Antioxidant and anticancer effects of extracts from fermented *Haliotis discus hannai* with *Cordyceps militaris* mycelia

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Abstract In this study, *Haliotis discus hannai* (*H. discus hannai*) was fermented using *Cordyceps militaris* (*C. militaris*) and was investigated for the improvement of its antioxidant and anticancer potential after fermentation. Different radical scavenging activities of the extracts from fermented *H. discus hannai* with *C. militaris* mycelia (FHCM) were assessed by electron spin resonance. The antioxidant potential of FHCM was also determined on the basis of a ferric reducing antioxidant power assay and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity. Higher antioxidant and radical scavenging activities were observed in FHCM than in the unfermented *C. militaris* mycelia or *H. discus hannai* alone. FHCM demonstrated an anticancer activity against melanoma B16F10 cell line. In addition, FHCM co-treatment with doxorubicin showed an increased anticancer effect in both *in vitro* and *in vivo*. Therefore, the present study suggests that the mycelial fermentation on *H. discus hannai* is highly suitable for pharmaceutical applications.

Keywords: *Haliotis discus hannai*, *Cordyceps militaris* mycelia, antioxidant activity, anticancer activity

Introduction

Natural products are promising sources of medicine for human ailments. They have been extensively studied against many diseases. Antioxidants are vital substances that have the potential to protect the human body from the adverse effects of free radical-induced oxidative stress. Thus, the potential of natural products as antioxidants has been evaluated by measuring the free radical scavenging activity. Antioxidants can reduce or prevent the oxidative process by removing free radical intermediates as desired. It was reported that the excessive generation of reactive oxygen species may lead to carcinogenesis (1). Therefore, the antioxidants derived from natural resources have been reported as one of the excellent sources of anticancer therapeutic agents (2). Cancer is one of the most threatening human diseases in the world. Anticancer agents are generally causing various side effects on human being such as hair loss, tiredness, reduction in the number of blood cells. Chemoprevention using natural products is an important tool to inhibit cancer as well as to reduce the side effects of anticancer drugs (3–5). Several studies have demonstrated the anticancer activity of natural resources (6,7). Moreover, the administration of synthetic drugs with the combination of natural drugs may reduce or avoid the toxic side effects of

synthetic drugs. Recently, mushrooms are gaining popularity because of their bioactivities toward cancer-prevention, immunomodulation, and tumor inhibition activities (8,9). *Haliotis discus hannai* (*H. discus hannai*), also known as disk abalone, is a large algivorous marine mollusk and one of the most important commercial aquaculture species of gastropods in Asia (10,11). *H. discus hannai* has long been used as food for its rich nutraceutical values. It has also been reported for antioxidant (12), anti-inflammatory (13), anticancer, and immunomodulatory effects (14), hepatoprotective activity (15), and so on. The *C. militaris* has been used as a potential drug source for many diseases (16). Therefore, this study tried to improve the bioactive potential of *H. discus hannai* by fermentation using *C. militaris* mycelia (FHCM) and evaluated the enhancements on its bioactivities. Several methods have been used to improve the functional properties of natural resources. In this extend, fermentation is one of the best methods to enhance the active compounds in natural materials. Puupponen-Pimiä *et al.* (17) reported that fermentation technologies are widely used in the process of plant-based materials to enhance texture, flavor, and bioactivity of food and pharmaceutical products. This is a pioneer attempt to evaluate the fermentation effects of natural materials and synergistic effects of their derivatives with a commercial anticancer compound

(doxorubicin). The present study has applied two steps to enhance the bioactive potential of *H. discus hannai*. The first one is the fermentation method conducted using mushroom mycelia, and the second step is evaluation of the synergic effects of both fermented *H. discus hannai* extract and doxorubicin. Therefore, the antioxidant activity of the FHCM was compared with that of *H. discus hannai* and *C. militaris* mycelia alone. A series of antioxidant assays have been tested including 2,2-diphenyl-1-picrylhydrazyl, hydroxyl, alkyl, 2,2'-azinobis-(3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) radical scavenging activity, and ferric reducing antioxidant power (FRAP). Moreover, the anticancer activity of FHCM and that of FHCM in combination with doxorubicin using *in vitro* and *in vivo* model was determined.

Materials and Methods

Materials *H. discus hannai* was purchased from a local market (Wando, Korea). *C. militaris* was generously provided by Dr. Jeong (Korea National University of Transportation, Jeungpyeong, Korea). 5,5-dimethyl-1-pyrroline N-oxide (DMPO), 2,2-azobis(2-amidinopropane) hydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), (4-pyridyl-1-oxide)-N-tert-butyl nitron (4-POBN), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade.

Preparation of the extract of fermented *H. discus hannai* with *C. militaris* mycelia The detailed procedures of fermentation were described in our previous study (18). The *H. discus hannai* was inoculated with *C. militaris* mycelia and cultured at 25°C for approximately 20 days. To prepare the extracts, fermented *H. discus hannai* (FHCM) was boiled with 10 times of distilled water than the FHCM for 2 h. The extracts were filtered with Whatman No. 41 filter paper, concentrated, and lyophilized. The extraction yields from FHCM are shown in Table 1. FHCM was dissolved in distilled water. Moreover, doxorubicin was dissolved in dimethyl sulfoxide (DMSO) and then diluted with distilled water.

Various radical scavenging activities by electron spin resonance (ESR) measurement

DPPH radical scavenging activity: DPPH radical scavenging activity

was estimated as described by Nanjo *et al.* (19). Briefly, 60 µL of each sample at various concentrations were added to 60 µL of DPPH (60 µM) in a methanol solution. After the solution was mixed for 10 s, it was transferred into a 100-µL Teflon capillary tube, and the DPPH radical scavenging potential of each sample was measured using an ESR spectrometer (JEOL Ltd., Tokyo, Japan). The experimental conditions were as follows: central field, 3475 G; modulation of frequency, 100 kHz; modulation of amplitude, 2 G; microwave power, 5 mW; gain, 6.3×10^5 ; and temperature, 298 K.

Hydroxyl radical scavenging activity: Hydroxyl radicals were generated by an iron-catalyzed Haber–Weiss reaction (i.e., a Fenton-driven HaberWeiss reaction), and the hydroxyl radicals were rapidly reacted using a nitron spin-trap DMPO. The resultant DMPO-OH adducts was detected by an ESR spectrometer. Briefly, 0.2 mL of each sample at various concentrations was mixed with 0.2 mL of DMPO (0.3 M), 0.2 mL of FeSO₄ (10 mM), and 0.2 mL of H₂O₂ (10 mM) in a phosphate buffer solution (pH 7.2), and the resulting solution was transferred into a 100-µL Teflon capillary tube. After 2.5 min, the ESR spectrum was observed by a JES-FA ESR spectrometer (JEOL Ltd.). The experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 ; and temperature, 298 K.

Alkyl radical scavenging activity: Alkyl radicals were generated by AAPH. The PBS (pH 7.4) reaction mixtures contained 0.1 mL of 10 mM AAPH, 0.1 mL of 10 mM 4-POBN, and 0.1 mL of the respective concentrations of the tested samples and were incubated at 37°C in a water bath for 30 min. The samples were then transferred to 100-µL Teflon capillary tubes, and the spin adduct was recorded using an ESR spectrometer. The measurement conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 1 mW; gain, 6.3×10^5 ; and temperature, 298 K.

Superoxide radical scavenging activity: Superoxide radicals were generated by UV irradiation of a riboflavin/ethylenediaminetetra acetic acid solution. The reaction mixtures contained 0.1 mL of 0.8 mM riboflavin, 0.1 mL of 1.6 mM EDTA, and 0.1 mL of 800 mM DMPO, and 0.1 mL of various concentrations of samples were irradiated for 1 min under a UV lamp at 365 nm. The mixtures were transferred to a 100-µL quartz capillary tube and the spectrum was measured using an ESR spectrometer. The experimental conditions were as follows: central field, 3475 G; modulation frequency, 100 kHz; modulation amplitude, 2 G; microwave power, 10 mW; gain, 6.3×10^5 and temperature, 298 K.

Table 1. Various radical scavenging activities (DPPH, alkyl, hydroxyl, and superoxide) of the FHCM extracts by ESR measurement

Samples	Yields (%, w/w)	DPPH radical (IC ₅₀ , mg/mL)	Alkyl radical (IC ₅₀ , mg/mL)	Hydroxyl radical (IC ₅₀ , mg/mL)	Superoxide radical (IC ₅₀ , mg/mL)	TEAC ²⁾ (mM Trolox eq./mg extract)	FRAP (mM FeSO ₄ eq./mg extract)
<i>H. discus hannai</i>	15.22	0.896±0.001 ¹⁾	0.534±0.002	1.346±0.003	1.465±0.001	0.081±0.02	0.016±0.01
<i>C. militaris</i>	2.15	0.318±0.002	0.372±0.002	0.515±0.001	0.866±0.002	0.116±0.03	0.053±0.01
FHCM	83.98	0.013±0.001	0.019±0.002	0.405±0.003	0.166±0.001	0.657±0.02	0.597±0.01

¹⁾Values represent mean±SD (n=3).

²⁾TEAC, Trolox equivalent antioxidant capacity; FRAP, Ferric reducing antioxidant power

FRAP assay To measure the antioxidant capacity of different FHCM extracts, the FRAP method was performed using the method of Benzie and Strain (20) with minor modifications. In these experiments, a 3 mL aliquot of FRAP reagent, a mixture of 0.3 M acetate buffer containing 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride (10:1:1 v/v/v), were mixed with 1 mL of FHCM extract. To determine the antioxidant capacity of the samples, the absorbance values were compared with those obtained from the standard curves of FeSO_4 (0–10 mM). The antioxidant capacity values were expressed as mM of FeSO_4 equivalent per mg of extract (mM FeSO_4 eq./mg extract).

ABTS radical scavenging activity The total antioxidant activity of the FHCM extract was measured using the ABTS $^{\cdot+}$ radical cation decolorization assay (21). ABTS was dissolved in water at a concentration of 7 mM, and the ABTS radical cation (ABTS $^{\cdot+}$) was produced from the ABTS stock solution with the addition of 2.45 mM potassium persulfate. The mixture was then allowed to stand in the dark at room temperature (RT) for 14 h before use. Oxidation of the ABTS commenced immediately; however, the absorbance was not maximal and was stable for more than 6 h or more than 2 days when stored in the dark at RT. After the mixture was kept in the dark at RT for 14 h to allow complete radical generation, it was diluted with ethanol until its absorbance was 0.70 ± 0.02 at 734 nm. To determine the scavenging activity, 0.9 mL of ABTS reagent was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm after 6 min of reaction at RT using ethanol as a control. The antioxidant activities of the FHCM extracts were expressed by trolox equivalents antioxidant capacity (TEAC), as mM of trolox equivalent per mg of extract (mM Trolox eq./mg extract).

Cell culture Murine melanoma B16F10 cell line was purchased from American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM (Gibco, Grand Island, NY, USA) by supplementing with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g}/\text{mL}$) under 5% CO_2 in a humidified incubator at 37°C.

Cell viability assay Cell viability was assessed using an MTT assay. Briefly, B16F10 cells (4×10^3 cells/well) were seeded in 96-well culture plates. The cells were treated with samples for 24 h and the MTT solution was added into each well, and the cells were further incubated for 4 h. The medium was discarded and the intracellular formazan product was dissolved in 150 μL DMSO with shaking for 10 min. The absorbance was measured at 540 nm using a microplate reader (Tecan, Salzburg, Austria). Cell viability was expressed as a percentage of the control.

Cell cycle analysis To analyze the cell cycle regulation, the cellular DNA content was estimated by flow cytometry. Briefly, the cells (1×10^5) were seeded in six-well plates and allowed to attach overnight. Cells were treated doxorubicin (0.5 μM) and FHCM extract (0.125,

0.25, and 0.5 mg/mL)+doxorubicin (0.25 μM) for 24 h. The cells were then harvested by trypsin treatment, washed with cold phosphate-buffered saline (PBS, pH 7.4), and then stained with PI solution (50 $\mu\text{g}/\text{mL}$ of propidium iodide, 10 $\mu\text{g}/\text{mL}$ RNase, and 0.5% Tween 20 in PBS). The stained cells were analyzed for DNA histograms and cell cycle phase distribution by flow cytometry (FACSCalibur, BD Bioscience, Franklin Lakes, NJ, USA). Data from 10,000 cells per sample were collected and analyzed with CellQuest software (BD Bioscience).

Annexin V/PI double staining To estimate the apoptotic way of cell death, the annexin V-FITC apoptosis detection kit was used according to the manufacturer's instructions. 1×10^5 cells/well were incubated and FHCM extract (0.125, 0.25 and 0.5 mg/mL)+doxorubicin (0.25 μM). After 24 h of incubation, cells were harvested, washed with PBS buffer and annexin buffer (1:10), and double-stained with Annexin V-FITC solution. After incubation, 300 μL of annexin buffer was added, and fluorescence was analyzed by flow cytometry.

Animals care Male C57BL/6 mice that were 5–6 weeks old were purchased from OrientBio (Seongnam, Korea) and housed in polycarbonate cages under laboratory conditions with a 12 h day–night cycle, room temperature of $22 \pm 2^\circ\text{C}$, and humidity of 50–60%. The animals were fed with standard pelleted rats chow and had free access to water during the experiment. The animal experiments were approved by the Konkuk University, Institutional Animal Care and Use Committee (KU-IACUC). The license number for using experimental animals was KU13138.

Tumor models and *in vivo* treatment regimen B16F10 mouse melanoma cells were collected from cell cultures by trypsinization and then injected subcutaneously (4×10^5 cells) into the right flank region of mice. After 8 days, tumors reached 100 mm^3 in volume. The experimental animals were randomly divided into five groups of five animals each. The animals were segregated as control (water), FHCM (500 mg/kg body weight, daily), doxorubicin (4 mg/kg body weight, on day 1, 3, and 5; total three times), and the doxorubicin combination group (2 mg/kg body weight, on day 1, 3, and 5; total three times) by intraperitoneal injection: receiving FHCM (300 mg/kg body weight, daily) and FHCM (500 mg/kg body weight, daily) by oral gavage for 5 days. Animal body weight and food and water consumption were monitored three times in a week. The tumor volume was assessed by measuring two perpendicular dimensions (long and short) using a caliper and calculated using the formula $(a \times b^2)/2$, where a is the larger and b is the smaller dimension of the tumor (22). After the treatment period, the animals were sacrificed, and the tumors were removed and immediately weighed.

The tumor inhibitory rates were calculated using the following formula: tumor inhibitory rate (%) = (mean tumor weight of the control mice – mean tumor weight of the treated mice) / average tumor weight of the control mice $\times 100\%$.

Hematological analysis After the overnight fasting, the mice were anesthetized, and blood was collected from the heart on the last day and used to determine the hematological indexes. Hemoglobin (Hb), white blood cell count (WBC), red blood cell count (RBC), platelet (PLT) count, hematocrit (Hct), WBC differential count, and RBC index were analyzed using an automatic blood analyzer (MEK-7222; Nihon Kohden Corp., Tokyo, Japan).

Statistical analysis Statistical analyses were assessed using Tukey's test and a one-way analysis of variance (ANOVA), which is followed by post-doc Duncan's multiple range tests. Graph Pad Prism software version 5.00 (Graph Pad Software Inc., San Diego, CA, USA) was used. The data are expressed as the mean±standard deviation (SD). Results were considered significant at $p<0.05$.

Results and Discussion

Radical scavenging activity of FHCM extracts using an ESR spectrophotometer The results of the present study indicate that the mycelial fermentation played a vital role in the molecular conversion of a sample and consequently showed higher activities. The FHCM extracts (IC_{50} , 0.013 ± 0.001 mg/mL) showed higher DPPH radical scavenging activities than *H. discus hannah* (IC_{50} , 0.896 ± 0.002 mg/mL) or *C. militaris* mycelia (IC_{50} , 0.318 ± 0.001 mg/mL) (Table 1).

The alkyl radical spin adduct of the 4-POBN/free radical generated by AAPH was potentially scavenged by FHCM extracts. The FHCM extracts showed higher alkyl radical scavenging activities (IC_{50} , 0.019 ± 0.002 mg/mL) than the *H. discus hannah* (IC_{50} , 0.534 ± 0.002 mg/mL) or *C. militaris* mycelia (IC_{50} , 0.372 ± 0.002 mg/mL) (Table 1). Hydroxyl radicals generated in the Fe^{2+}/H_2O_2 system were trapped by a DMPO forming spin adduct, which could be detected using an ESR spectrometer. It was reported that hydroxy radical scavenging capacity of the FHCM extracts (IC_{50} , 0.405 ± 0.003 mg/mL) was higher than that of its original counterparts, unfermented *H. discus hannah* (IC_{50} , 1.346 ± 0.003 mg/mL) and *C. militaris* mycelia alone (IC_{50} , 0.515 ± 0.001 mg/mL) (Table 1). Moreover, superior superoxide radical scavenging activity was reported by the FHCM extracts (IC_{50} , 0.166 ± 0.001 mg/mL) than unfermented *H. discus hannah* (IC_{50} , 1.465 ± 0.001 mg/mL) and *C. militaris* mycelia (IC_{50} , 0.866 ± 0.002 mg/mL) alone. These overall results demonstrated that the FHCM extracts had a significantly higher activity on various radical scavenging activities than the original samples. Free radicals can cause deleterious effects on protein, DNA, and lipid molecules. Consequently, these damages lead to cancer, aging, cardiovascular diseases, atherosclerosis, and inflammatory diseases (23-25). Many studies have shown that antioxidants play a potential role in anticancer effects (26-28). Cellular events associated with carcinogenesis phases such as tumor initiation, promotion and progression are shown to be affected by the antioxidants leading to promote apoptosis and cell cycle arrests

(29-31). Furthermore, *in vivo* models have demonstrated that the antioxidants could prevent carcinogenesis and cancer advancement. Therefore, we hypothesized that antioxidant produced using FHCM could relate with the anticancer effect.

FRAP assay The FRAP assay has been used to measure the antioxidant effects of any substance in the reaction medium based on its reducing ability. The antioxidant capacities of FHCM extracts are shown in Table 1. The results showed that the FHCM extracts (0.997 ± 0.032 mM $FeSO_4$ eq./mg extract) had a higher FRAP antioxidant activity than *H. discus hannah* extracts (0.016 ± 0.014 mM $FeSO_4$ eq./mg extract) or *C. militaris* extracts (0.053 ± 0.018 mM $FeSO_4$ eq./mg extract).

ABTS radical scavenging activity The antioxidant activities of FHCM extracts were evaluated by measuring the scavenging activity of ABTS radical cations generated by addition of potassium persulfate, compared to the standard, Trolox and values were expressed as Trolox equivalent antioxidant capacity (TEAC). FHCM extract (0.657 ± 0.020 mM Trolox eq./mg) showed a higher TEAC value than its original compounds, unfermented *H. discus hannah* extracts (0.081 ± 0.022 mM Trolox eq./mg extract) and *C. militaris* extracts (0.116 ± 0.032 mM Trolox eq./mg extract) (Table 1). The higher TEAC values indicated that the FHCM can function as hydrogen donors and the oxidation process could be terminated by converting free radicals to stable forms (32). Therefore, these results showed that FHCM extracts display higher antioxidant activity by acting as hydrogen donors.

Effect of various FHCM extracts on cell viability The B16F10 cells were treated with different concentrations of *H. discus hannah*, *C. militaris* mycelia and FHCM extracts (0.125, 0.25, and 0.5 mg/mL), and the percentage of cell survival was assessed using an MTT assay. After the treatment of samples, the cell viability for B16F10 cells were significantly decreased, especially, for FHCM extracts (Fig. 1A). The cell viability of FHCM at 0.125, 0.25, and 0.5 mg/mL was 63.8, 59.4, and 52.9%, respectively. Then, we determined the effect of co-treatment of doxorubicin and FHCM extract on cell viability. As shown in Fig. 1B, treatment of $0.5\text{ }\mu\text{M}$ doxorubicin inhibited cell viability up to 57%. The co-treatment of doxorubicin ($0.25\text{ }\mu\text{M}$) and 0.5 mg/mL of FHCM showed similar cell viability (55%) as the treatment with doxorubicin ($0.5\text{ }\mu\text{M}$). The co-treatment of doxorubicin ($0.25\text{ }\mu\text{M}$) with 0.125 and 0.25 mg/mL of FHCM showed an increase in the inhibition of cell viability compared to FHCM. In the present study, FHCM showed an increased growth inhibition on B16F10 cells when administered with the combination of doxorubicin. These data indicate that the co-treatment of doxorubicin and FHCM could be used to reduce the concentration of doxorubicin. In addition, our previous work did not show any toxicity on up to 1.0 mg/mL of FHCM extract on Chang cells (data not shown).

Fig. 1. Cell viability. (A) B16F10 cells were treated with extracts of *H. discus hannai*, *C. militaris mycelia*, and *H. discus hannai* fermented with *C. militaris mycelia* (HFCM) for 24 h. Cell viability was measured using the MTT assay. (B) B16F10 cells were co-treated with extracts of FHCM and doxorubicine for 24 h. Results are expressed as mean±SD from three independent experiments. One-way ANOVA was used for to compare multiple group means followed by Dunnett's *t*-test (** $p < 0.001$, ** $p < 0.01$ compared to control).

Effects of FHCM extracts on the cell cycle distribution in B16F10 cells

To evaluate the mode of cell death induced by the combined treatment, the cells were examined by flow cytometry. When the subG1 phase was determined using propidium iodide staining in B16F10 cells and the apoptosis was observed as 7.31% by doxorubicin (0.5 μM), followed by 13.67, 10.60, and at 7.33% by co-treatment of doxorubicin (0.25 μM) and FHCM (0.125, 0.25, and 0.5 mg/mL) respectively (Table 2). However, 0.44% of the subG1 phase was observed in the control. The S phase of doxorubicin increased up to 15.71% when compared to control (8.85%). Moreover, the co-treatment with 0.125, 0.25, and 0.5 mg/mL of FHCM provided S phases of 10.73, 11.37, and 12.13% respectively. Co-administration

of FHCM with doxorubicin showed a cell cycle arrest in the subG1 phase and S phase. Anticancer potential of FHCM could be attributed to the factors involved in the cell cycle regulation, cell proliferation, apoptosis, and necrosis.

Annexin V/PI double staining

In many reports, fluorescent Annexin V and PI dual staining have been used to assess the apoptotic and necrotic cell death, in which Annexin V-positive/PI-negative cells were indicated the early apoptotic cells and PI-positive stained cells were indicated the necrotic cell death (33). After 24 h of incubation with doxorubicin or co-treatment of FHCM induced cell death indicated the involvement of both apoptotic and necrosis

Table 2. Effects of co-treatment doxorubicin and FHCM on cell cycle distribution in B16F10 cells

Group	Number of cell (%)			
	Sub G1	G1	S	G2/M
Control	0.44±0.12	45.73±2.21	8.85±1.23	31.90±2.65
Doxorubicin (0.5 µM)	7.31±2.13**	37.91±3.12**	15.71±2.13**	33.33±2.34
Doxorubicin (0.25 µM)+FHCM (0.125 mg/mL)	13.67±1.32**	33.42±2.28**	10.73±1.36**	33.60±3.12
Doxorubicin (0.25 µM)+FHCM (0.25 mg/mL)	10.60±1.23**	39.39±2.38**	11.37±1.26**	32.85±2.68
Doxorubicin (0.25 µM)+FHCM (0.5 mg/mL)	7.33±1.23**	41.51±3.69	12.13±2.13**	29.67±2.14

The cells were treated with the indicated concentration of samples for 24 h and stained with PI for flow cytometry analysis. The percentages of cells in each phase of the three independent experiments are presented. ** $p < 0.01$ compared to control.

pathways. Apoptotic and necrosis pathways displayed an increased percentage of Annexin V positive cells and PI positive cells when compared to the control group (Table 3). In some situations, the cell can trigger the apoptosis process. Subsequently, the ATP depletion is followed by the inactivation of caspases, ended with necrosis (34,35). Our result showed that the co-treatment of FHCM and doxorubicin favored both apoptosis and necrotic pathways.

Animal body weight and Hematological analysis As compared with the control group, animal body weight and food and water consumption had moderate changes in FHCM groups and positive group, but these were not significant (data not shown). The effects of experimental treatments on the hematological profile is summarized in Table 4. The WBC counts in doxorubicin treated group were significantly lower than the control group. Similarly, the sample-treated group also showed significant decreases in WBC counts. The counts of PLT and segment neutrophil in doxorubicin treatment were significantly higher than control group. However, the percentage of RBC, hemoglobin, and hematocrit and ratio of blood MCV, MCH, and MCHC did not differ markedly among all groups.

Effect of FHCM extract on the growth of B16F10 melanoma cells in syngeneic C57BL/6 mice The average tumor volume of the

Table 3. Effects of co-treatment doxorubicin and FHCM on annexin V and PI double staining in B16F10 cells

Group	Positive cells (%)	
	Annexin V	PI
Control	2.18±0.12	0.41±0.26
Doxorubicin (0.5 µM)	21.93±3.51**	71.23±2.95**
Doxorubicin (0.25 µM)+FHCM (0.125 mg/mL)	33.22±3.26**	41.07±1.87**
Doxorubicin (0.25 µM)+FHCM (0.25 mg/mL)	18.48±2.32**	63.20±2.51**
Doxorubicin (0.25 µM)+FHCM (0.5 mg/mL)	5.43±1.32**	72.34±3.28**

The cells were treated with the indicated concentration of samples for 24 h, annexin V and PI double stained for flow cytometry analysis. Three independent experiments are presented. ** $p < 0.01$ compared to control.

experimental and control groups are shown in Fig. 2. The average tumor volume of the control group was $1032 \pm 162.4 \text{ mm}^3$, which is followed by $549.2 \pm 117.6 \text{ mm}^3$ of the average tumor volume in the doxorubicin treated group (4 mg/kg) on 7th day. The low concentration of FHCM (300 mg/kg) co-treatment with doxorubicin (2 mg/kg) showed $594.8 \pm 133.3 \text{ mm}^3$ of tumor volume. However, at a higher concentration (500 mg/kg) of the FHCM co-treated with doxorubicin

Table 4. Blood hematological analysis

Blood parameters	Hb ²⁾ (g/dL)	Hct (%)	RBC ($\times 10^6$)/ µL	WBC ($\times 10^3$)/ µL	PLT ($\times 10^3$)/ µL	WBC differential count				RBC index					
						Segment- neutrophil (%)	Lympho- cyte (%)	Monocyte (%)	Basophil (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	MPV (fL)	PDW (fL)
Control	11.37± 2.22 ¹⁾	41.88± 7.36	7.32± 1.33	7.48± 1.56	650± 302.03	15.17± 11.69	79.67± 15.03	4.83± 3.66	0.33± 0.52	57.25± 0.79	15.48± 0.33	27.07± 0.75	16.13± 1.21	8.07± 0.23	8.57± 0.78
Doxorubicin 4 mg/kg	12± 1.90	42.72± 6.46	7.82± 1.21	4.79± 1.87**	1997.40± 314.97**	31.60± 9.84**	61.40± 7.47*	6.40± 2.61**	0.60± 1.34	54.68± 1.73	15.34± 0.15	28.06± 0.74	15.14± 0.68	8.46± 0.27	9.66± 0.62
Doxorubicin 2 mg/ kg+FHCM 300 mg/kg	12.47± 0.61	44.98± 2.18	8.14± 0.38	5.05± 1.51**	1529.67± 206.97**	27± 8.76**	69.83± 8.86*	3.17± 2.14*	0±0	55.23± 0.52	15.30± 0.18	27.70± 0.24	15.40± 0.99	8.48± 0.31	9.40± 0.77
Doxorubicin 2 mg/ kg+FHCM 500 mg/kg	13.25± 0.90	47.52± 3.03	8.52± 0.52	4.33± 0.93**	1273.50± 197.13**	29.83± 5.74**	67.50± 6.16*	2.33± 1.21**	0.33± 0.52	55.80± 1.04	15.57± 0.30	27.88± 0.24	15.38± 1.13	8.32± 0.29	9.15± 0.71
FHCM 500 mg/kg	13.52± 2.98	49.85± 9.72	8.69± 1.87	6.29± 1.54	764.33± 113.60	11.83± 3.54	86.50± 3.27*	1.67± 0.82**	0±0	57.70± 1.95	15.53± 0.24	26.95± 1.02	16.43± 0.32	8.15± 0.24	8.77± 0.66

¹⁾Results are expressed as the mean±SD (n=5). * $p < 0.05$, ** $p < 0.01$ compared to the control group.

²⁾Hb, Hemoglobin; Hct, Hematocrit; RBC, Red blood cells; WBC, white blood cells; MCV, Mean cell volume; MCH, Mean cell hemoglobin; MCHC, Mean cell hemoglobin concentration; RDW, Red cell distribution width; MPV, Mean platelet volume; PDW, Platelet distribution width.

Fig. 2. Images of the mouse tumor of all groups. (A) Control, (B) doxorubicin 2 mg/kg+FHCM 300 mg/kg, (C) doxorubicin 2 mg/kg+FHCM 500 mg/kg, (D) FHCM 500 mg/kg, (E) doxorubicin 4 mg/kg.

(2 mg/kg), we observe $475.9 \pm 90.36 \text{ mm}^3$ of the average tumor volume. A higher concentration of FHCM (500 mg/kg) alone treated group showed $635.5 \pm 69.8 \text{ mm}^3$. The tumor weights of control group, doxorubicin treated group (4 mg/kg), doxorubicin 2 mg/kg+FHCM 300 mg/kg, doxorubicin 2 mg/kg+FHCM 500 mg/kg, and FHCM 500 mg/kg were 909.0 ± 183.6 , 367.9 ± 289.8 , 390.8 ± 67.8 , 250.9 ± 125.1 , and $447.4 \pm 195.3 \text{ mg}$, respectively. The results obtained emphasize that the drug treated groups significantly inhibited the tumor growth when compared to the control group. The tumor growth inhibitory rate of doxorubicin was 59.6% ($p < 0.05$). Furthermore, co-treated groups had the highest tumor growth inhibition of 57 and 72.3% in doxorubicin 2 mg/kg+FHCM 300 mg/kg and doxorubicin 2 mg/kg+FHCM 500 mg/kg, respectively. Figure 2 depicted the effective tumor growth inhibition in groups that are co-treated with FHCM and doxorubicin. In conclusion, FHCM successfully demonstrated the potential to be used as an antioxidant and to inhibit cancer in both *in vitro* and *in vivo* models. From the results obtained from the experiments, we observe that the fermented *H. discus hannai* using *C. militaris* (FHCM) showed higher antioxidant activity than the other samples tested. The FHCM also enhanced the anticancer potential of the commercial drug doxorubicin in B16F10 cells in both *in vivo* and *in vitro* conditions by synergic effects. Therefore, the present study concluded that the fermented *H. discus hannai* using *C. militaris* mycelia is the best method to improve the bioactivity toward pharmacological applications. However, further studies are required to understand its active compounds behind these activities.

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