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Evaluation of *Hirsutella sinensis* Mycelium on Food Safety and Anti-hepatoma Activity in an Animal Model

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Abstract. *There is evidence that Hirsutella sinensis may have antitumor activity. The aim of the present study was to determine the anti-hepatoma effects and food safety assessment of Hirsutella sinensis mycelium in vivo and in vitro. Effects on mutagenicity were determined using a bacterial reverse mutation assay employing the Salmonella typhimurium strains TA98, TA100, TA102, TA1535 and TA1537. There were no dose-dependent increases or decreases in the number of colonies both with and without metabolic S9 activation in Ames tests. Mice were inoculated with SK-Hep 1 cells and those developing tumors were treated with three different concentrations of Hirsutella sinensis mycelium. After six weeks, blood samples were collected and liver pathology was determined. Aspartate aminotransferase levels were significantly different only in the low-dose treatment group (106±27 IU/l, p=0.048), compared to the control group (162±80 IU/l). The tumor weight was significantly different only in the low-dose treatment group. We found that necrosis, hemorrhage and calcifications were presented in both control and experimental groups. Inhibition of tumor growth was observed only at the lowest dose.*

Cancer is a major public health problem and its incidence and mortality rates continue to increase worldwide. A 2012 report of the World Health Organization (WHO) estimated that 14.1 million people were diagnosed with cancer and 8.2 million people died from cancer all over the world. The WHO predicts that by 2030 an estimated 21.4 million new cases of cancer and 13.2 million cancer deaths will occur annually around the world (1, 2). Liver cancer is the sixth most common type of cancer in the world, with 745,000 new cases diagnosed in 2012. This accounted for about 6% of the total number of cases of cancer in 2012. Mongolia has the highest rate of liver cancer, followed by the Gambia and Taiwan (1, 2). It has been shown that cancer treatment using herbal medicines in combination with chemo- or radiotherapy can enhance the efficacy of and diminish the side-effects and complications caused by such therapy (3). The use of complementary and alternative medicine has gained greater acceptance among patients with cancer in Western countries, with use being as high as 80% (4, 5). Traditional Chinese medicine (TCM), and

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herbal medicines in particular, have been used in the treatment of cancer for thousands of years in China, Japan, and other Asian countries. These medicines are now widely accepted as current forms of complementary and alternative medicine in cancer treatment in the United States and Europe (6, 7).

Mushrooms have been used as an important nutritional food and therapeutic agent throughout the world (8). Mushroom extracts are common sources of immunological, hypocholesterolemic, antiviral, antibacterial, anticarcinogenic, anti-inflammatory and antiparasitic activities (9). There are many reports on mushrooms containing more than one polysaccharides with antitumor activity. The mushrooms credited with success against cancer belong to the geni *Phellinus*, *Pleurotus*, *Agaricus*, *Ganoderma*, *Clitocybe*, *Antrodia*, *Trametes*, *Cordyceps*, *Xerocomus*, *Calvatia*, *Schizophyllum*, *Flammulina*, *Suillus*, *Inonotus*, *Inocybe*, *Funlia*, *Lactarius*, *Albatrellus*, *Russula*, and *Fomes* (10). The anticancer compounds found in mushrooms have multiple effects such as anti-mitotic activity, induction of reactive oxygen species, and inhibition of mitotic kinase and topoisomerase, leading to a reduction in cancer proliferation (10-18).

Recent studies indicate that the mushroom *Hirsutella sinensis* has a wide range of biological activities, including antitumor, immunomodulatory, anti-inflammatory, antioxidant, anti-infection, and anti-aging properties (19).

Hirsutella sinensis was found to have antitumor activity as seen in studies on prostate (PC3), breast (MCF7), hepatocellular (HepG2, Hep3B), colorectal (HT-29) and HCT 116), and HL-60 cells (20-22). There have not been any published reports on SK-Hep 1 hepatoma cells in SCID mice. The aim of the present study was to examine whether *H. sinensis* mycelium was effective against tumor-bearing mice and to determine whether the treatment effect was dependent on the concentration of *H. sinensis* mycelium.

Materials and Methods

Preparation of H. sinensis mycelium solution for Ames test. H. sinensis mycelium powder (500 mg) manufactured by Chang Gung Biotechnology Corporation, Ltd., Taipei, Taiwan, R.O.C., and 10 ml distilled water were mixed thoroughly and filtered (0.22 μm pore size) to provide a solution with a concentration of 50 mg/ml. A series of concentrations was prepared from this stock solution by dilution, namely 3 mg/ml, 6 mg/ml, 12 mg/ml, 25 mg/ml and 50 mg/ml.

Bacterial strains. Bacterial strains were provided by the Food Science Institute, Hsinchu, Taiwan. The strains used were *Salmonella typhimurium* TA98, TA100, TA102, TA1535, and TA1537. Strains were prepared by preculturing for 8 h at 37˚C in a nutrient broth. Strain properties, including their susceptibility to mutagens, were confirmed prior to use in the assays by the National Taiwan University College of Medicine Animal Medicine Center, Taipei, Taiwan.

Preparation of liver S9 fractions. Rats treated with enzyme-inducing agent β-naphthoflavone were sacrificed by spinal dislocation. Briefly, rat livers were removed, placed in beakers on ice, rinsed with ice-cold homogenization KCl (1.15%) buffer, minced with scissors and then placed in 4 volumes of ice-cold KCl buffer and homogenized with a tissue grinder. The homogenate was transferred to a close-fitting (0.045 mm clearance) Perspex [poly (methyl methacrylate)]/glass homogenizer and homogenized. After diluting the homogenate to 10% with the homogenization buffer and centrifuging at 9000 × *g*, the microsomal pellets were suspended in KH_2PO_4 buffer (pH 7.4) and stored at −80˚C.

Bacterial reverse mutation (Ames) assay. The Ames test was used to examine the mutagenicity of *H. sinensis* mycelium. For the plate incorporation method, without metabolic activation, 0.1 ml of the test solutions of different concentrations of *H. sinensis* mycelium, 0.1 ml of fresh bacterial broth and 0.5 ml of sterile buffer were mixed with 2.0 ml of overlay agar. For the assay with metabolic activation, 0.5 ml of metabolic activation mixtures containing an adequate amount of post-mitochondrial fraction was mixed with the overlay agar (2.0 ml), together with the bacteria and test solution. The contents of each tube were mixed and poured over the surface of a plate with minimal glucose agar. The overlay agar was allowed to solidify before incubation. The plate was incubated for 48 h at 37˚C and the number of reverting colonies was then counted. All plates in a given assay were incubated at 37˚C for 48 h. After the incubation period, the number of reverting colonies per plate was counted. Agar solvent was used as a negative control. The positive control without S9 fraction consisted of 0.5 μg/plate of 4-nitro-o-phenylenediamine for TA98; 4 μg/plate of sodium azide for TA100 and TA 1535 strains; 0.5 μg/plate of mitomycin C for TA102 strain; and 80 μg/plate of 9-aminocridine for TA1537 strain; for any with S9 fraction (S9 is a metabolic activation system consisting of the postmitochondrial fraction in rat liver), 5 μg/plate of benzo[a]pyrene was used for TA98, TA102 and TA1537 strains, and 8 μg/plate of 2-aminoanthracene for TA100 and TA1535 strains. Mutagenicity was evaluated based on the rule reported previously by Claxton *et al*. (23) *i.e*. the value of the positive control should be significantly higher than that of the negative control. Mutagenicity was judged to be positive when the revertants in the test plates increased by more than two-fold compared with those of the negative control.

Preparation of H. sinensis mycelium at three different doses for anti-hepatoma activity. We suspended *H. sinensis* mycelium powder (manufactured by Chang Gung Biotechnology Corporation, Ltd. In Taipei, Taiwan, R.O.C.), in 0.2 ml distilled water at 50˚C for 10 min, then cooled it to room temperature and left it for 1 h with 200 rpm stirring to form three different doses of treatment solution (low dose 184.5 mg/kg/day; medium dose 369 mg/kg/day; high dose 553.5 mg/kg/day).

Animals. 25 g SCID male mice aged six weeks and used in the present study were maintained according to the recommendations of the guidelines approved up by the National Science Council of the Republic of China and the Ethical and Health Research Committee of the Institute of Biosciences. Experiments were performed according to law, regulations and guidelines for animal experiments in Taiwan. The experimental protocol used complied with the principles for Institutional Animal Care and Use Committee of Chen Hsin General Hospital (Taipei, Taiwan, R.O.C.). SCID mice

Table I. *The number of total colony−forming units, including spontaneous revertant colonies, that appeared on a plate were determined by Salmonella typhimurium reverse mutation test under different concentrations of Hirsutella sinensis mycelium.*

Strain	Mix	Positive control	Hirsutella sinensis mycelium (mg/plate)					Negative control
			5	2.5	1.2	0.6	0.3	
TA98	−S9	154±24	46±6	43±4	36±8	43±2	46±6	48±2
	+S9	264±30	37±3	32±7	35±4	31±3	34±3	32±3
TA100	−S9	601±38	139±2	124±18	139±2	145±4	145±3	137±9
	+S9	1779±391	159±7	145±2	151±12	151±12	161±13	145±6
TA102	−S9	1315±200	244±3	244±4	248±11	242±2	247±7	206±61
	+S9	2323±147	259±4	258±4	257±4	246±8	252±5	251±10
TA1535	−S9	339±23	29±13	32±5	25±3	25±5	22±4	24±4
	+S9	238±23	25±11	20±3	16±1	15±2	19±6	17±2
TA1537	−S9	63±13	13±2	16±4	13±2	13±3	13±4	13±2
	+S9	84±12	9±4	9±4	12±4	9±6	7±2	11±3

S9 is a metabolic activation system consisting of the postmitochondrial fraction in rat liver.

were obtained from the BioLASCO Taiwan Co., Ltd. Animals were earmarked, housed in an air conditioned animal room at 22±3˚C, relative humidity 55±15%, and kept under a 12:12-hour light/dark cycle (light from 9 a.m. to 9 p.m.) for a 2-week acclimatization period before the experimental treatments. Mice recived autoclaved water and laboratory pellet chow ad libitum (24).

Study design and treatment with H. sinensis mycelium. Mice were subcutaneously (*s.c.*) inoculated with SK-Hep 1 cells (3×10^7 cells/mouse) in the hind limb dorsal area. After 2-3 weeks (week 0) of cell implantation, mice with tumors of 1-3 mm in diameter were divided into four groups of 10 mice per group. Group 1 was the control group without any treatment. Groups 2-4 were administered low (184.5 mg/kg/day), medium (369 mg/kg/day) or high (553.5 mg/kg/day) dose of *H. sinensis* mycelium respectively. After six weeks, surviving animals were sacrificed. Levels of GOT (glutamate oxaloacetate transaminase), GPT (Glutamic Pyruvic Transaminase) and vascular endothelial growth factor (VEGF) were determined in serum. Tumor properties (size, weight, pathology) were determined.

Determination of serum parameters. Peripheral venous blood samples were drawn into sterile glass tubes (Vacutainer, Becton Dickinson, USA) in the morning around 8 or 9 h after an overnight fasting. Blood samples were allowed to coagulate at room temperature for 30 minutes then centrifuged at 1,800 ×*g* for 10 min. Serum was separated and stored at −70˚C until assayed. A DxC 800 clinical chemistry analyzer and reagents manufactured by Beckman, Inc. (Brea, CA, USA) were used to determine serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (T-bil), albumin (ALB), total protein (TP), total cholesterol (T-CHO), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C) levels. Murine serum Vegf concentrations were determined using a commercially available enzyme-linked immunoassay kit (900-M99; PeproTech Rocky Hill, NJ, USA) according to the manufacturer's instructions

Tissue processing and histology. At the end of the experiments (after six weeks' treatment), the survival rate was assessed. Tumors were rapidly removed and fixed in formalin. The fixed tissue was embedded in paraffin wax, and sliced into transverse sections. Tissue samples were rinsed with 0.9% saline solution, fixed in 10% formalin and processed as follows: i) 10% neutral buffered formalin for 1 h, twice; ii) 70% alcohol for 1.5 h; iii) 80% alcohol for 1.5 h; iv) 90% alcohol for 1.5 h; v) absolute alcohol for 1.5 h, twice; vi) xylene for 1.5 h, twice; vii) in molten wax at 65˚C for 2.5 h. The processed tissue was embedded in paraffin and sectioned at 4 μm thickness, placed on frosted glass slides and dried on a hot plate at 70˚C for 30 min. The tumor slices were stained using the Hematoxylin and eosin stain (H&E) stains and visualized using a Leica TP1020 microscope (Singapore).

Statistical analysis. Statistical analysis for comparison between two groups was performed using the Student's *t*-test. Data are expressed as means±standard deviations (SD). The level of significance used was $p<0.05$.

Results

Table I shows the mean number of revertants/plate, after treatment with the five concentrations of *H. sinensis* mycelium, observed in *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA 1537in the presence and absence of metabolic activation by the Ames test. There was no effect on bacterial growth and mutagenic activity was considered negative.

After six-week treatment with *H. sinensis* mycelium of the mice, the survival rates were: control 100%; low-dose 100%; medium-dose 80%; and high-dose 80%.

Following SK-Hep 1 inoculation to initialize hepatoma (Figure 1A), mice were orally-treated with different doses of *H. sinensis* mycelium. After 6 weeks of treatment, blood specimens were collected from all survivors which were then sacrificed, and tumor characteristics were determined (Figure 1B).

AST was significantly different only in the low-dose treatment group (106±27 IU/l, $p=0.048$) compared to the control group (162±80 IU/l) after the 6-week treatment.

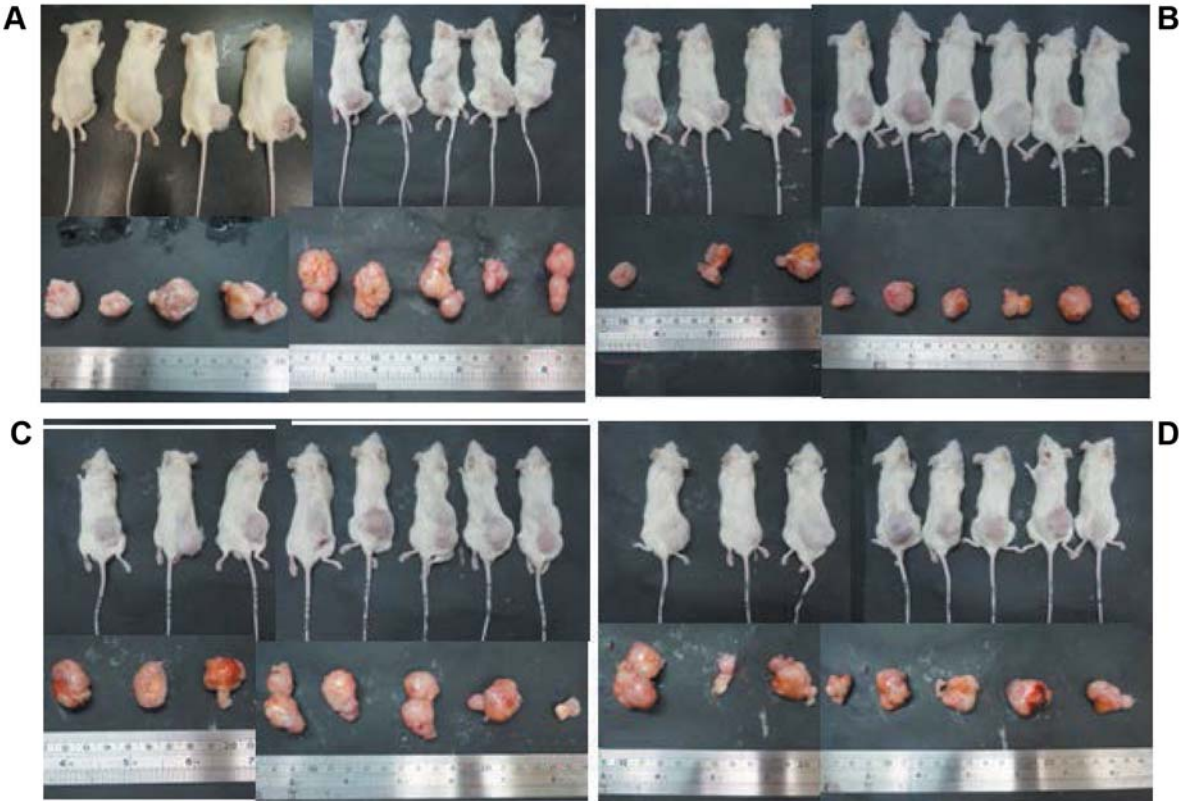

Figure 1. *After 2-3 weeks of dorsal area injection of SK-Hep-1 cells to initialize hepatoma in SCID mice, each mouse had a palpable tumor measuring about 1-3 mm in diameter. Mice of experimental groups were orally administered different doses of Hirsutella sinensis mycelium: control (A), low dose (B), medium dose (C) and high dose (D). After six weeks' treatment, all the survivors were sacrificed, and the size of liver tumor was assessed. Live mice and representative tumors are presented. Only the low-dose treatment evidently reduced tumor growth by comparison with the control group.*

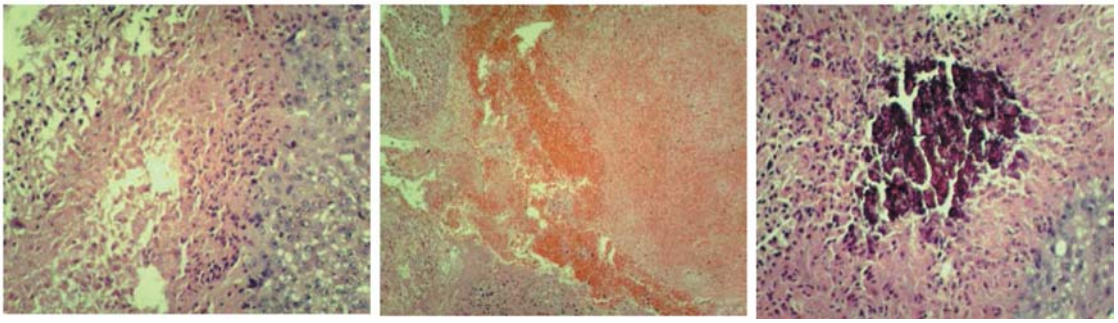

Figure 2. *Representative photomicrographs of tumors from mice treated with Hirsutella sinensis mycelium, compared with those of the control group. After treatment, tumor sections were stained with H&E and revealed necrosis on account of a good number of cavities. Tumor sections also showed dark eosinophilic cytoplasm and darkly stained nuclei because of extensive cancer cell proliferation. Irregular shapes of focal necrotic areas and loss of normal architecture were characterized necrotic cells with eosinophilic cell debris and peripheral viable tissues. Foci of hemorrhage and scarlet calcification were frequently observed in the center of some necrotic areas.*

Although AST differed in the control and medium-dose-treated (117±38 IU/l) group, this was not statistically significant. Mice treated with increasing doses of *H. sinensis* mycelium did not exhibit any differences in serum ALT and T-bil concentrations compared to control mice (Table II). Albumin levels were higher in the *H. sinensis* mycelium-treated mice than in the control group (Table II). The blood TP concentrations were significantly lower in the low high

Table II. *Mean serum biochemical values of mice which were inoculated with SK−Hep 1 cells and then administered different doses of Hirsutella sinensis mycelium.*

Dose (mg/kg/day) parameter	Control	Low dose (0.5 mg/ml)	Medium dose (25 mg/ml)	High dose (50 mg/ml)
Aspartate aminotransferase (IU/l)	162±80	106±27* p=0.048	117±38	153±73
Alanine aminotransferase (IU/l)	17±4	15±4	14±4	13±3
Total bilirubin (mg/dl)	0.334±0.148	0.334±0.086	0.369±0.057	0.414±0.127
Albumin (g/dl)	1.01±0.13	1.17±0.12* p=0.0172	1.12±0.06* p=0.0427	1.18±0.17* p=0.0336
Total serum protein (g/dl)	4.14±0.13	3.90±0.24* p=0.0126	4.06±0.17	3.99±0.16* p=0.0431
Total cholesterol (mg/dl)	97±15	75±13* p=0.0022	79±14* p=0.0122	74±14* p=0.0030
Triglyceride (mg/dl)	147±83	34±14* p=0.0019	39±17* p=0.0020	36±12* p=0.0021
High-density lipoprotein cholesterol (mg/dl)	74±10	64±9* p=0.0250	69±12	65±13
Low-density lipoprotein cholesterol (mg/dl)	17±6	16±3	17±3	14±3
Vascular endothelial growth factor (ng/ml)	0.572±0.054	0.556±0.150	0.608±0.113	0.528±0.087

Significantly different from the control group at *p<0.05.

Table III. *At the end of experiment, tumors were removed and sections from each tumor mass were stained with H&E. Necrosis, hemorrhage and calcifications were present in some animals.*

		Mouse no.										
Treatment		1	2	3	4	5	6	7	8	9	10	Average
Control	Necrotic rate	0.6	0.4	0.3	0.3	0.25	0.6	0.6	0.4	0.3		0.42±0.15
	Hemorrhage	−	−	−	−	−	+	−	+	−		2/9
	Calcification	−	−	−	−	−	+	+	−	−		2/9
Low dose (0.5 mg/ml)	Necrotic rate	0.3	0.4	0.2	0.2	0.2	0.2	0.3	0.4	0.3	0.4	0.29±0.09
	Hemorrhage	−	+	−	+	+	+	−	+	+	+	7/10
	Calcification	−	−	−	−	−	−	−	−	−	−	0/10
Medium dose (25 mg/ml)	Necrotic rate	0.2	0.2	0.3	0.5	0.2	0.3	0.2	0.3	0.2		0.27±0.10
	Hemorrhage	−	+	+	+	+	+	+	−	+		7/9
	Calcification	−	−	−	+	+	−	+	−	−		3/9
High dose (50 mg/ml)	Necrotic rate	0.3	0.2	0.4	0.2	0.2	0.2	0.3	0.3	0.2	0.4	0.27±0.08
	Hemorrhage	+	−	+	+	+	+	+	+	+	+	9/10
	Calcification	−	−	−	−	−	−	−	−	−	−	0/10

+: Feature present; −: feature absent.

dose groups than the control group (Table II). *H. sinensis* mycelium treatment also reduced serum T-CHO and TG concentrations. HDL-C and LDL-C concentrations were generally not affected, except for there being a lower HDL-C level at the low-dose treatment (p=0.0250) compared to control mice. VEGF levels were not altered by *H. sinensis* mycelium treatment (Table II).

The average tumor weights of control, low-, medium- and high-dose treatment groups were 4.81±1.84 g, 2.66±1.48 g, 3.62±1.83 g and 3.59±2.23 g, respectively. *H. sinensis* mycelium treatment reduced tumor weight at all dose but only that of mice treated with the low dose was significantly different from that of the control group (p=0.0078).

Representative parts of tumors were taken and stained with H&E. The tumor cells were arranged in trabecular, glandular or island patterns, with hyperchromatic coarse chromatin and eosinophilic cytoplasm. Variable percentages of scattered necrotic areas characterized by eosinophilic cells debris palisaded by viable tumor cells were observed. Areas of recent and old hemorrhage and calcification were also found in the center of certain necrotic areas in some experimental groups (Table III).

Discussion

Compounds used in commercial products are evaluated *via* the Ames test as potential carcinogens. Some identified mutagens were considered possible carcinogens, and previous studies showed that 90% of known carcinogens may be identified by this test (25). The test is useful as a screening tool for setting priorities because it is an inexpensive and quick way to help single-out chemicals that

should be subjects of further testing. We strongly suggest that mutagens identified in the Ames test require further tests such as chromosome aberration tests, micronucleus test and mouse lymphoma assay because *Salmonella typhimurium* is not a eukaryote, and therefore it is not considered to be a perfect model for humans. In order to mimic mammalian metabolic conditions, rat liver S9 fraction was applied but was previously limited by its availability. Fortunately, it is now commercially available and therefore its use may be more feasible.

Cancer is the largest single cause of death in both men and women, claiming over six million lives each year worldwide. Anticancer drugs such as 5-fluorouracil derivatives, cisplatin, mitomycin, adriamycin, and taxol, have been used extensively for the treatment of certain types of cancer. However, these drugs are associated with side-effects such as severe gastrointestinal toxicity, leucopenia and immune suppression, that can impact on patient compliance. After the removal of a malignant tumor by surgical operation, radiation therapy or adjuvant therapy with cancer chemotherapy drugs may be curative. However, the removal of certain types of cancer, for example, breast carcinoma, colon carcinoma and osteogenic sarcoma, may be followed by the rapid growth of distant metastases to lung, liver *etc*. Therefore, it is necessary to develop new anticancer agents with antitumor and antimetastatic activities but without the adverse effect, often associated with cancer chemotherapeutic drugs.

Effectiveness is not only important for TCM, but for all medical systems. Clinical research in TCM is a relatively new phenomenon. Largely due to the many existing methodological defects of TCM trials, the efficacy of TCM remains controversial (26, 27). All these issues have created a bottleneck in the development of TCM. There is growing recognition that more attention has been paid to enhancing the quality in clinical research of TCM. Especially in this past decade, a new discipline, clinical evaluation of TCM, has come into being and its development has been supported by the Chinese Major Science and Technology Projects. Owing to concerted efforts from a panel of experts with multidisciplinary background, the quality of clinical research of TCM has been improved, in terms of the construction of clinical research platforms and the introduction of process management.

In the present study, experiments were carried out to examine the effects of *H. sinensis* mycelium on inhibition of tumor growth in tumor-bearing mice. We observed that only the low-dose treatment was capable of inhibiting tumor growth compared to higher doses. This study failed to find evidence that *H. sinensis* mycelium is capable of inhibiting tumors induced by SK-Hep 1 cells in a dose-dependent manner. Treatment with high doses may in fact cause the opposite effect. Considering that medicinal herbs contain complex mixtures of thousands of components that can act alone or synergistically, we may say that not all individual components of *H. sinensis* mycelium are be good for human health. For instance, although *Agaricus* blazei Murrill extract is traditionally used to treat cancer, it contains a number of aromatic hydrazines. Among these, the most abundant is agaritine, β-N-(γ-L(+) glutamyl)-4-(hydroxymethyl) phenylhydrazine (28, 29), which has been shown to induce adenomas and adenocarcinomas in the lungs of mice (30).

There are several natural and cultured *Cordyceps* species, however, by using carbohydrate gel electrophoresis and high-performance thin layer chromatography, different species of natural and cultured *Cordyceps* can be differentiated based on the saccharide mapping (31). We cannot jump to the conclusion whether different species are able to inhibit tumor growth or not.

There are a variety of liver medicines. Unfortunately, few of them are actually successfully used in humans (32). Over the past several years, we have tried our best to find herbal therapies to cure patients with liver cancer, which has a high prevalence rate in Taiwan. From the above, we may conclude that proper dosing is required to inhibit tumor growth. Dedicated research should be undertaken for isolation, purification and structural investigation of novel anticancer and immune-stimulatory compounds. Studies to date have identified a number of compounds and elucidated underlying mechanisms. However, further research is required to elucidate the different roles of multiple active compounds and the pathways involved. The present results and data might provide new insights into the possible therapeutic uses of *H. sinensis* mycelium and helpful suggestions for the design of antitumor drugs from *H. sinensis* mycelium to combat cancer.

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