

The effect-enhancing and toxicity-reducing activity of *Hypericum japonicum* Thunb. extract in murine liver cancer chemotherapy

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Received September 18, 2012; Accepted December 11, 2012

DOI: 10.3892/mco.2012.52

Abstract. Chinese herbs are potential sources of antitumor drugs with immunoregulatory activity and few adverse effects. In the present study, we investigated whether the *Hypericum japonicum* Thunb. (HJT) extract enhanced the efficacy of 5-fluorouracil (5-FU) treatment in murine liver tumor xenografts and reduced toxicity of chemotherapy in hepatoma H22-bearing mice. Tumor weight and inhibition rate, thymus and spleen indices, as well as white blood cell (WBC) count were calculated. The phagocytic function of macrophages was assessed by observing peritoneal macrophages phagocytized chicken red blood cells (RBC). Body weight and toxic reactions of the chemotherapeutic and life prolongation rate were investigated in the mice. Results demonstrated that the HJT extract significantly enhanced the tumor inhibition rate of 5-FU, improved the immune function, reduced the toxic effects and prolonged the survival time in the tumor-bearing mice. Taken together, these results indicated that the HJT extract has a synergistic tumor-inhibiting effect with 5-FU, is able to reduce the toxic side effects and is likely to be safe and efficacious for use in antitumor therapy.

Introduction

Liver cancer is a malignant disease with a high incidence and mortality, and is often diagnosed at an advanced stage (1). Common therapies, such as surgical resection, percutaneous or transarterial interventions are of limited efficacy (2), as chemotherapy, one of the main measures for a comprehensive treatment, may induce adverse effects such as bone marrow

suppression, gastrointestinal reactions and lowered immunological function.

Several Chinese herbs have been identified as potential sources of antitumor drugs (3-5). The effect-enhancing and toxicity-reducing activity of Chinese herbs in radiotherapy and chemotherapy of tumors was previously confirmed (6,7). However, no studies focusing on the antitumor and toxicity-reducing effects of *Hypericum japonicum* Thunb. (HJT) have been reported thus far. Therefore, the present investigation was conducted to ascertain whether the HJT extract enhanced the efficacy of 5-fluorouracil (5-FU) treatment in murine liver tumor xenografts, and reduced toxicity of chemotherapy in tumor-bearing mice.

Materials and methods

Reagents. RPMI-1640 medium, fetal bovine serum (FBS), penicillin G and streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA, USA). 5-FU was obtained from the Shanghai Xudong Haipu Pharmaceutical Co., Ltd. (Shanghai, China). Murine H22 hepatoma cells were purchased from the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China).

Animals. Institute of Cancer Research (ICR) mice (male/female, 50-50%; weight, 20±2 g) were purchased from the Experimental Animal Center, Zhengzhou University (Zhengzhou, China). The animals were kept in a standard laboratory, under a 12-h light/dark cycle, at room temperature (20±2°C), in a humid (75±15%) and filtered laminar air flow-controlled room in the animal facility of the Experimental Animal Center of the Zhengzhou University. Mice were raised and cared for, given autoclaved water and fed *ad libitum* with laboratory pellet chow. Experiments were performed according to the regulations and guidelines for animal experiments in China. This study was approved by the Ethics Committee of Zhengzhou University, Zhengzhou, China.

Cell culture. H22 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 1x10⁵ U/l penicillin and 100 mg/l streptomycin in a humidified atmosphere with

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Key words: antitumor, *Hypericum japonicum* Thunb., hepatoma, chemotherapy, mice

Table I. Effect of the HJT extract on body and tumor weight of the H22-bearing mice [mean \pm standard deviation (SD), n=10].

Group	Dose (g/kg/day)	Body weight (g)		Variation of body weight (g)	Tumor weight (g)	Tumor inhibition rate (%)
		Pre-treatment	Post-treatment			
Normal control	-	20.05 \pm 2.07	24.35 \pm 3.24	4.30 \pm 3.47 ^{a,b}	-	-
Tumor control	-	20.11 \pm 1.94	21.07 \pm 2.02	0.96 \pm 2.13	2.70 \pm 1.13	-
5-FU	0.02	19.98 \pm 2.03	20.55 \pm 1.86	0.57 \pm 1.89 ^a	1.59 \pm 0.59 ^a	41.11
	0.02+3.00	20.01 \pm 2.25	21.49 \pm 1.94	1.48 \pm 1.65 ^{a,b}	1.23 \pm 0.54 ^{a,b}	54.44
5-FU + HJT	0.02+6.00	20.07 \pm 2.06	22.15 \pm 1.75	2.08 \pm 2.03 ^{a,b}	1.13 \pm 0.42 ^{a,b}	58.15
	0.02+12.00	19.96 \pm 1.95	23.24 \pm 2.05	3.28 \pm 2.19 ^{a,b}	0.91 \pm 0.63 ^{a,b}	66.29

^aP<0.05 vs. tumor control group; ^bP<0.05 vs. the 5-FU group. HJT, *Hypericum japonicum* Thunb.

5% CO₂ incubator at 37°C. The cells were subcultured until logarithmic growth phase was reached.

Preparation of the HJT extract. HJT extract was obtained from Xi'an Kanger America Biotechnology Co., Ltd. (Xi'an, China). Air-dried HJT samples were ground and mixed with double-distilled water at 100°C for 10 min, then cooled to room temperature. HJT was then stored at 4°C in dark bottles and kept sterile until instilled intragastrically in the mice.

Experimental design. ICR mice were randomized into A-F groups (n=10). The animals were subcutaneously injected with H22 cells (1 \times 10⁷ cells/mouse) into the armpit of the right hind limb, with the exception of group A (normal group), which received sodium suspension (0.9%). Group B served as the tumor control. In groups C, D, E and F, 5-FU (20 mg/kg) was injected into the abdominal cavity, once daily. Groups D (low-dose HJT group), E (medium-dose HJT group) and F (high-dose HJT group) received perfusion of HJT extract intragastrically at 3, 6 and 12 g/d per kg, administered 24 h after the tumor inoculation, once daily for 10 days.

Determination of antitumor activity in vivo. The general condition of mice such as movement, fur, eating, drinking, body weight increase and toxic responses was examined. Twenty four hours after the last dose, blood was collected from the animals for the white blood cell (WBC) count by retro-orbital puncture under slight anesthesia (diethyl ether). Mice from each group were sacrificed under anesthesia using CO₂. The mice and the separated tumor were weighed. Antitumor activity was evaluated by tumor weighing. The tumor inhibition rate was calculated as: Tumor inhibitory rate (%) = (1-average tumor weighing of administration group/average tumor weighing of the tumor control group) \times 100% (9). Simultaneously, the thymus and spleen were excised and weighed. Indices were calculated as: Thymus index = the thymus weight (mg)/body weight (g) and spleen index = the spleen weight (mg)/body weight (g) (10).

Determination of the phagocytic function of macrophage. After the last dose and 12-h fasting, chicken-red cells (1 ml, 5%) were injected intraperitoneally into each group. After 12 h, the mice were sacrificed and normal saline (2 ml) was

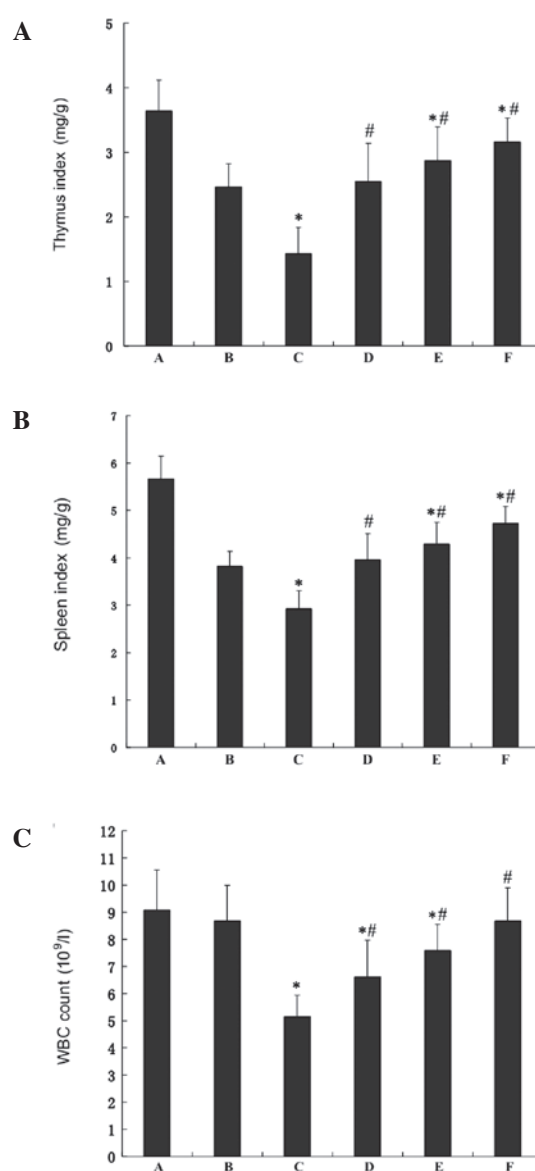


Figure 1. Effect of *Hypericum japonicum* Thunb. (HJT) extract on immune organs and white blood cell (WBC) count of H22-bearing mice. Group A, normal control; Group B, tumor control; Group C, 5-FU; Group D, 5-FU + low-dose HJT; Group E, 5-FU + medium-dose HJT; Group F, 5-FU + high-dose HJT. Results are presented as the mean \pm standard deviation for the animals in each group. *P<0.05 vs. tumor control group; #P<0.05 vs. 5-FU group.

Table II. Effects of the HJT extract on the life prolongation rate in the H22-bearing mice [mean \pm standard deviation (SD), n=10].

Group	Dose (g/kg/day)	Survival time (day)	Life prolongation rate (%)	
			vs. tumor control group	vs. 5-FU group
Tumor control	-	15.98 \pm 4.85	-	-
5-FU	0.02	16.23 \pm 4.03	1.56	-
	0.02+3.00	20.13 \pm 3.89 ^{a,b}	25.97	24.03
5-FU + HJT	0.02+6.00	22.97 \pm 4.31 ^{a,b}	43.74	41.53
	0.02+12.00	25.93 \pm 5.07 ^{a,b}	62.27	59.77

^aP<0.05 vs. tumor control group; ^bP<0.05 vs. the 5-FU group. HJT, *Hypericum japonicum* Thunb.

injected into the abdominal cavity. Peritoneal fluid (1 ml) was then drawn for glass slides. Following incubation for 30 min, peritoneal fluid was fixed with a mixture of acetone/methanol (1:1, v/v) and stained with 4% Giemsa. After drying, peritoneal macrophages were counted by microscopy. The effect of the HJT extract on phagocytosis of enterocoelia macrophage was evaluated by the chicken-red cell phagocytic rate and index, calculated using the formulas: phagocytic index = chicken-red cells phagocytized/total macrophages and phagocytic rate = (macrophages that phagocytized red blood cells (RBC)/total macrophages) \times 100% (11).

Determination of life prolongation rate in the H22-bearing mice. The grouping and drug administration for this experiment were the same as described above (n=10). H22 cells (1×10^7 cells/mouse) were injected into the abdominal cavity of each mouse. The survival time of the mice was recorded for 35 days after terminating drug administration. The experiment was considered invalid if the mortality rate in the control group during the experiment was >20%, or the survival time of 20% mice was >5 weeks. The life prolongation rate was determined as (the mean living days of the mice in the treatment group/the mean living days in the tumor control group-1) \times 100%.

Statistical analysis. Values were expressed as the mean \pm standard deviation (SD). Statistical analysis was performed with one-way analysis of variance (ANOVA) using the statistical software SPSS 17.0. P<0.05 was considered to indicate a statistically significant difference.

Results

Antitumor and toxicity-reducing activity of the HJT extract on hepatoma. H22-bearing mice in the tumor control group were not lively, with dark fur, and exhibited loss of appetite as well as slow weight gain. Additionally, mice in the 5-FU group died prior to the last administration. By contrast, mice in the HJT extract groups were vigorous, with shiny fur and a rapid body weight increase, showing a good condition. The toxic effects in the 5-FU group were severe, including anorexia, abdominal distention and emaciation, while the mice in the HJT extract groups showed no or little toxicity.

No statistically significant difference was detected in the body weight between each group prior to treatment (P>0.05) (Table I). Following the experiment, the body weight of the tumor-bearing mice in the groups increased to varying degrees. The body weight increase was significantly higher in each HJT group compared with the tumor control group (P<0.05), whereas the increase in the 5-FU group was significantly lower compared with the other groups (P<0.05). The tumor weight of three HJT groups and the 5-FU group was significantly lower compared with the tumor control group (P<0.05). Compared with the 5-FU group, the tumors of three HJT groups showed a significant decrease in size, with the HJT dose group increasing (P<0.05). The tumor inhibitory rates of the 5-FU group and HJT groups were 41.11, 54.44, 58.15 and 66.29%, respectively.

Effect of the HJT extract on immune organs and WBC count of H22-bearing mice. As shown in Fig. 1, compared with the normal control group, the thymus and spleen indices were significantly lower in the tumor control group and further decreased in the 5-FU group (P<0.05). In the HJT extract groups, atrophy of the immune organs induced by 5-FU was improved (P<0.05). Compared with the tumor control group, the WBC count showed a significant decrease in the 5-FU group (P<0.05), and showed an increasing tendency in the HJT extract groups.

Effect of the HJT extract on the phagocytic function of macrophages in H22-bearing mice. Compared with the tumor control group, 5-FU decreased the phagocytic index and rate significantly (P<0.05) (Fig. 2). However, the phagocytic function of the macrophages of mice was increased in the HJT groups. The phagocytic indices of the low-, medium- and high-dose HJT groups were increased 16.32, 53.31 and 81.63%, respectively, compared with the 5-FU group. The phagocytic rates of the low-, medium- and high-dose HJT groups were increased by 35.07, 54.47 and 83.31%, respectively, compared with the 5-FU group.

Effects of the HJT extract on the life prolongation rate in H22-bearing mice. As shown in Table II, the survival time of the ascites tumor mice in the HJT groups was significantly higher compared with those of the tumor control (P<0.05) and

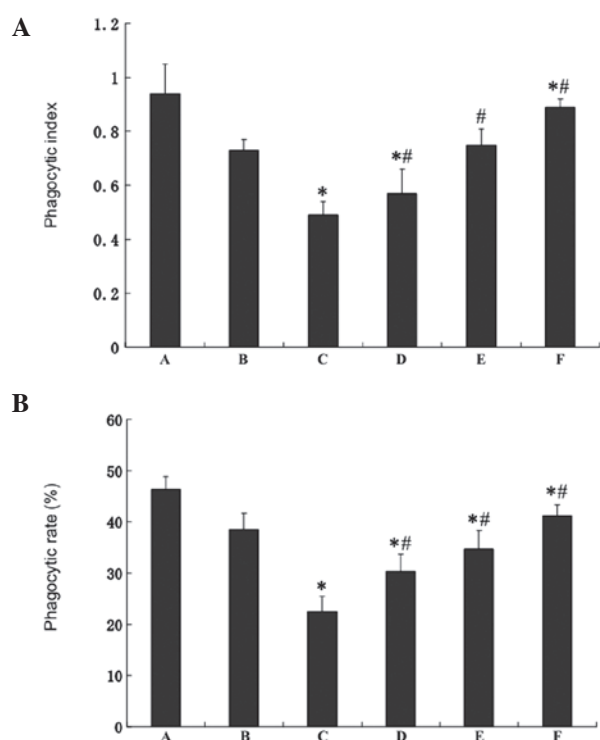


Figure 2. Effect of *Hypericum japonicum* Thunb. (HJT) extract on the phagocytic function of macrophages in H22-bearing mice. Group A, normal control; Group B, tumor control; Group C, 5-FU; Group D, 5-FU + low-dose HJT; Group E, 5-FU + medium-dose HJT; Group F, 5-FU + high-dose HJT. Results are presented as the mean \pm standard deviation for the animals in each group. * $P < 0.05$ vs. tumor control group; # $P < 0.05$ vs. 5-FU group.

5-FU groups ($P < 0.05$). Survival time of the 5-FU group was slightly higher compared with that of the tumor control group, with no significant difference being detected between the two groups. The life prolongation rates in the low-, medium- and high-dose HJT groups were 25.97, 43.74 and 62.27%, respectively, which were significantly higher compared with those of the 5-FU group.

Discussion

Previous studies have noted the bioactivities of the *Hypericum* genus including the antitumor, antioxidant, antimicrobial and natural killer (NK) cell-activating activity (8-12). HJT, an annual herb from the genus *Hypericum* L., known in traditional Chinese medicine as Tian-ji-huang, grows mainly throughout southern China. HJT has been used in a Chinese herbal compound formula for many years for the treatment of bacterial diseases; infectious, acute and chronic hepatitis; gastrointestinal disorder; internal hemorrhage and tumors (13,14). Data in the present study demonstrated that the HJT extract significantly inhibited the growth of H22-transplanted tumors and had a synergistic tumor-inhibiting effect with the 5-FU group.

Reduction of the chemotherapy-induced side effects in cancer treatment has gained increasing attention. The results of this study demonstrated that the HJT extract exhibited an improved efficacy of supporting treatment in chemotherapy. The increase in body weight markedly reduced toxic reactions

and the higher life prolongation rate demonstrated that the HJT extract had toxicity-reducing activity.

The immune function is important in the course of radiotherapy and chemotherapy for patients with malignant tumors. Findings of the present study have shown that the thymus and spleen indices, WBC count and the phagocytic function were elevated in the HJT groups, indicating that the HJT extract efficaciously antagonized the immunosuppression caused by 5-FU and may be used as an immune response modifier in chemotherapy. It is possible to estimate that HJT likely improves the immune function of patients suffering from a malignant disease.

The antitumor and toxicity-reducing effects of HJT extract are potentially associated with components such as flavonoids, xanthenes, glucoside and embelin in HJT, which also contribute to the immune activity on the tumor cells (15-18). However, in the present study, the whole herb extract was used, while the concrete mechanism of action remains to be elucidated.

Acknowledgements

This study was supported by the Talent Recruitment Research Grant of the Zhengzhou University, Zhengzhou, China.

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