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Anticancer potential of *Hericium erinaceus* extracts against human gastrointestinal cancers

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ABSTRACT

Ethnopharmacological relevance: Hericium is a genus of mushrooms (fungus) in the *Hericiaceae* family. *Hericium erinaceus* (HE) has been used for the treatment of digestive diseases for over 2000 years in China. HE possesses many beneficial functions such as anticancer, antiulcer, antiinflammation and antimicrobial effects, immunomodulation and other activities. The aim of the studies was to evaluate the anticancer efficacy of two extracts (HTJ5 and HTJ5A) from the culture broth of HE against three gastrointestinal cancers such as liver, colorectal and gastric cancers in both of *in vitro* of cancer cell lines and *in vivo* of tumor xenografts and discover the active compounds.

Materials and methods: Two HE extracts (HTJ5 and HTJ5A) were used for the studies. For the study of chemical constituents, the HTJ5 and HTJ5A were separated using a combination of macroporous resin with silica gel, HW-40 and LH-20 chromatography then purified by semipreparative high-performance liquid chromatography (HPLC) and determined by nuclear magnetic resonance (NMR) spectra. For the *in vitro* cytotoxicity studies, HepG2 and Huh-7 liver, HT-29 colon, and NCI-87 gastric cancer cell lines were used and MTT assay was performed to determine the *in vitro* cytotoxicity. For *in vivo* antitumor efficacy and toxicity studies, tumor xenograft models of SCID mice bearing liver cancer HepG2 and Huh-7, colon cancer HT-29 and gastric cancer NCI-87 subcutaneously were used and the mice were treated with the vehicle control, HTJ5 and HTJ5A orally (500 and 1000 mg/kg/day) and compared to 5-fluorouraci (5-FU) at the maximum tolerated dose (MTD, 25–30 mg/kg/day) intraperitoneally daily for 5 days when the tumors reached about 180–200 mg (mm^3). Tumor volumes and body weight were measured daily during the first 10 days and 2–3 times a week thereafter to assess the tumor growth inhibition, tumor doubling time, partial and complete tumor response and toxicity.

Results: Twenty-two compounds were obtained from the fractions of HTJ5/HTJ5A including seven cycli dipeptides, five indole, pyrimidines, amino acids and derivative, three flavones, one anthraquinone, and six small aromatic compounds. HTJ5 and HTJ5A exhibited concentration-dependent cytotoxicity *in vitro* against liver cancer HepG2 and Huh-7, colon cancer HT-29, and gastric cancer NCI-87 cells with the IC_{50} in 2.50 ± 0.25 and 2.00 ± 0.25, 0.80 ± 0.08 and 1.50 ± 0.28, 1.25 ± 0.06 and 1.25 ± 0.05, and 5.00 ± 0.22 and 4.50 ± 0.14 mg/ml; respectively. For *in vivo* tumor xenograft studies, HTJ5 and HTJ5A showed significantly antitumor efficacy against all four xenograft models of HepG2, Huh-7, HT-29 and NCI-87 without toxicity to the host. Furthermore, HTJ5 and HTJ5A are more effective than that of 5-FU against the four tumors with less toxicity.

Conclusion: HE extracts (HTJ5 and HTJ5A) are active against liver cancer HepG2 and Huh-7, colon cancer HT-29 and gastric cancer NCI-87 cells *in vitro* and tumor xenografts bearing in SCID mice *in vivo*. They are more effective and less toxic compared to 5-FU in all four *in vivo* tumor models. The compounds have the potential for development into anticancer agents for the treatment of gastrointestinal cancer used alone and/or in combination with clinical used chemotherapeutic drugs. However, further studies are required to

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find out the active chemical constituents and understand the mechanism of action associated with the super *in vivo* anticancer efficacy. In addition, future studies are needed to confirm our preliminary results of *in vivo* synergistic antitumor efficacy in animal models of tumor xenografts with the combination of HE extracts and clinical used anticancer drugs such as 5-FU, cisplatin and doxurubicin for the treatment of gastrointestinal cancers.

1. Introduction

Gastrointestinal (GI) cancers such as liver, gastric and colorectal cancers are one of the most common forms of cancers and accounted for about 25% of all cancers from the estimate by the United States (US) National Cancer Institute (Chuang et al., 2009). Liver cancer is the fifth most common cancer and the third most common cause of cancer-related deaths in the world. China has the highest rate of liver cancer in the world and accounts for more than 55% of all cases of primary liver cancer (Chuang et al., 2009). Although liver cancer is uncommon in the US, it is the fastest increasing cause of cancer related deaths and has more than tripled during the past two decades. Gastric (stomach) cancer is the fourth common cancer and the second most common cause of cancer-related deaths in the world (Siegel et al., 2011). Similar to liver cancer, gastric cancer has a considerable high incidence and mortality rates in China (~50% death in the world from China) (Siegel et al., 2011). Colorectal cancer (CRC) is one of the most common and leading cause of cancer-related mortality in the Western world, ranked third in prevalence and lethality (Siegel et al., 2011). CRC is usually diagnosed later in life with most patients presenting after the age of 50. The incidence of CRC in China is lower than that in the Western countries, but has significantly increased in recent years, particularly in the more developed areas. The treatment plan for patients with GI cancers may include surgery, chemotherapy, radiation therapy and immunotherapy. Chemotherapy is the mainstay of treatment for the patients with GI cancers although the treatment remains mainly palliative. However, drug resistance and dose-limiting toxicity limit the success. Therefore, discovery and development of novel anticancer drugs with more efficacy and/or less toxicity are urgently needed.

Mushrooms have been used as edible and medicinal resources for thousands of years and antitumor substances such as polysaccharides have been identified in many mushroom species (Ikekawa et al., 1969; Mizuno et al., 1995; deVere White et al., 2002; Wasser, 2002; Zhang et al., 2007; Ferreira et al., 2010). *Hericium erinaceus* (HE) is an edible mushroom which has been used as a traditional Chinese medicine (TCM) for the treatment of digestive diseases for over 2000 years in China. HE polysaccharides have been widely studied and exhibited anticancer, immune stimulation, lowering cholesterol, and stimulating neurite outgrowth activities (Mizuno, 1995; Park et al., 2002; Zhang et al., 2007; Choi et al., 2010). Besides HE polysaccharides, a series of *erinacines* are regarded to have nerve regenerating property and able to pass through the blood brain barrier to heal on myelin or nerve tissue (Mori et al., 2008; Ma et al., 2010). HE also possesses many other beneficial functions such as anticancer and anti-metastasis (Mizuno et al., 1992; Kim et al., 2011; Kim et al., 2013), anti-ulcer (Abdulla et al., 2008), anti-inflammation and antimicrobial (Okamoto et al., 1993; Okwulehie and Odunze, 2004; Kim et al., 2012), immunomodulation (Xu et al., 1994), improving liver function (Lindequist et al., 2005; Zhang et al., 2012a, 2012b), anti-aging (Zhang et al., 2012a, 2012b), lower blood sugar and lipids (Yang et al., 2003; Wang et al., 2005; Hiwatashi et al., 2010), and improving the body hypoxia tolerance, increasing cardiac blood output and improving the body's blood circulation (Chen et al., 1996).

In the present studies, we investigated the chemical constituents of HTJ5/HTJ5A by separating them with a combination of macroporous resin consisting of silica gel, HW-40 and LH-20 chromatography and purified by semipreparative high-performance liquid chromatography (HPLC) and determined by nuclear magnetic resonance (NMR) spectra. We further evaluated the *in vitro* cytotoxic effect of HTJ5 and HTJ5A on HepG2 and Huh-7 liver, HT-29 colon, and NCI-87 gastric cancer cell lines by MTT assay and *in vivo* antitumor efficacy and toxicity in animal models of SCID mice bearing liver HepG2 and Huh-7, colon cancer HT-29 and gastric cancer NCI-87 tumor xenografts subcutaneously and compared to the effect of 5-FU.

2. Materials and methods

2.1. Collection, extraction and isolation of extracts of HE: HTH5 and HTJ5A

The solid cultures of HE (200 g) were obtained from Hunan Xinhui Pharmaceutical Co., Ltd. (Changsha, China) and dispersed in 1000 ml water, extracted with Herbal Blitzkrieg Extractor under 30°C for 20 min and centrifugalized at 3000 rpm/min for 10 min. The precipitations were extracted and centrifugalized for 10 min. The supernatants were concentrated under reduced pressure and lyophilized to obtain HTJ5 (54 g).

The HTJ5 was dissolved in 200 ml water, the polysaccharides and proteins were separated from HTJ5 by adding 600 ml ethanol, the ethanol solution was filtered and concentrated under reduced pressure to obtain a brown crude extract HTJ5A (20 g).

2.2. Chemical constituent study

Thin layer chromatography (TLC) and column chromatography (CC) were performed on plates precoated with silica gel GF254, silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, China), Sephadex LH-20 (General Electric Healthcare Life Sciences, Piscataway, NJ, USA) and Toyopearl HW-40 (Tosoh Corporation, Tokyo, Japan), respectively. Nuclear magnetic resonance (NMR) spectra were taken on a BRUKER AV-400 and a BRUKER AV-500 spectrometers (Bruker Corporation, Billerica, MA, USA) using Dimethyl sulfoxide-d_6 (DMSO-d_6) as solvent and tetramethylsilane (TMS) as internal standard. Semipreparative high-performance liquid chromatography (HPLC) was performed using an ODS column (YMC Triart C_{18}, 5 μm, 20 mm × 250 mm, YMC America Inc., Allentown, PA, USA) and an Agilent 1200 series system consisting of degasser, quad pump, and variable wavelength detector.

The HTJ5A gradient elution on macroporous resin with water, 20%, 40%, and 95% alcohol, obtained the fractions A, B, C, and D, respectively. Each fraction was separated by the combination of silica gel column chromatography, gel HW-40, and LH-20 column, and purified by semi-preparation.

For determination of the contents of HTJ5 and HTJ5A, the polysaccharides were determined by the modified phenol–sulfuric acid method (Dong et al., 1996), while the nitrogenous compounds were determined by Kjeldahl method (Castillo et al., 1962).

2.3. Cell culture

HepG2 and Huh-7 liver, HT-29 colon, and NCI-87 gastric cancer cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS, Atlanta Biological, Lawrenceville, GA, USA) and penicillin (100 units/ml)/streptomycin (0.1 μg/ml) (Invitrogen, Grand Island, NY, USA). The cells were cultured in a 5% CO_2 incubator at 37 °C and renewed with new medium every 3–5 days. All cell lines are mycoplasma-free confirmed by MycoSensor PCR Assay kit (Stratagene, La Jolla, *CA*, USA).

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The MTT assay is used to determine the effect of HTJ5 and HTJ5A on the cell growth and survival. A total of 3500 cells were added to a 96 well plate. After 24 h incubation, the cells were treated with various concentrations of HTJ5 and HTJ5A in a 100 μl final volume and incubated for 72 h. Then 10 μl of MTT (Sigma Aldrich, St Louis, USA) in PBS (5 mg/ml) was added into each well and the cells were further incubated for 4 h. The MTT metabolic product formazan was solubilized by adding 200 μl of DMSO to each well. Absorbance was measured at 570 nm with Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Experiments were performed in triplicate and analyzed for statistics.

2.5. Animal and tumors

Eight to 12-week-old female SCID mice (body weight 18–22 g) were obtained from Roswell Park Cancer Institute (Buffalo, NY, USA) and kept five mice/cage with water and food *ad libitum* according to an institutionally approved animal protocol (#1181M). HepG2 and Huh-7 human hepatocellular carcinoma, HT-29 human colon adnocarcinoma, and NCI-87 gastic carcinoma, were used for the studies. The tumor xenografts were initially established by injecting subcutaneously 10^6 cultured cells and tumors were passed several generations by transplanting ~50 mg non-necrotic tumor (2–3 pieces) via a trocar from the passage tumors when the tumors reach to 1000–1500 mg as described previously (Cao et al., 1999).

2.6. Drug preparation and treatment

HTJ5 and HTJ5A were dissolved in pure DMSO first and diluted with sterile saline (0.9% NaCl) at 50 mg/ml with the final DMSO concentration at 10%. 5-FU was purchased from Hoffmann-La Roche, Inc (Nutley, NJ, USA) as a solution of 50 mg/ml in 10-ml vials and diluted with sterile saline. Sterile saline contained 10% DMSO was used as vehicle control.

HTJ5 and HTJ5A were given by oral route (p.o.) at 200–1000 mg/kg/day and 5-FU by intraperitoneal injection (i.p.) at 25–30 mg/kg/day once a day for 5 days (daily ×5). The reason for 5-FU giving i.p. is due to its poor bioavailability. Treatment was initial 7 days after tumor transplantation when the tumor reached ~180–200 mg. The mice in the control group will receive the vehicle (10% DMSO) orally at 200 μl per 20 g mouse body weight with the same treatment schedule. Five mice were used for each experimental group and most experiments were repeated once.

2.7. Tumor measurement

The method for tumor measurement was described previously (Cao et al., 1999, 2004). In brief, two axes (mm) of tumor (L, the longest axis; W, the shortest axis) were measured with the aid of a Vernier caliper. Tumor weight (mg) was estimated as a formula: tumor weight (mg) or tumor volume = ½($L \times W^2$) (mg or mm^3, 1 mg = 1 mm^3). Tumor measurements were taken daily during drug treatment and 3–4 times a week thereafter.

2.8. Maximum tolerated dose (MTD) and toxicity evaluation

The MTD was defined as the highest drug dose could be administrated to the mice that do not cause drug-related lethality in mice with a body weight loss $< 20\%$ of original body weight and toxicities were reversible (Cao et al., 1999, 2005). The kinetics of drug-induced toxicities (body weight loss, diarrhea, and lethality) were determined daily for the first 10 days after starting treatment and every 2 days thereafter. To determine the MTD for HTJ5 and HTJ5A, the mice were treated with 200, 500, and 1000 mg/kg/day orally daily for 5 days. None of the animals died or showed any signs of adverse reaction to the treatment. It was thus determined that it was safe to use any of the doses of 500 mg/kg and 1000 mg/kg for the efficacy study.

2.9. Antitumor activity

Antitumor activity was assessed by tumor growth inhibition (TGI) which is mean tumor weight of treated group compared with vehicle control group at the same time or later time if control mice were sacrificed due to large tumor (TGI = TWTG − TWCG) ÷ TWCG × 100%). The tumor doubling time (TDT) was defined as the mean time for the tumor to reach twice its initial weight. Tumor response was expressed as partial tumor response (PR) when tumor size (weight) was reduced at least 50% initial tumor size (weight) and eventually the tumor relapsed, and complete tumor response (CR) was defined as the inability to detect tumor palpitation at the initial site of tumor appearance (tumor complete disappearance or cure, Cao et al., 1999, 2005).

2.10. Statistical analysis

Statistical significance of the data was analyzed by unpaired two-tailed Student's *t* test for independent samples with a minimum significance level set at $p < 0.05$. The average mean of the different samples was calculated and the data represented as mean ± SD.

3. Results

3.1. The chemical constituent study of HTJ5/5A and its fractions

The contents of polysaccharides and nitrogenous compounds are 9.8% and 27.6% in HTJ5 and 5.8% and 37.8% in HTJ5A, respectively. Twenty-two compounds were obtained from the fractions A, B, C and D of HTJ5A by the combination of silica gel column chromatography, gel HW-40, and LH-20 column and purified by semipreparative HPLC. These compounds include seven cycli dipeptides: cyclo(Val–Tyr) (Birkinshaw and Mohammed, 1962), cyclo(Leu–Tyr) (Scopel et al., 2013), cyclo(Phe–Tyr) (Huang et al., 2012), cyclo(Phe–Phe) (Kanzaki et al., 2000), cyclo(Leu–Leu) (Shen et al., 2011), cyclo(Leu–Ala) (Caesar et al., 1969), and cyclo(Val–Ala) (Li et al., 2005); five indole, pyrimidines, amino acids and derivative: 5-hydroxy-2-pyridinecarboxylic acid (Ding et al., 2009), 3-formylindole (Chowdhury and Chakraborty, 1971), uracil (Hu et al., 2005), 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole-3-carboxylic acid (Goda et al., 1999), and tryptophan (Jia et al., 2011); three flavones (flavonoid glycoside): neoliquirtin (Shen et al., 2012), liquiritigenin (Li et al., 2006), and calycosin (Liu et al., 2007); one anthraquinone: emodin (Xu et al., 2008); and six small aromatic compounds: 4-hydroxy-3-methoxybenzoic

Table 1
In vitro cytotoxicity of HTJ5 and HTJ5A against selected human cancer cell lines with MTT assay.

Cancer cell line.	IC50 (mg/ml)	
	HTJ5	HTJ5A
HepG2	2.50 ± 0.25*	2.00 ± 0.25
Huh-7	0.80 ± 0.08	1.50 ± 0.28
HT-29	1.25 ± 0.06	1.25 ± 0.05
NCI-87	5.00 ± 0.22	4.50 ± 0.14

* Mean ± SD.

acid, 4-hydroxy-3-methoxycinnamic acid, hydroxy-benzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzaldehyde and syringic acid.

3.2. Antiproliferation activity of HTJ5 and HTJ5A

We evaluated the antiproliferation activity of HTJ5 and HTJ5A with various concentrations (0.156–20.0 mg/ml) against human cancer cell lines utilizing MTT assay and the results are presented in Table 1. Both HTJ5 and HTJ5A showed activity against the tested cancer cells with the most potent activity observed against the liver cancer cells Huh-7 while it was least active against the gastric cancer cells NCI-87. The IC_{50} values of HTJ5 and HTJ5A are 2.50 ± 0.25 and 2.00 ± 0.25, 0.80 ± 0.08 and 1.50 ± 0.28, 1.25 ± 0.06 and 1.25 ± 0.05l, and 5.00 ± 0.22 and 4.50 ± 0.14 mg/ml against HepG2, Huh-7, HT-29 and NCI-87 cells, respectively.

3.3. Antitumor efficacy and toxicity of HTJ5 and HTJ5A and compared with 5-FU in SCID mice bearing human HepG2 and Huh-7 liver, HT-29 colon and NCI-87 gastric cancer xenografts

Next, we established tumor xenografts in SCID mice from cultured cells with the same human cancer cell lines tested for the *in vitro* cytotoxicity of HTJ5 and HTJ5A. Then, we evaluated the antitumor efficacy and toxicity of HTJ5 and HTJ5A and compared to 5-FU, a most widely used anticancer drug for the treatment of gastrointestinal cancers clinically.

We initially evaluated the antitumor efficacy and toxicity of HTJ5 and HTJ5A at 500 mg/kg/day while 5-FU at 30 mg/kg/day with the schedule of daily for 5 days in SCID mice bearing HepG2 liver cancer xenograts and the kinetics of tumor growth inhibition and body weight loss were illustrated in Fig. 1. Treatment was initiated 7 days after tumor transplantation when the tumors reached ~180–200 mg (mm^3). 5-FU was administrated by i.p. due to its poor oral bioavailability. While HTJ5, HTJ5A and vehicle control were treated by oral route (p.o.). We previously compared the antitumor activity of HTJ5 and HTJ5A at 200 and 500 mg/kg/day for 5 days with i.p. and p.o. against FaDu head and neck cancer xenografts and similar antitumor efficacy was observed with both administration routes but it was less toxic with the p.o. route (data not shown). The data indicates that 5-FU at 30 mg/kg/day by i.p. for daily ×5 schedule is active against HepG2 liver cancer xenografts but is highly toxic to the host. Both HTJ5 and HTJ5A are similar or more active and much less toxic than 5-FU (Fig. 1). Then, we performed a second experiment with HepG2 tumors to increase the doses of HTJ5 and HTJ5A from 500 mg/kg/day to 1000 mg/kg/day to see if it could further increase the antitumor activity and reduce the dose of 5-FU from 30 mg/kg/day to 25 mg/kg/day to see how active and toxicity at low dose with the same administration route and schedule and the data are presented in Fig. 2. The combined data from the two independent experiments are summarized in Table 2. The data showed that the growth of HepG2 xenografts were relatively slow with a tumor doubling time 9.0 ± 0.7 days, while 5-FU at 25 mg/kg/day had only limited

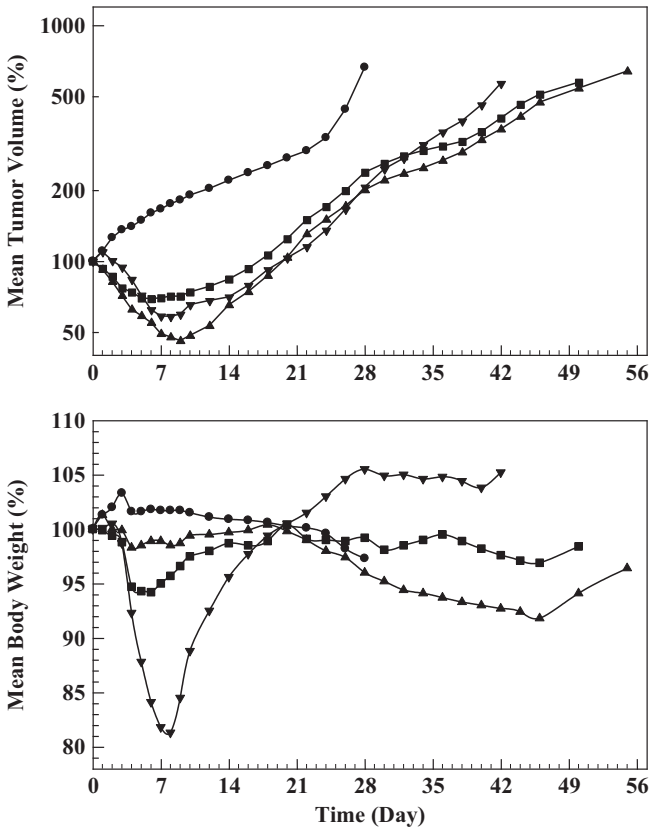

Fig. 1. Kinetics of antitumor activity and toxicity of human HepG2 liver cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. ● Control-vehicle (p.o.); ▼ 5-FU 30 mg/kg/day (i.p.); ▲ HTJ5 500 mg/kg/day (p.o.); and ■ HTJ5A 500 mg/kg/day (p.o.). Each treatment group had 5 mice.

antitumor activity with 49 ± 5.7% tumor growth inhibition, 8.6 ± 0.4 days tumor doubling time without partial response (PR) or complete response (CR) and with 15.1 ± 4.5% body weight loss. 5-FU at 30 mg/kg/day increased antitumor activity to 71.4 ± 22% of tumor growth inhibition, increased the tumor doubling time to 17.6 ± 4 days and achieved 40% PR with 19.6 ± 1.2% body weight loss. Therefore, 5-FU showed obviously dose-dependent manner against HepG2 xenografts. HTJ5 at 500 mg/kg/day achieved tumor growth inhibition of 70%, prolonged tumor growth delay with doubling tumor 21.0 ± 7.2 days and produced 40% PR and 20% CR. HTJ5A at 500 mg/kg/day had similar or slight more activity than HTJ5 with tumor growth inhibition of 77.6 ± 15.5%, prolonged tumor growth delay with doubling tumor 19.0 ± 6.1 days and produced 80% PR. Surprisingly, both HTJ5 and HTJ5A at higher dose (1000 mg/kg/day) did not further increase the antitumor efficacy nor increase toxicity.

We then evaluated the antitumor efficacy and toxicity of HTJ5 and HTJ5A at 1000 mg/kg/day and 5-FU at 25 and 30 mg/kg/day in SCID mice bearing another liver tumor xenografts: Huh-7 which showed more sensitive *in vitro* cytotoxicity response to HTJ5 and HTJ5A compared to HepG2 cells, with the same schedule and administration route used in HepG2 model and the results are presented in Figs. 3 and 4 and Table 3 from the two combined independent experiments. The data indicates that the growth of Huh-7 xenografts were slight fast than that of HepG2 xenografts with a doubling time 7.0 ± 0.3 days. Although 5-FU at 25 mg/kg/day was initially active against the tumors with 72.1 ± 3.9 growth inhibition (vs. 49% for HepG2 tumors) and the tumors quickly relapsed after termination of treatment. No significant tumor growth delay was observed with a tumor doubling time 7.8 ± 0.4 (via 7.0 days of control group, $P > 0.05$) but induced significant

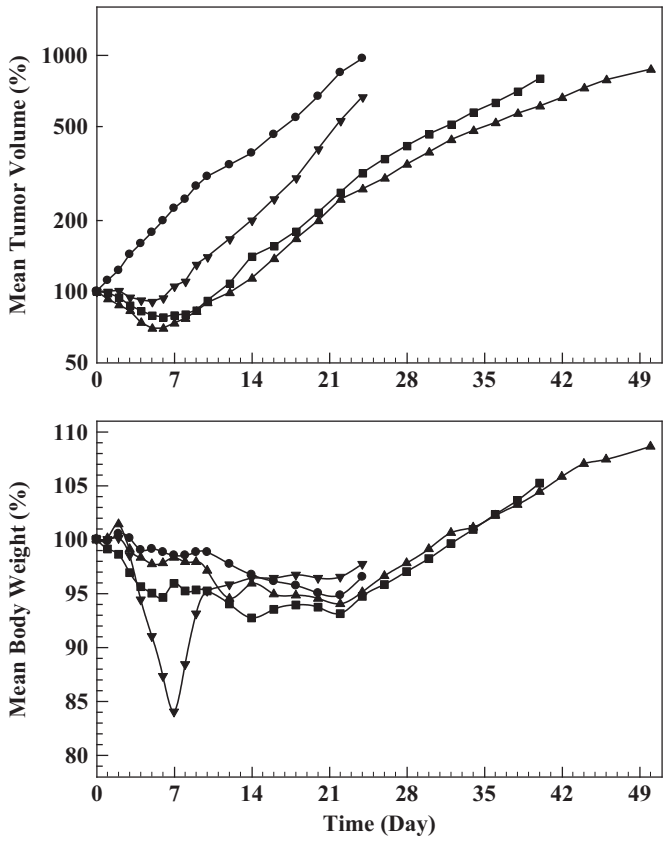

Fig. 2. Kinetics of antitumor activity and toxicity of human HepG2 liver cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. • Control-vehicle (p.o.); ▾ 5-FU 25 mg/kg/day (i.p.); ▴ HTJ5 1000 mg/kg/day (p.o.); and ■ HTJ5A 1000 mg/kg/day (p.o.). Each treatment group had 5 mice.

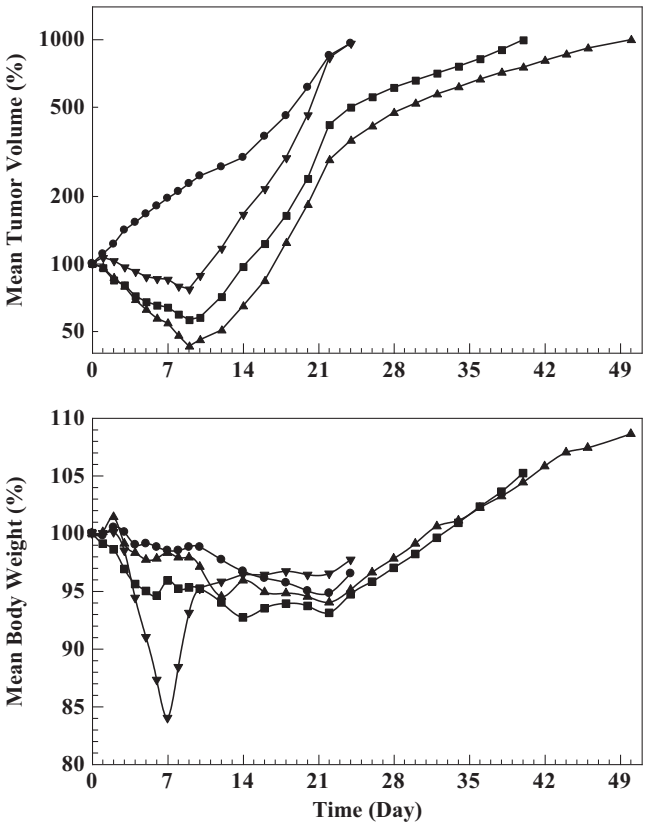

Fig. 3. Kinetics of antitumor activity and toxicity of human Huh7 liver cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. • Control-vehicle (p.o.); ▾ 5-FU 25 mg/kg/day (i.p.); ▴ HTJ5 1000 mg/kg/day (p.o.); and ■ HTJ5A 1000 mg/kg/day (p.o). Each treatment group had 5 mice.

Table 2
Antitumor activity and toxicity of 5-FU, HTJ5, and HTJ5A in SCID mice bearing human HepG2 liver cancer xenografts.

Treatment	Antitumor activity.				Toxicity (%).	
	TGI (%)	TDT (day)	PR (%)	CR (%)	MWL	Lethality
Control-vehicle	–	9.0 ± 0.7	0	0	5.3 ± 3.8	0
5-FU 25 mg/kg/day	49.0 ± 5.7	8.6 ± 0.4	0	0	15.1 ± 4.5	0
5-FU 30 mg/kg/day	71.4 ± 22.0	17.6 ± 4.0	60	0	19.6 ± 1.2	0
HTJ5 500 mg/kg/day	70.0 ± 23.3	21.0 ± 7.2	40	20	9.7 ± 3.6	0
HTJ5 1000 mg/kg/day	66.8 ± 13.0	14.2 ± 1.8	20	0	8.2 ± 2.6	0
HTJ5A 500 mg/kg/day	77.6 ± 15.5	19.0 ± 6.1	80	0	8.9 ± 3.5	0
HTJ5A 1000 mg/kg/day	70.4 ± 12.6	16.3 ± 1.6	20	0	6.4 ± 4.5	0

Five mice were used for each experimental group and repeated once, ten mice in total for each group. The treatment was initiated on day 7 after tumor transplantation when the tumors reached ~180–220 mg (mm^3). 5-FU was administered by i.p. and vehicle control, HTJ5 and HTJ5A by p.o. once a day for 5 days. TGI: tumor growth inhibition; TDT: tumor doubling time; PR: partial response; CR: complete response.
*Mean ± SD.

toxicity with body weight loss of 15.1 ± 4.5%. 5-FU at 30 mg/kg/day achieved similar tumor growth inhibition but slight prolonged tumor doubling time of 8.9 ± 0.6 days with even more toxicity compared to 5-FU at 25 mg/kg/day. HTJ5 and HTJ5A at 1000 mg/kg/day by p.o. were more active against Huh-7 tumors compared to 5-FU at 25 and 30 mg/kg/day by i.p., particularly with tumor growth delay with the tumor growth doubling time 14–15 days ($P < 0.01$) without induced host toxicity. More important 50–60% PR was achieved with HTJ5 and HTJ5A, respectively. Interestingly, Huh-7 liver xenografts were more initially response to 5-FU, HTJ5 and HTJ5A but with less duration of tumor growth inhibition compared to HepG2 tumors.

We also evaluated the antitumor activity and toxicity of HTJ5, HTJ5A and 5-FU in SCID mice bearing HT-29 colon cancer xenografts. First, we used 5-FU at 30 mg/kg/day by i.p. and HTJ5 and HTJ5A at 500 mg/kg/day by p.o. and the results are showed in Fig. 5. Once again, the data indicates that both HTJ5 and HTJ5A had similar antitumor activity against HT-29 tumors and were more active and much less toxic compared to 5-FU. Then, we increased the dose of HTJ5 and HTJ5A to 1000 mg/kg/day while kept 5-FU at the same dose to see if higher dose of HTJ5 and/or HTJ5A could be more active against the xenografts and the results are shown in Fig. 6. The data showed that higher dose of HTJ5 and HTJ5A did not further increase the antitumor activity against HT-29 tumors from the similar observation with the results of HepG2 tumors. The two independent experiments were summarized in Table 4 and the data indicate that the growth of HT-29 xenografts were moderate with a doubling time of 7.6 ± 0.6 days. 5-FU at 30 mg/kg/day had moderate antitumor activity against HT-29 xenografts with the tumor growth inhibition 53.0 ± 8% and prolonged the tumor doubling time compared to the control (12.3 ± 1.8 vs. 8.7.6 ± 0.6 days, $P < 0.05$), however, 5-FU also induced significant toxicity with maximum body weight lose about 20% from the similar observation obtained with liver cancer models. HTJ5 and HTJ5A at 500 mg/kg/day and 1000 mg/kg/day had similar antitumor efficacy against the HT-29 xenografts and were more active and much less toxic than 5-FU against HT-29 xenograft with the tumor

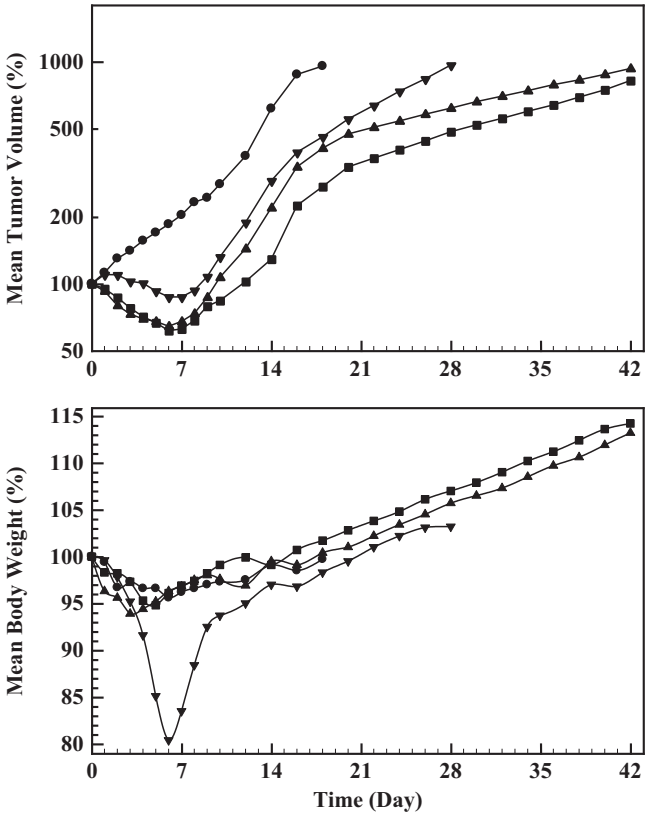

Fig. 4. Kinetics of antitumor activity and toxicity of human Huh7 liver cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. ● Control-vehicle (p.o.); ▼ 5-FU 30 mg/kg/day (i.p.); ▲ HTJ5 1000 mg/kg/day (p.o.); and ■ HTJ5A 1000 mg/kg/day (p.o). Each treatment group had 5 mice.

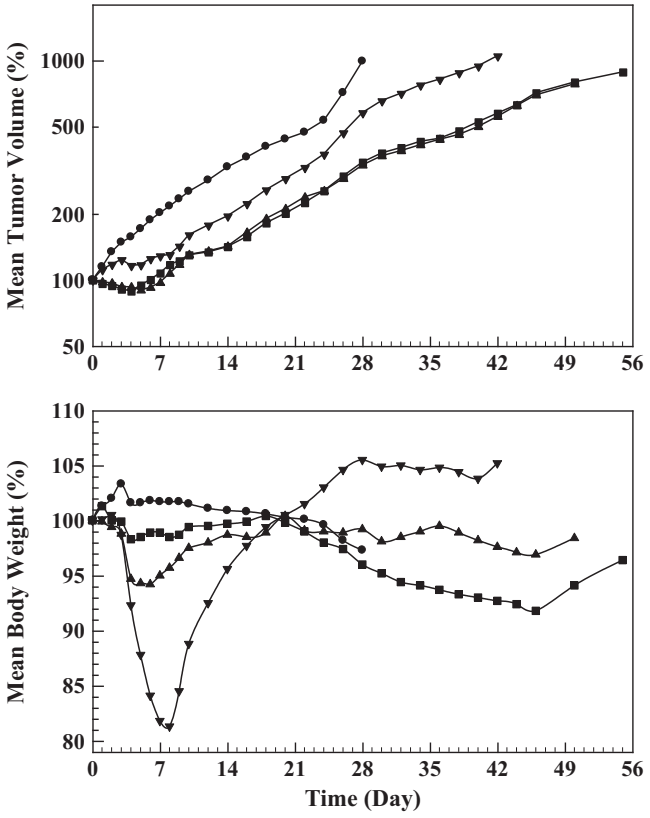

Fig. 5. Kinetics of antitumor activity and toxicity of human HT-29 colon cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. ● Control-vehicle (p.o.); ▼ 5-FU 30 mg/kg/day (i.p.); ▲ HTJ5 500 mg/kg/day (p.o.); and ■ HTJ5A 500 mg/kg/day (p.o.). Each treatment group had 5 mice.

Table 3
Antitumor activity and toxicity of 5-FU, HTJ5, and HTJ5A in SCID mice bearing human Huh-7 liver cancer xenografts.

Treatment	Antitumor activity				Toxicity (%).	
	TGI (%)	TDT (day)	PR (%)	CR (%)	MWL	Lethality
Control-vehicle	–	7.0 ± 0.3	0	0	5.8 ± 3.3	0
5-FU 25 mg/kg/day.	72.1 ± 3.9	7.8 ± 0.4	0	0	15.1 ± 4.5	0
5-FU 30 mg/kg/day	67.6 ± 20.5	8.9 ± 0.6	0	0	19.6 ± 2.7	0
HTJ5 1000 mg/kg/day	79.9 ± 7.5	14.4 ± 1.3	50	0	7.1 ± 3.9	0
HTJ5A 1000 mg/kg/day	81.4 ± 5.6	14.7 ± 1.1	60	0	6.0 ± 2.7	0

Five mice were used for each experimental group and repeated once, ten mice in total for each group. The treatment was initiated on day 7 after tumor transplantation when the tumor reached ~180–220 mg (mm^3). 5-FU was administered by i.p. and vehicle control, HTJ5 and HTJ5A by p.o. once a day for 5 days. TGI: tumor growth inhibition; TDT: tumor doubling time; PR: partial response; CR: complete response.
*Mean ± SD.

growth inhibition of about 65.0% and significantly delay tumor growth with prolonged tumor doubling time of up to 19.3 days. We are not sure HTJ5 and HTJ5A are really not dose dependent and at the dose 500 mg/kg or higher doses or individual tumor had different response to treatment or different drug batch may have different antitumor efficacy.

We further evaluated the antitumor activity and toxicity of vehicle control, HTJ5 and HTJ5A at 1000 mg/kg/day and 5-FU 30 mg/kg/day in SCID mice bearing human gastric NCI-87 from two independent experiments with the same administration route and schedule of liver and colon xenograft models. The data are shown in Fig. 7 and Table 5. The data indicate that the growth of NCI-87 xenografts was relatively slow compared to other xenograft models with a doubling time 11.5 ± 0.5 days. 5-FU at 30 mg/kg/day is active against NCI-87 xenograft in term of tumor growth inhibition (69.2 ± 8.6%), however, it only slight prolonged tumor doubling time with 14.2 ± 1.1 days. 5-FU induced significant and unacceptable toxicity with the body weight loss as high as 21.1 ± 1.1 in treated mice 2–4 day after the treatment but the mice recovered eventually. HTJ5 is active against the NCI-87 tumors with the tumor growth inhibition of 80.5 ± 6.4% and significantly delay tumor growth with prolonged tumor doubling time of 29.9 ± 2.8 days and produced 50% PR. HTJ5A is even slight more active against the NCI-87 tumors compared to HTJ5 with the tumor growth inhibition of 83.5 ± 5.9% with prolonged tumor doubling time of 31.2 ± 4.3 days and achieved 60% but there is no significant difference statistically between HTJ5A and HTJ5 ($P < 0.05$). However, there is a significant difference statistically compared to the groups of HTJ5 and HTJ5A to the groups of control and 5-FU ($P < 0.01$). Interestingly, both HTJ5 and HTJ5A at 1000 mg/kg/day induced no host toxicity.

4. Discussion

The results obtained from the current study demonstrated that the extracts from culture of HE, HTJ5 and HTJ5A, exhibited *in vitro* cytotoxicity and particularly high *in vivo* efficacy against gastrointestinal cancers such as liver, colon, and gastric cancers

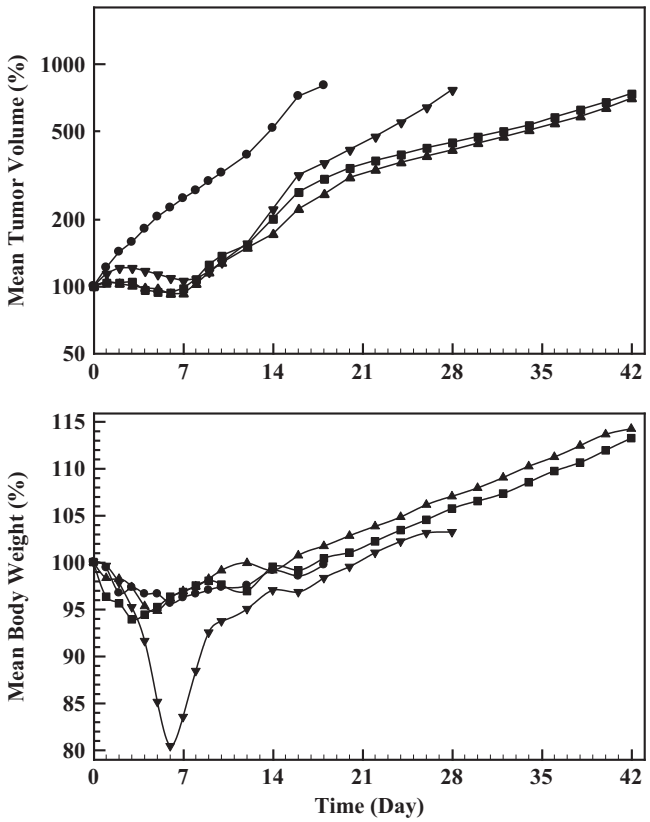

Fig. 6. Kinetics of antitumor activity and toxicity of human HT-29 colon cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. ● Control-vehicle (p.o.); ▾ 5-FU 30 mg/kg/day (i.p.); ▴ HTJ5 1000 mg/kg/day (p.o.); and ■ HTJ5A 1000 mg/kg/day (p.o.). Each treatment group had 5 mice.

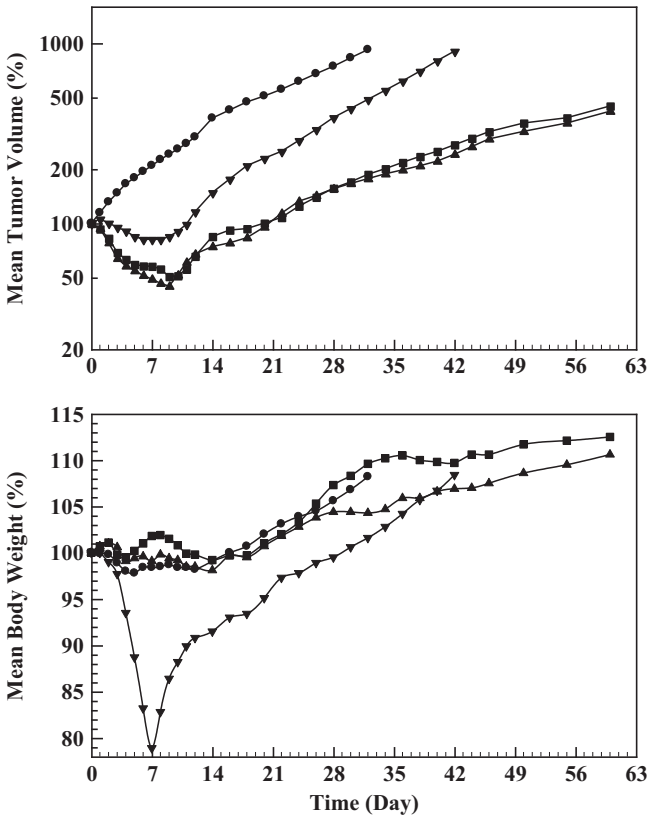

Fig. 7. Kinetics of antitumor activity and toxicity of human NCI-87 gastric cancer xenografts treated with vehicle control, 5-FU, HTJ5 and HTJ5A with daily ×5 schedule. ● Control-vehicle (p.o.); ▾ 5-FU 30 mg/kg/day (i.p.); ▴ HTJ5 1000 mg/kg/day (p.o.); and ■ HTJ5A 1000 mg/kg/day (p.o.). Each treatment group had 10 mice from two independent experiments.

Table 4
Antitumor activity and toxicity of 5-FU, HTJ5, and HTJ5A in SCID mice bearing human HT-29 colon cancer xenografts.

Treatment	Antitumor activity				Toxicity (%)	
	TGI (%)	TDT (day)	PR (%)	CR (%)	MWL	Lethality
Control-vehicle	–	7.6 ± 0.6*	0	0	5.3 ± 4.1	0
5-FU 30 mg/kg/day.	53.0 ± 8.0	12.3 ± 1.8	0	0	19.6 ± 2.0	0
HTJ5 500 mg/kg/day	65.0 ± 17.3	19.3 ± 4.9	0	0	9.7 ± 3.6	0
HTJ5 1000 mg/kg/day	66.8 ± 4.6	16.2 ± 1.3	0	0	5.9 ± 4.3	0
HTJ5A 500 mg/kg/day	66.2 ± 17.8	19.2 ± 3.3	0	0	8.9 ± 3.5	0
HTJ5A 1000 mg/kg/day	64.2 ± 2.9	15.6 ± 1.7	0	0	6.3 ± 2.0	0

Ten mice were used for the groups of control and 5-FU and five mice for the groups of HTJ5 and HTJ5A. The treatment was initiated on day 7 after tumor transplantation when the tumor reached ~180–220 mg (mm^3). 5-FU was administered by i.p. and vehicle control, HTJ5 and HTJ5A by p.o. once a day for 5 days. TGI: tumor growth inhibition; TDT: tumor doubling time; PR: partial response; CR: complete response.

* Mean ± SD.

Table 5
Antitumor activity and toxicity of 5-FU, HTJ5 and HTJ5A in SCID mice bearing human NCI-87 gastric cancer xenografts.

Treatment	Antitumor activity.				Toxicity (%)	
	TGI (%)	TDT (day)	PR (%)	CR (%)	MWL	Lethality
Control-vehicle	–	11.5 ± 0.5*	0	0	2.4 ± 1.8	0
5-FU 30 mg/kg/day	69.2 ± 8.6	14.2 ± 1.1	0	0	21.1 ± 1.1	0
HTJ5 1000 mg/kg/day	80.5 ± 6.4	29.9 ± 2.8	50	0	2.4 ± 1.2	0
HTJ5A 1000 mg/kg/day	83.5 ± 5.9	31.2 ± 4.3	60	0	2.2 ± 1.4	0

Ten mice were used for each experimental group from two independent experiments. The treatment was initiated on day 7 after tumor transplantation when the tumor reached ~180–220 mg (mm^3). 5-FU was administered by i.p. and vehicle control, HTJ5 and HTJ5A by p.o. once a day for 5 days. TGI: tumor growth inhibition; TDT: tumor doubling time; PR: partial response; CR: complete response.

* Mean ± SD

(Tables 1–5 and Figs. 1–7). In addition, other extracts of HE such as HTJ2, HTJ4, HTJ6, HTJ7, HTJ8 and HTJ9 also showed anticancer activity *in vitro* and/or *in vivo* against gastrointestinal cancers (data not shown). Therefore, the extracts of HE have therapeutic potential to be developed as anticancer drugs against gastrointestinal cancers such as liver, colon, and gastric cancers.

HE, as a well-known traditional edible mushroom, contains valuable constituents including polysaccharides, lectins, proteins, lipids, hericenone, erinacol, erinacine, and terpenoids (Kawagishi et al., 1994; Wang and Ng, 2004; Ko et al., 2005). Previous studies have demonstrated that HE polysaccharides possesses antitumor activities (Wang et al., 2001; Lee and Hong, 2010). However, it needs to be further studied to determine whether the other constituents possess anticancer activity. Our data from the

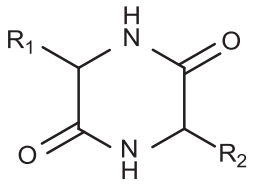

Fig. 8. The general structure of 2, 5-diketopiperazine, where R1 and R2 represent the substituting amino acid residues.

in vivo studies demonstrated that HTJ5A which contains less polysaccharides than HTJ5 is similar or slight more active than HTJ5 against Huh-7 liver cancer xenografts and NCI-87 gastric cancer xenografts. Thus, it's a reasonable presumption that not only polysaccharides of HE but also some other small molecular constituents may contribute to the anticancer activity of HE and they could be developed as potential anticancer drugs.

Interestingly, we discover a lot of chemical compounds include cyclic dipeptides (also known as 2, 5-diketopiperazines or DKPs), flavones, liquiritigenin, calycosin, and others compounds from the extract of HE, which some have not been reported previously for their antitumor activity. Cycli dipeptides are heterocyclic compounds comprising of two amino acid residues linked to a central DKP ring structure. The general structure for DKPs is illustrated in Fig. 8. The DKP backbone is conformationally restrained by a six-membered ring with side chains that are oriented in a spatially defined manner. The DKP ring contains two hydrogen bond-accepting centers and two hydrogen-bond-donating sites, which are important for potential interactions between the lead compounds and receptor sites (Rhee, 2004; Milne and Kilian, 2010). DKPs have already been shown to exhibit antitumor activity (Milne et al., 1998; Graz et al., 2000). For example, cyclo(Phe–Tyr) was proved to be effective against various cancer cell lines and at the concentration of 100 μM produced 60.6%, 73.4% and 75.6% of inhibitory rates against HT-29, Hela, and MCF-7 cells, respectively (Kilian et al., 2005). Cyclo (Trp–Tyr) at the concentration of 2 mM inhibited cell growth of 90.77% and 62.19% against Hela and MCF-7 cells, respectively (Versluis, 2002).

Graz et al. (2000) studied the effect of nine cyclic dipeptides on HT-29 cancer cells. The respective cyclic dipeptides were shown to have great specificity for the cancer cells, with little effect on human normal gastrointestinal mucosa, thereby limiting severe adverse effects with great selectivity. However, the mechanism action of cyclic dipeptides associated with anticancer activity is still unclear. Rhee (2004) has explored the various mechanisms of cyclic dipeptides. Cyclic dipeptides may have anticarcinogenic activity by activating cellular systems to intercept and detoxify carcinogens, or may stimulate DNA damage repair, and/or eradicate heavily damaged cells through apoptosis. Inhibition of DNA topoisomerase I activity has also been proposed as one potential molecular target for cyclic dipeptides.

Liquiritigenin could effectively inhibit pituitary adenoma tumor growth and induce cell apoptotic death via Ras/ERKs and ROS-dependent mitochondrial pathways (Wang et al., 2014). it could also inhibit the migration of lung adenocarcinoma A549 cells through suppression on PI3K/Akt signaling pathway (Wang et al., 2012) and mimic hypoxic-induced HIF-1α protein accumulation in HeLa cells (Xie et al., 2012).

Calycosin could markedly inhibit the growth of human immortalized myelogenous leukemia K562 cells with an IC_{50} of 130.32 μg/ml (Zhang et al., 2013) and human hepatocellular carcinoma BEL-7402 cells by blocking the cells in the G1 phase (Zhang et al., 2013).

Therefore, these compounds may play an important role in the anticancer activity of HE extracts. However, the chemical constituents of HE extracts such as HTJ5 and HTJ5A are very complicated and further studies are needed to confirm their role and possible mechanism. The works are underway and some progresses have already been made in our laboratories.

The study of *in vitro* cytotoxicity of HTJ5 and HTJ5A showed that HTJ5 and HTJ5A exhibited the most potent cytotoxicity observed against the liver cancer cells Huh-7 while it was least cytotoxic against the gastric cancer cells NCI-87. However, the *in vivo* antitumor efficacy studies showed that the agents were similar or even more active against NCI-87 and HepG2 tumors than against Huh-7 tumors although they had initial good response to the treatment but the duration of tumor inhibition was short. While HT-29 xenografts were the least response tumors to the treatment of HTJ5 and HTJ5A *in vivo*. Therefore, the *in vitro* cytotoxicity may not accurately predict the *in vivo* antitumor efficacy for an anticancer agent.

Interestingly, we did not find the dose-dependent manner with HTJ5 and HTJ5A with the tumor xenografts of HepG2 liver cancer and HT-29 colon cancer. Because we used different bath of HTJ5 and HTJ5A and studied at different time with different passage for tumor transplantation with different experiments so we are not sure that HTJ5 and HTJ5A do not really have dose dependency and reached the maximum antitumor efficacy at the dose 500 mg/kg/day or individual tumor had different response to treatment or different drug batch may have different antitumor efficacy. Future studies will be performed to further increase the dose of HTJ5 and HTJ5A to 1500–2000 mg/kg/day with 3–4 different doses at the same time against the same tumor xenografts to validate the finding.

We also studied the effect of HTJ5 and HTJ5A in combination with clinically widely used anticancer drugs for gastrointestinal cancer such as 5-FU, cisplatin and doxorubicin against the four tumor xenografts. Our preliminary data showed that synergistic or additive antitumor effect without increased toxicity was observed with the combinations (data not shown) and more studies are needed to confirm the results. HE extracts may also be developed as the modulators for anticancer drugs used in combination for the treatment of gastrointestinal cancer clinically.

5. Conclusion

Our studies demonstrated that HE extracts (HTJ5 and HTJ5A) are active against liver cancer HepG2 and Huh-7, colon cancer HT-29 and gastric cancer NCI-87 cells *in vitro* and tumor xenografts bearing in SCID mice *in vivo*. They are more effective and less toxic compared to 5-FU in all four tested *in vivo* tumor models. The compounds have the potential to be developed into the anticancer agents for the treatment of gastrointestinal cancer used alone and/or in combination with clinically used chemotherapeutic drugs. However, further studies are required to find out the active contents and understand the mechanism of action associated with the super *in vivo* anticancer efficacy. Moreover, more studies will be performed to confirm our preliminary results of *in vivo* synergistic antitumor efficacy of HE extracts in combination with clinical used anticancer drugs such as 5-FU, cisplatin and doxorubicin for the treatment of gastrointestinal cancers against tumor xenograft models.

Acknowledgments

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