracil (99% pure, a kind gift from Vimta laboratories ltd, Hyderabad, India) was used as a standard drug which was procured from the local market.

Animals

The study was carried out using Swiss albino male mice weighing 20 ± 2 g. They were obtained from the National Institute of Nutrition, Hyderabad, and Aandhra Pradesh. The mice were grouped and housed in polyacrylic cages with not more than 8 animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with a 14/10 h dark/light cycle. They were allowed free access to standard dry pellet diet (National Institute of Nutrition, Hyderabad, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before starting the experiment. All procedures were accepted by the University Animals Ethical Committee [Ref No: Ucpsc/K.U/01/2007].

Tumor cells

An EAC mouse was initially obtained from Vel's College of Pharmacy, Pallavaram, Chennai, India. EAC cells were collected (Mazumder *et al*. 1997) form that mouse and maintained by intraperitonial (i.p.) inoculation of 2×10^6 cells administered to other mice at the Pharmacology Research Lab, University College of

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Antitumor activity using EAC mice

Swiss albino mice were than divided in to 4 groups ($n = 8$). All the groups were injected i.p. with EAC cells (0.3 ml of 2×10^6 cells/ mouse). This was day zero. The EAC-control group [group 1] animals did not receive any drug. Standard drug 5-florouracil (Kavimani and Manisenthil kumar 2000), methylhesperidine and chrysin (20 mg/kg per day) were administered i.p. to the mice in groups 2, 3 and 4 respectively for 14 days. All mice were weighed on the day of tumor inoculation and every day before treatment. After a last dose and 18-h fasting, blood samples were collected form tail vein of mice and estimated for different hematological and biochemical parameters (Mazumder *et al*. 1997). Tumor growth response was measured by studying MST and ILS. Most tumor-induced animals died after 4 weeks. So we extended our study up to 5 weeks or 35 days. The duration of the study was thus 35 days. Results were expressed as the mean ± standard deviation. Statistical evaluation was done by ANOVA followed by Newman-Keul's test and the difference was considered statistically significant at $P < 0.05$. Graphpad prism software (Version 7, Lajolla, USA) was used for statistical analysis.

Tumor growth response

The tumor growth response of flavonoids was assessed by a change in body weight, ascites tumor volume, packed cell volume, viable and non viable tumor cell count, MST and %ILS. MST of each group was monitored by recording the mortality daily for 5 weeks and %ILS was calculated using following equation (Mazumder *et al*. 1997).

MST= (Day of first death+ Day of last death)/2

ILS (%) = [(Mean survival time of treated group/mean survival group of control group)-1] ×100.

Hematological profile

The blood samples were send to the physiology lab for the estimation of hemoglobin content, RBC and WBC count and Differential leukocyte count using Neubauer slide (Wintrobe *et al*. 1958; D'Armour *et al*. 1965).

Biochemical parameters

After the collection of blood samples SGOT, SGPT, serum creatinine levels were estimated in the biochemistry laboratory using standard SGOT, SGPT, serum creatinine kits [Marketed products of Dr. Reddy's Laboratories Ltd., Hyderabad, India].

RESULTS

The results of the present study indicate that methylhesperidine and chrysin showed significant antitumor activity in EAC-bearing mice. The effect of flavonoids at a dose of 20 mg/kg on MST, %ILS, tumor volume, packed cell volume, and tumor cell count (viable and non viable) are shown in **Table 1**.

Effect on mean survival time (MST)

In the EAC control group, the MST was 14.5 ± 0.16 days, while it increased to 21.5 ± 2.27 days with methyl hesperidine, where as standard drug (5-flourouracil)-treated group had MST of 29 ± 3.47 days. No change was observed in MST with chrysin when compare with control mice (**Table 1**).

Effect on tumor growth

Treatment with methyl hesperidine and chrysin significantly ($P < 0.01$) reduced the packed cell volume and the viable tumor cell count as compared to that of the EAC control group. Furthermore non viable tumor cell count was significantly ($P < 0.05$) increased by methyl hesperidine (**Table 1**).

Effect on hematological parameters

Hematological parameters of flavonoids treated mice on day 14 showed some changes when compared with the EAC mice (**Table 2**). RBC, neutrophil and platelet count increased with methyl hesperidine. The differential count of WBC showed that the neutrophil, platelet count increased while the RBC and hemoglobin decreased.

Effect on SGOT, SGPT, and Serum creatinine

Some changes found in the SGOT, SGPT and serum createnine levels of flavonoids treated mice when compared with EAC mice (**Table 3**). Treatment with methyl hesperidine significantly increased the SGOT levels. The serum createnine levels were found to decrease significantly when compared with the EAC-control group. There are no significant changes were observed in SGPT levels.

Table 1 Effect of the flavonoids on mean survival time, %ILS, tumor volume, packed cell volume and viable and non viable tumor cell count of EAC-bearing mice.

Parameters	EAC control (2×10^6 cells/ml/mouse)	Std (5-fu) (20 mg/kg + EAC)	Methyl hesperidine (20 mg/kg + EAC)	Chrysin (20 mg/kg + EAC)
Body weight (g)	33.61 ± 1.44	27.78 ± 1.41	30.83 ± 2.17	32.63 ± 0.44
Mean survival time (days)	14.5 ± 0.16	29 ± 3.47 ***	21.5 ± 2.27 **	14.5 ± 0.12
Increased life span (%)	--	100	48.3	0
Tumor Volume (ml)	13 ± 0.70	3.6 ± 0.23***	10 ± 0.70	10.38 ± 0.68
Packed cell volume (ml)	2.63 ± 0.23	0.13 ± 0.12 ***	1.5 ± 0.20 ***	1.08 ± 0.14***
Viable tumor cells (X 10^7 cells / ml)	6.64 ± 0.02	0.11 ± 0.11 ***	6.16 ± 0.02**	5.44 ± 0.18***
Non via tumor cell (X 10^7 cells / ml)	0.06 ± 0.018	0.02 ± 0.022	0.12 ± 0.11**	0.11 ± 0.004

Data are reported as mean ± SEM (n=8). ***$P < 0.001$, EAC standard group compared with control group. ** $P < 0.05$ flavonoids treated group compared with EAC control group.

Table 2 Effect of the flavonoids on hematological parameters of EAC- bearing mice.

Parameters	EAC control (2×10^6 cells/ml/mouse)	Std (5-fu) (20 mg/kg + EAC)	Methyl hesperidine (20 mg/kg + EAC)	Chrysin (20 mg/kg + EAC)
Hemoglobin (g %)	13.87 ± 1.14	12.62 ± 0.88	12 ± 1.07	13.7 ± 1.31
RBC (ml/cm)	7.39 ± 0.79	7.38 ± 0.61	7.91 ± 0.73	7.70 ± 1.22
WBC (T)/cm	30.47 ± 13.29	23.55 ± 4.92	40.55 ± 16.71	13.31 ± 2.05
Nutrophils (%)	38 ± 3.1	31.5 ± 7.12	48.25 ± 11.59	31.25 ± 4.49
Lymphocytes (%)	62 ± 3.1	68.5 ± 7.12	51.75 ± 11.59	68.75 ± 4.49
Platelet count X 10^5/ml	12.19 ± 3.16	9.70 ± 1.64	31.84 ± 19.49	12.75 ± 1.51

Data are reported as mean ± SEM (n=8).

Table 3 Effect of the flavonoids on the body weight and different biochemical parameters of EAC- bearing mice.

Parameters	EAC control (2×10^6 cells/ml/mouse)	Std (5-fu) (20 mg/kg + EAC)	Methyl hesperidine (20 mg/kg + EAC)	Chrysin (20 mg/kg + EAC)
SGOT (U/L)	73.55 ± 24.39	167.25 ± 30.56	311 ± 72.98 **	229.5 ± 24.92
SGPT (U/L)	77.33 ± 30.57	67.03 ± 8.16	59.98 ± 12.39	35.05 ± 6.7
Serum creatinine (mg/dl)	0.64 ± 0.23	0.15 ± 0.04 **	0.18 ± 0.04 **	0.14 ± 0.04 **

Data are reported as mean ± SEM (n=8). ** $P < 0.05$ flavonoids treated group compared with EAC control group.

DISCUSSION

Many components isolated from plants have been approved to be potent anti-cancer agents. Plant-derived polyphenolic compounds are promising nutraceuticals for control of various disorders and cancer. These compounds may be the future developing anticancer drugs with no side effect and low cost for people all around the world. The much lower risk of colon, prostate and breast cancers in Asians, who consume more vegetables, fruits and tea than populations in the western hemisphere, raises the role of flavonoids components as protective factors against carcinogenesis (Rand *et al*. 2009).

The observations of the past studies were saying that the methanol extract of *Caesalpinia bonducella* leaves treated animals significantly inhibited the tumor volume, packed cell volume, tumor cell count, and brought back to normal levels. The extract also restored the hepatic lipid peroxidation and free radical scavenging enzyme GSH as well as antioxidant enzymes such as SOD and CAT in tumor-bearing mice to near normal levels. The pharmacological activity of MECB is due to the presence of flavonoids (Gupta *et al*. 2004).

In another previous study the effect of various natural flavonoids, cinnamic acid derivatives, and a series of synthetic flavones on cell proliferation was evaluated *in vitro* in a panel of established human and murine tumor cell lines. The *in vitro* activity of different natural flavonoids, cinnamic acid derivatives, and a series of synthetic flavones in established cancer cell lines was studied. Analysis of each group of compounds indicated that apigenin, caffeic acid *n*-butyl ester, and 2′-nitroflavone possess the most potent antiproliferative activities (Mariano *et al*. 2006). LYG-202 significantly decreases tumor growth in mice inoculated with S180 sarcoma cells, compared with the control group. Meanwhile, the viabilities of various kinds of tumor cells were inhibited by LYG-202 with IC_{50} values in the range of 4.80 to 27.70 μM (Zeng *et al*. 2009).

In other study, the polyphenolic extract (PPE) of leaves of *Ichnocarpus frutescens* was evaluated for antitumor activity *in vivo*. A murine Ehrlich ascites carcinoma (EAC) model was used to assess PPE antitumor activity *in vivo*. Results of *in vivo* study showed a significant decrease in tumor volume, viable tumor cell count and a significant increase of life span in the PPE treated group compared to untreated one: the life span of PPE treated animals increased by 53.41% (50 mg PPE/kg) and 73.95% (100 mg PPE/kg) (Kumarappan and Subhash 2007).

Santoshkumar *et al*. (2007) reported that the methanolic extract of *H. hookerianum* Wight and Arnott stem (MEHH) exhibited potent *in vitro* cytotoxic activity against various cancerous cell lines. 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.

Flavones, flavonols, isoprenoid-substituted flavonoids, benzophenones, xanthones, anthraquinones, phenylbutazone glucoside, stilbene glucoside, coumarin derivatives, hydroxyketones, stylrylchromones, dihydroisoxazole and isoxazole derivatives showed low to moderate tumor-specificity. There was no strict relationship between the tumor-specific cytotoxicity and apoptosis induction (Sakagami *et al*. 2007). Based on the results of the previous studies, it was proved that the methyl hesperidine and chrysin like flavonoids present in the plant extracts were responsible for some of the important biological activities of plant extracts.

So the present study was carried out to evaluate the *in vivo* antitumor effect of bioflavonoids methylhesperidine and chrysin in EAC-bearing mice. The flavonoids treated animals at the dose 20 mg/kg significantly inhibited the tumor volume, packed cell volume, tumor cell count and brought back the hematological parameters to normal levels. The flavonoids also restored the SGOT, SGPT and serum creatinine levels. During 14 days the test compounds did not exhibit any adverse effects like alopecia, weight reduction and vomiting.

In EAC–bearing mice, a regular rapid increase in ascites tumor volume was noted. Ascites fluid is the direct nutritional source for tumor cells and a rapid increase in ascetic fluid with tumor growth would be a means the nutritional requirement of tumor cells (Prasad and Giri 1994; Rajkapoor *et al*. 2004). Treatment with methyl hesperidine significantly increased the mean survival time. Methyl hesperidine and chrysin significantly decreased the packed cell volume, viable tumor cell count which shows the tumor protective activity of both flavonoids. Usually in cancer chemotherapy the major problems that are encountered are myelosuppression and anemia. The anemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage, and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions. Treatment with methyl hesperidine, chrysin brought back the hemoglobin content, RBC (mil/cmm), and WBC (T/cmm) count more or less to normal levels. This indicates that the test drugs possess protective action on hemopoietic system. SGOT levels were significantly increases with methyl hesperidine. Serum creatinine levels were significantly decreases with methyl hesperidine, chrysin. This indicates the hepato and renal protective activity of both flavonoids (**Table 3**).

The present study demonstrates that flavonoids methyl hesperidine and chrysin increased the life span of EAC-tumor bearing mice and decreased the non viable tumor cells. The above parameters are responsible for the anti tumor activity of the methyl hesperidine, chrysin.

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