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Anti-tumor activity of the fermentation broth of *Cordyceps militaris* cultured in the medium of *Radix astragali*

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Abstract

This study used *Radix astragali* (RA) as the medium for culturing *Cordyceps militaris* to investigate the anti-tumor activity of the fermentation broth. It was found that the product from culturing *C. militaris* in RA medium had a better anti-tumor activity than that culturing in synthetic medium. The fermentation broth inhibited the growth of four tumor cells including human gastric cancer AGS cells, human breast cancer MCF-7 cells, human hepatocellular carcinoma Hep G2 cells and murine colorectal adenocarcinoma CT26 cells with IC_{50} 465 μg/mL, 37 μg/mL, 25 μg/mL, and 20 μg/mL, respectively. Although cordycepin was the major bioactive component with the strongest anti-tumor activity in the fermentation broth of *C. militaris* in RA medium, there were other constituents which enhanced the anti-tumor activity of fermentation broth synergistically. To validate the anti-tumor activity, the BALC/c mice were implanted with CT26 cells and then fed with various dosages of the fermentation product. It was found that 20 mg/kg body weight (BW)/day group had no significant anti-tumor activity as compared to the control group. The dosage of 100 mg/kg BW/day and 200 mg/kg BW/day group inhibited the tumor volume by 43.81% and 48.89%. Tumor weight was also reduced by 31.21% and 39.48% compared to the control group. Besides, the fermentation broth had low cytotoxicity against primary rat hepatocytes, and did not impose serious side effect on the vital organs of the mice as compared to the chemotherapeutic drug 5-FU.

Keywords: *Cordyceps militaris*; *Radix astragali*; Fermentation; Cordycepin; Anti-tumor activity; BALB/c male mice

1. Introduction

Cordyceps militaris, a caterpillar-shaped Chinese traditional mushroom, is an entomopathogenic fungus, which belongs to the class Ascomycetes and DongChongXiaCao group in Chinese herbs [1]. Recent studies have pointed out that *C. militaris* has many pharmacological functions such as inhibition of human glomerular mesangical cell proliferation induced by native LDL [2], antifibrotic effect on fibrotic rats induced by BDL/S [3], antiangiogenetic effect [4,5], anti-inflammatory [5], improvement of insulin resistance and insulin secretion [6], and growth inhibition of U937 leukemia cells through induction of apoptosis [7]. The major bioactive compound of *C. militaris* is cordycepin which is reported to possess many biological and pharmacological activities such as immunological stimulating, anti-virus, and anti-cancer activities [8–12].

Radix astragali (RA) is the dried root of *Astragalus membranaceus Bge. Var. mongholicus* and used as a tonic in the traditional Chinese medicine. RA has been used extensively as an adjuvant in cancer treatment and as a phytochemical immune modulator to strengthen the host defense system [13–17]. In the last decade, many researchers found that RA can reduce radical-mediated injury to renal tubules [18], protect the inflammation in human amnion [19], induce cell differentiation/death in K562 and HEL cells [20], and activate the cytotoxicity of lymphocytes to increase the secretion of IL-2 and IFN-γ [21]. *R. astragali* contains several bioactive constituents such as isoflavonoids, saponins, and polysaccharides [22,23]. The saponins of RA show several therapeutic effects in the progress of diabetic nephropathy [24], prominent IL-2 inducing activity [25], and protective effect against myeloid Graffi tumor [26]. The major isoflavonoid, calycosin, is an active component in *R. astragali*, and it displayed beneficial effects such as anti-tumor activity [27], protection of

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endothelial cells from hypoxia-induced barrier impairment [28], and inhibition of lipid peroxidation by reactive oxygen species [29]. The RA polysaccharides can activate mouse B cells and macrophages [30].

Fermentation is a useful tool for producing biological materials with health promoting properties. The culture medium is very important to the yield of bioactive products because these nutrients are directly related to cell proliferation and metabolite biosynthesis [31–35]. When the carbon and nitrogen sources in the medium are not enough, it might stimulate the microorganisms to go to a different pathway and produce different biosynthesis metabolites. Many investigators have tried to use different medium such as chitosan, wheat bran and bean powder to obtain more bioactive compounds and mycelium [36–38]. Plant oils and fatty acids were used to stimulate the exo-biopolymer production in *C. militaris* [39]. The *R. astragali* (RA) has been used in the medium for culturing *Ganoderma lucidum* for producing bioactive polysaccharides during solid-state fermentation [40]. Because both of RA and *C. militaris* possess anti-tumor property, in this study, we used *R. astragali* as the medium to cultivate *C. militaris* and investigate the anti-tumor activity of the fermentation product.

2. Materials and methods

2.1. Microorganism and culture

The fungal strain used (deposited at the Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Hsinchu, Taiwan) was *C. militaris* (BCRC 32219). The strain was maintained on potato dextrose agar (PDA) plates at 4 °C in a refrigerator. Before the experiment, the active PDA slant of the mycelia was prepared by culturing at 25 °C for 8 days, and then the seed culture was transferred from the active slant grown on PDA medium in a Petri dish at 25 °C for another 8 days. The inoculant was prepared by punching out 1 cm of the PDA plate culture with a sterilized cylindrical cutter. Starter flasks (250 mL) of YM broth were inoculated with mycelia mat (ca. 1 cm^2) from a stock culture and incubated on a shaker at 175 rpm for 5 days at 25 °C. The starter cultures (20 mL) were transferred to 500 mL Erlenmeyer flasks with 200 mL medium and incubated at 25 °C. For comparison, *C. militaris* was cultured in either RA suspension or synthetic medium. The RA used in this study was *Astragalus membranaceus* (*Fisch.*) *Bunge*. The RA medium contained 20 g RA powder and 180 mL water. Synthetic medium was composed of 5% glucose, 3% yeast extract, and 0.5% peptone. After fermentation, the broth was centrifuged at 10,000 rpm for 20 min. The supernatant was filtrated by Whatman no. 1 paper, subjected to freeze-drying, and stored at −20 °C in a freezer for further experiments. All fermentation experiments were performed in triplicate to ensure the reproducibility.

2.2. Cell cultivation and viability assay

Human gastric cancer AGS cell line, murine colorectal adenocarcinoma CT26 cell line, human hepatocellular carcinoma Hep G2 cell line and human breast cancer MCF-7 cell line were purchased from the Bioresource Collection & Research Centre (Food Industry Research and Development Institute, Hsinchu, Taiwan). AGS and CT26 cells were cultured in RPMI1640 medium (Gibco, Grand Island, NY). Hep G2 and MCF-7 cells were cultured in DMEM medium (Gibco, Grand Island, NY). All medium were supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT) at 37 °C in a humidified 5% CO_2 incubator, passaged every 2–3 days with trypsin-EDTA-glucose (TEG) solution (0.25% trypsin, 0.1% EDTA and 0.05% glucose in Hanks' balanced salt solution), and maintained in exponential growth.

The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) colorimetric method was used to determine the cell viability. In brief, tumor cells were cultured in a 96-well microplate (10^5 cells per well in 100 mL medium) for 24 h, and then incubated with different concentrations of sample for 72 h. At the end of incubation, tetrazolium dye was added as an indicator in order to convert tetrazolium salts to a colored product, formazan. The formazan concentration was measured by spectrophotometer at 570 nm.

2.3. Cytotoxicity test using primary rat hepatocytes

The primary rat hepatocytes were obtained from anesthetized rats treated with intraperitoneal injection of sodium pentobarbital (100 mg/kg body weight). Cell viability was confirmed to be more than 90% via trypan blue exclusion. After washed, the isolated hepatocytes were resuspended in L-15 cell culture medium (pH 7.6) which supplemented with 18 mM HEPES, 2.5% FBS, 5 mg/L of insulin, 5 mg/L of transferrin, 28 mM galactose, 1 M dexamethasone, 100,000 IU/L penicillin, and 100 mg/L streptomycin at a density of 5×10^8 cells/L. Cells (2.5×10^6) were plated on 60 mm collagenprecoated plastic tissue culture dish (Falcon Labware) and incubated at 37 °C in a humidified incubator (NUAIRE, USA). After 4 h plating, the medium was changed by the new medium that contained the same components but replaced FBS with 2.0 g/L BSA. After 16 h, medium was renewed and testing sample of various dosages was added. After 24 h, the medium was removed to stop the reaction and cells were washed with phosphate buffered saline. Then cells were removed with a cell scraper for analysis [41].

2.4. HPLC analysis

Both of the freeze-dried sample and standard cordycepin (Sigma–Aldrich Co., St. Louis, MO) were dissolved in distilled water before being analyzed by HPLC. The Agilent ZORBAX ODS C18 column (4.6 mm × 250 mm, 5 mm particle size) was used for separation. The mobile phase was 0.02 M KH_2PO_4, which was dissolved in methanol/distilled water (15:85). Elution was performed at a flow rate of 0.7 mL/min with column temperature at 25 °C. The UV wavelength of 254 nm was monitored by a tunable absorbance detector (model: 1100 series, Agilent, USA) [9]. Syringe of 5 μL was used for injection. The calibration curve obtained by the standard cordycepin was used for determining the concentration of cordycepin in the sample.

2.5. Separation and identification of the bioactive constituents in the broth

The freeze-dried sample was dissolved in distilled water with the concentration of 1000 ppm, treated with 90% (v/v) acetone, and centrifuged for 10 min at 7000 rpm. The precipitate was collected and dried by lyophilization and the filtrate was dried by rotary evaporator. The semi-preparative column (Hypersil HS C18 column, 10 mm × 250 mm, 5 mm particle size) was used for constituent separation. Mobile phase was methanol/distilled water (15:85). Elution was performed at a flow rate of 10 mL/min with column temperature 25 °C. The UV wavelength of 254 nm was monitored by a tunable absorbance detector (model: 1100 series, Agilent, USA) [9]. Syringe of 75 μL was used for injection. The eluate was collected and dried by vacuum evaporator for further bioassay. Four fractions were selected and diluted to analyze their anti-tumor activities for CT26 cell line. In proportional to their peak areas (1:1:6:47), fractions were also mixed to test anti-tumor activities for CT 26 cell line of different combinations.

2.6. Animal model

BALB/c male mice (6–8 weeks old) were obtained from the National Laboratory Animal Center (Taipei, Taiwan) and housed in a rodent facility at 22 ± 1 °C with a 12-h light/12-h dark cycle. All experiments were performed in accordance with the regulations of the NIH Guide for the Care and Use of Laboratory Animals (DHHS publication No. NIH 85-23, revised 1996). 5-Fluorouracil (5-FU) was purchased from Sigma–Aldrich Co. There were six groups of mice in the animal model including normal group, control group, experimental groups (20 mg/kg BW/day, 100 mg/kg BW/day and 200 mg/kg

BW/day), and the positive control (5-FU) group. Each group had 8 mice. Besides the normal group, mice were implanted with CT26 cells (1×10^5 cells) by subcutaneous injection into the right gluteal region. The normal group and control group received PBS (NaCl 8 g, KCl 0.2 g, Na_2HPO_4 1.44 g and KH_2PO_4 0.24 g dissolved in 1000 mL distilled water, pH 7.4) orally with feeding needle everyday. The experimental groups received daily oral injections of sample with 20 mg/kg BW/day, 100 mg/kg BW/day, and 200 mg/kg BW/day. The 5-FU group received intra-peritoneal (i.p.) 15 mg/kg BW/day of 5-FU everyday after inoculation.

2.7. Evaluation of tumor volume, leukocyte count, tumor and visceral organ weight

The total body weight of each mouse and size of implanted tumors were determined everyday by a single observer. Calipers were used to measure the largest (a) and smallest (b) diameter, and the tumor volume was estimated according to the formula $0.5ab^2$. The leukocyte count was estimated by retro-orbital blood sampling every 4 days from the day before mice were injected with CT26 tumor cells. Animals were sacrificed on the 21 days after inoculation. After mice were sacrificed, the tumor and visceral organ weights were measured.

2.8. Statistical analysis

All values are means of at least three replicates ± S.D. Statistical analysis was performed using ANOVA and Duncan test (SAS Institute Inc., Cary, NC, USA) to determine significant differences among means ($p < 0.05$).

3. Results and discussion

3.1. Effect of fermentation temperature on anti-tumor activity

Using anti-tumor activity as marker, we tested four different fermentation temperatures on four tumor cell lines (AGS, MCF-7, Hep G2, and CT26). It was observed that *C. militaris* grew successfully at 15, 20 and 25 °C. When the culture temperature was 30 °C, the *C. militaris* did not grow well and the broth had little anti-tumor activity. This result agreed with the reports in the literatures that the optimal temperatures for the growth of *Cordyceps* mycelial and exopolysaccharide production ranged from 20 to 28 °C [42–44].

The IC_{50} of the fermentation broth obtained at 25 °C for AGS, MCF-7, Hep G2, CT26 cell lines were 465.17, 36.9, 24.63, and 20.28 μg/mL, respectively (Table 1). Wu et al. used the ethyl acetate extract of *Cordyceps sinensis* mycelium to treat cancer cells, and they found that IC_{50} for MCF-7 was 44.7 μg/mL [45]. In our study, we used the crude fermentation broth without purification but still observed a similar strength of anti-tumor activity as Wu's research. The best cultivation temperature for *C. militaris* in RA medium appeared to be 25 °C. The fermentation broth cultivated at 25 °C was also tested on the primary rate hepatocytes to examine its toxicity against normal cells. The IC_{50} for the primary hepatocytes was 359.85 ± 17.14 μg/mL as compared to 24.63 ± 0.77 μg/mL for hepatoma cells, Hep G2. It appeared that the fermentation broth possessed strong anti-tumor activity but was much less toxic to the normal cells. Therefore, it may be a potential remedy for cancer treatment.

The murine colorectal adenocarcinoma CT26 cell line was most susceptible to the *C. militaris* broth. In fact, no matter the *C. militaris* was cultured at 15, 20, or 25 °C, the anti-tumor activity of the broth for the cancer cells was in the same order of CT26 > Hep G2 > MCF-7 > AGS (Table 1). Since the CT26 was the most sensitive cell line for the *C. militaris* broth, this cell line was used for the subsequent studies.

Table 1
Effect of cultivation temperature on IC_{50} (μg/mL) of *Cordyceps militaris* fermentation broth for different tumor cell lines

Cell line[a]	Temperature (°C)			
	15	20	25	30
AGS	660.61 ± 56.91[b]	698.00 ± 23.67[b]	465.17 ± 63.29[a]	>1000
MCF-7	40.19 ± 0.47[b]	38.32 ± 0.06[ab]	36.90 ± 3.72[a]	>1000
Hep G2	28.44 ± 2.09[b]	28.31 ± 0.39[b]	24.63 ± 0.77[a]	>1000
CT26	25.83 ± 2.95[b]	28.25 ± 0.56[c]	20.28 ± 2.65[a]	>1000

AGS, Human gastric cancer cell line; CT26, murine colorectal adenocarcinoma cell line; Hep G2, human hepatocellular carcinoma cell line; MCF-7, human breast cancer cell line.

[a] Means with different letters (a–c) within a row are significantly different ($p < 0.05$).

3.2. Effect of agitation and cultivation time on CT26 cells

Many investigations have shown that the aeration and agitation will influence the mycelial morphology of *C. militaris* [46,47]. The effect of agitation and cultivation time on the IC_{50} of *C. militaris* fermentation broth for CT26 cells was shown in Table 2. The anti-tumor activity of the fermentation broth without agitation increased with the cultivation time, and there was a sharp increase in anti-tumor activity from 3 to 4 weeks of cultivation. For the fermentation at 100 rpm, practically no anti-tumor activity was found in the broth after 2 weeks of cultivation. After 3 weeks of cultivation with agitation, the IC_{50} of the fermentation broth was approximately 700 μg/mL. These results clearly showed that the static culture of *C. militaris* had better anti-tumor activity than the agitated culture. From the morphological observation, the mycelium of *C. militaris* matted together on the surface of medium during static cultivation, and the thickness and area of the mat increased with fermentation time. When the agitation speed was 100 rpm, the mycelium formed 2–3 mm pellets suspended in the broth. Masuda et al. [47] also demonstrated that in the shaking culture of *C. militaris* the cordycepin production was suppresses to 1/10 (buffled Erlenmeyer flask) ~1/3 (no-buffled) relative to the surface culture, and they suspected that shear stress might have adverse effect on the fungus. Contrarily, Mao et al. [48]

Table 2
Effects of cultivation time and agitation speed on IC_{50} (μg/mL) of *Cordyceps militaris* fermentation broth produced at 25 °C for CT26 cell line

Agitation speed[a]	Time			
	1 week	2 weeks	3 weeks	4 weeks
100 rpm	>1000	>1000	673.31 ± 75.01[a]	689.4 ± 79.65[a]
0 rpm	>1000	516.14 ± 169.33[c]	248.23 ± 9.08[b]	20.28 ± 2.65[a]

[a] Means with different letters (a–c) within a row are significantly different ($p < 0.05$).

demonstrated that the maximum cordycepin production was achieved under 110 rpm of agitation. Nevertheless, our study suggested that the optimal cultivation condition for *C. militaris* in RA medium was at 25 °C for 4 weeks without agitation.

3.3. Cultivation medium comparison

Composition of the medium is very important for fermentation. Different medium will stimulate the production of different secondary metabolites and active compounds. Using anti-tumor activity as a marker, we compared the bioactivity of the fermentation broth of *C. militaris* cultured with synthetic medium and RA medium. First of all, we found that the IC_{50} of RA medium without fermentation was higher than 1000 μg/mL, and practically did not have any anti-tumor activity. The IC_{50} of fermentation broth from RA medium and from synthetic medium for CT26 cell line were 20.28 μg/mL and 43.03 μg/mL, respectively (Table 3). This result indicated that the fermentation broth of *C. militaris* cultured in RA medium has stronger anti-tumor activity than that in synthetic medium. Cordycepin is commonly recognized as a major anti-tumor compound of *C. militaris* and usually used as marker during fermentation of *C. militaris*. Yoshikawa et al. [18] demonstrated that orally administered cordycepin 15 mg/kg BW inhibited melanoma cell growth in mice with no adverse effects. Cordycepin can inhibit cancer cell growth both *in vitro* and *in vivo*. In our study, the cordycepin content in RA and synthetic medium were 46.35 ppm and 112.62 ppm, respectively (Table 3). It is interesting to note that the fermentation broth from synthetic medium had more cordycepin content (Fig. 1) but less anti-tumor activity than that in RA medium.

3.4. Constituents analysis

From previous results, we concluded that cordycepin is not the only compound with anti-tumor activity in the fermentation broth. Because the fermentation broth contained one-fourth of protein, we used 90% acetone to precipitate protein and evaluated the anti-tumor activity of precipitate and filtrate. It was found that the precipitated protein had no anti-tumor activity, but the anti-tumor activity of the filtrate was significantly increased due to protein removal. The IC_{50} of filtrate for CT26 was 4.25 ± 0.86 μg/mL (Table 4), much higher than the crude broth (20.28 ± 2.65). By using semi-preparative column, we successfully separated major anti-tumor constituents from the filtrate. Four fractions were collected and analyzed for their anti-tumor activities (Fig. 2). Fraction 4 was identified to be cordycepin. The IC_{50} of fraction 1 to fraction 4 for CT26 were 10.12 ± 1.69, 23.34 ± 1, 50.34 ± 3.77, and 2.91 ± 0.17 μg/mL, respectively (Table 4). It is worth to note that the combinations of fractions 1 + 4 and 2 + 4 had significantly higher anti-tumor activity than fraction 4 ($p < 0.05$), the cordycepin. In particular, fraction 2 plus fraction 4 had the strongest anti-tumor activity with IC_{50} 2.05 ± 0.15 μg/mL, only 70% of the IC_{50} value of cordycepin (fraction 4). This result demonstrated that fraction 1 or 2 and fraction 4 had synergistic effect for anti-tumor activity. However, because of the complexity of the constituents found in the fermentation broth, purification and identification of these fractions, and their synergistic effects on anti-tumor activity need to be further investigated.

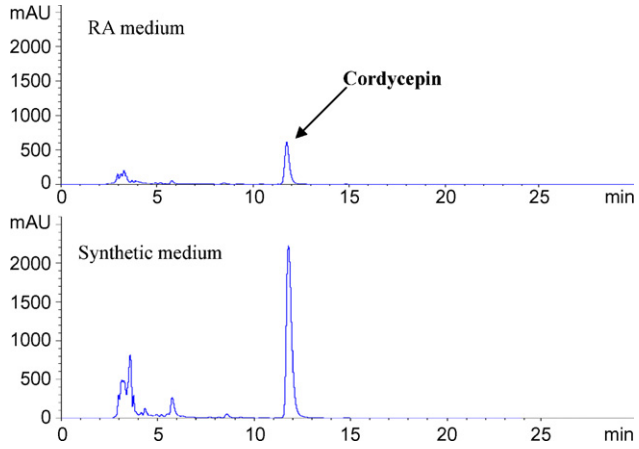

Fig. 1. The HPLC profiles of cordycepin from RA and synthetic mediums. The mediums were fermented by *C. militaris* at 25 °C for 4 weeks without agitation.

3.5. Changes in mice body weight and leukocyte count

The applied dosages of freeze-dried *C. militaris* broth were 20 mg/kg BW/day, 100 mg/kg BW/day and 200 mg/kg BW/day for the study of anti-tumor activity *in vivo*. It was found that

Table 3
Comparison of the cordycepin content and IC_{50} (μg/mL) for CT26 cells of *Cordyceps militaris* fermentation broths produced using synthetic medium and RA medium at 25 °C for 4 weeks without agitation

IC_{50} and cordycepin[a]	Medium	
	Synthetic medium	RA medium
IC_{50} for CT 26 (μg/mL)	43.03 ± 3.47^{b}	20.28 ± 2.65^{a}
Cordycepin content (ppm)	112.62 ± 3.9^{b}	46.35 ± 0.81^{a}

[a] Means with different letters (a and b) within a row are significantly different ($p < 0.05$).

Table 4
The IC_{50} of various fractions of the filtrate of *C. militaris* fermentation broth for CT 26 cells

Fractions	IC_{50} for CT26[a] (μg/mL)
Filtrate	4.25 ± 0.86
Fr. 1	10.12 ± 1.69
Fr. 2	23.41 ± 1.00
Fr. 3	50.34 ± 3.77
Fr. 4	2.91 ± 0.17^{c}
Fr. 1 + 2	13.09 ± 2.31
Fr. 1 + 3	35.21 ± 3.38
Fr. 2 + 3	23.44 ± 2.18
Fr. 1 + 4	2.33 ± 0.04^{b}
Fr. 2 + 4	2.05 ± 0.15^{a}
Fr. 3 + 4	2.76 ± 0.16^{c}

[a] Means with different letters (a–c) within a column are significantly different ($p < 0.05$).

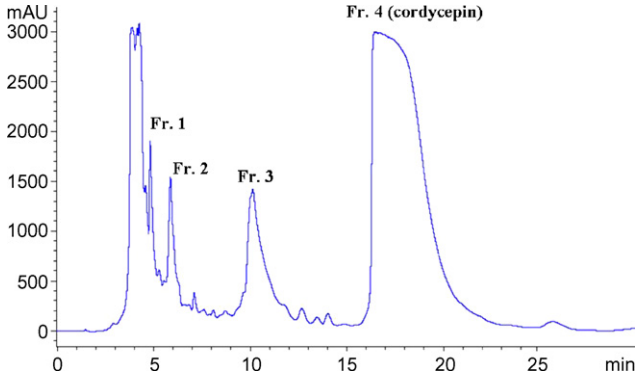

Fig. 2. The chromatogram of the filtrate of *C. militaris* fermentation broth after protein removal by acetone precipitation and separated by semi-preparative HPLC system.

except the positive control group, 5-FU group, there was no significant change in body weight in all treatment groups (Fig. 3). 5-FU is a fluorinated pyrimidine whose metabolites are believed to trigger apoptosis by depleting thymidine. This chemical is commonly used in the treatment of carcinomas of the colon, rectum, stomach, pancreas, breast, head and neck, anus, and gallbladder. The predominant side effects of 5-FU are diarrhea, anorexia, enteritis, hand–foot syndrome, and myelosuppression. After the treatment, mice in the positive control group were more angular than mice in other group, which was the normal side effect of 5-FU. The fact that all of the mice in the treatment groups did not show any loss in body weight suggested that the *C. militaris* broth did not affect the normal metabolism of the animals.

As shown in Fig. 4, the leukocyte count of mice in the positive control group decreased significantly. In other words, mice treated with 5-FU had weaker immune ability. Contrary to the positive control group, the leukocyte counts in other treatment groups increased slightly and there was no significant

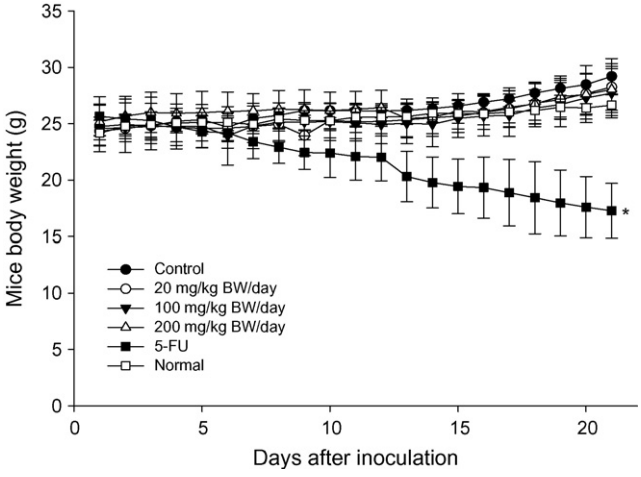

Fig. 3. Changes of body weight of CT26-implanted BALB/c mice administrated with different samples. The mice in 5-FU group were intraperitoneally administrated with 10 mg/kg BW/day, and the mice in normal and control groups were orally administrated with PBS. ●: Control, ○: 20 mg/kg BW/day, ▼: 100 mg/kg BW/day, △: 200 mg/kg BW/day, ■: 5-FU, □: Normal. *Significantly different at 0.05% level compared with normal group.

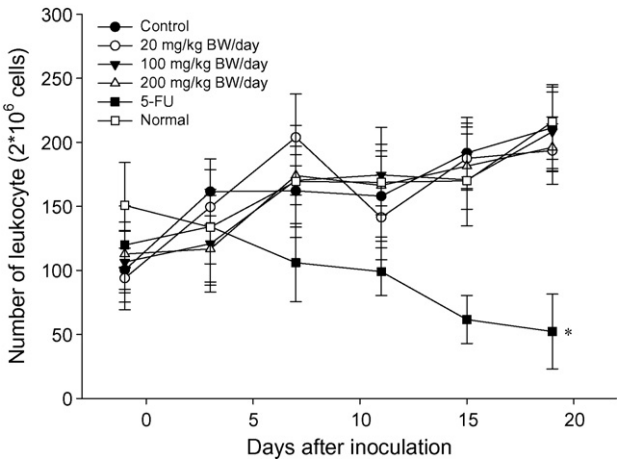

Fig. 4. Changes in leukocyte number of CT26-implanted BALB/c mice administrated with different samples. The mice in 5-FU group were intraperitoneally administrated with 10 mg/kg BW/day, and the mice in normal and control groups were orally administrated with PBS. ●: Control, ○: 20 mg/kg BW/day, ▼: 100 mg/kg BW/day, △: 200 mg/kg BW/day, ■: 5-FU, □: Normal. *Significantly different at 0.05% level compared with normal group.

difference between these groups. This result indicated that the immune system of mice were unaffected by the treatment of *C. militaris* fermentation broth.

3.6. Inhibition of tumor growth

As shown in Fig. 5, low-dose treatment (20 mg/kg BW/day) had no significant effect on tumor growth as compared to the PBS control group. However, 100 mg/kg BW/day and 200 mg/kg BW/day treatments showed a significant decrease in tumor size ($p < 0.05$). There was no significant difference between these two groups. After 21 days of inoculation, the inhibition of tumor size in 100 mg/kg BW/day and 200 mg/kg BW/day

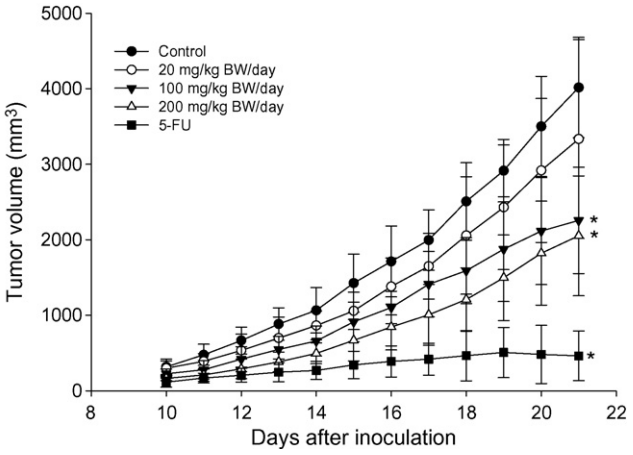

Fig. 5. Changes in tumor volume (mm^3) of CT26-implanted BALB/c mice administrated with different samples. The mice in 5-FU group were intraperitoneally administrated with 10 mg/kg BW/day, and the mice in normal and control groups were orally administrated with PBS. The tumor volume was determined by direct measurement with calipers and calculated by the formula: (length (mm) × width $(mm)^2$)/2. ●: Control, ○: 20 mg/kg BW/day, ▼: 100 mg/kg BW/day, △: 200 mg/kg BW/day, ■: 5-FU. *Significantly different at 0.05% level compared with control group.

Table 5
Changes of organ weight (g) of CT26-implanted BALB/c mice fed with various amount samples for 21 days

Group	Organ[a]				
	Heart	Liver	Lung	Kidney	Tumor
Normal	0.158 ± 0.006^{b}	1.846 ± 0.146^{b}	0.185 ± 0.022^{b}	0.479 ± 0.010^{b}	–
Control	0.145 ± 0.011^{b}	1.993 ± 0.224^{b}	0.209 ± 0.014^{bc}	0.488 ± 0.034^{b}	5.314 ± 0.968^{c}
20 mg	0.148 ± 0.015^{b}	1.998 ± 0.155^{b}	0.218 ± 0.023^{c}	0.461 ± 0.043^{b}	4.516 ± 1.026^{c}
100 mg	0.148 ± 0.007^{b}	1.936 ± 0.174^{b}	0.219 ± 0.035^{c}	0.469 ± 0.036^{b}	3.655 ± 0.813^{b}
200 mg	0.154 ± 0.023^{b}	1.884 ± 0.232^{b}	0.189 ± 0.022^{b}	0.465 ± 0.065^{b}	3.216 ± 1.153^{b}
5-FU	0.104 ± 0.022^{a}	0.981 ± 0.340^{a}	0.151 ± 0.028^{a}	0.279 ± 0.055^{a}	0.744 ± 0.583^{a}

[a] Means with different letters (a–c) within a column are significantly different ($p < 0.05$).

groups were 43.81% and 48.89%, respectively. This result indicated that *C. militaris* broth could effectively inhibit the tumor growth not only *in vitro* but also *in vivo*. Wu et al. [45] also found that 12 μg/mL of the ethyl acetate extract of *Cordyceps sinensis* mycelium could inhibit the proliferation of B16 cancer cell by 50%. When mice were treated with 500 mg/kg BW/day of the acetate extract for 27 days, the tumor weight was decreased by 48%; the tumor volume was reduced by 62%. In our study, the fermentation broth itself, without extraction, could achieve similar anti-tumor activity.

3.7. Changes of tumor and organs weight

When the mice were sacrificed on the 21 days after inoculation, it was found that the positive group had the minimum tumor weight of 0.744 g (Table 5). Although there was no significant difference in tumor weight between PBS control group and 20 mg/kg BW/day group, when the treatment dosage was increased to 100 and 200 mg/kg BW/day, the tumor weight were reduced significantly and the reduction of tumor weight were 31.21% and 39.48%, respectively. We suspect that further increase of dosage may be able to enhance the anti-tumor effect of the *C. militaris* fermentation broth. Since the weight of all of the vital organs of mice checked in this study were basically unaffected by the treatment, suggesting that the *C. militaris* fermentation broth did not have any serious side effect on mice's organ development. The results reported above demonstrated that the fermentation broth of *C. militaris* cultured in RA medium had strong anti-tumor activity both *in vitro* and *in vivo* without significant side effect.

4. Conclusion

This study found that using *R. astragali* as the medium could enhance the anti-tumor activity of the fermentation broth of *C. militaris* as compared to the synthetic medium. Although cordycepin is definitely the key compound responsible for the anti-tumor activity of the fermentation broth, there are other unknown compounds also contributing to the anti-tumor activity of the broth synergistically with cordycepin. Therefore, further experiments are needed in order to identify all of the bioactive compounds as well as the possible anti-tumor mechanism.

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