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Extracts from Fermented Black Soybean Milk Exhibit Antioxidant and Cytotoxic Activities

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Summary

In this study, ethanol extracts from 2-day fermented black soybean milk (FBE) by immobilized *Rhizopus oligosporus* NTU5 have been evaluated for both antioxidant and cytotoxic activities. The results reveal that a 2-day FBE had strong 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect (76 %). The extracts were further fractionated by silica gel column chromatography and an unknown compound, FBE5-A, was obtained, which exhibited strong antioxidant activity. IC_{50} of the DPPH scavenging effect of FBE5-A was 7.5 μg/mL, which is stronger than a commonly used antioxidant, vitamin E (α-tocopherol; 17.4 μg/mL), and similar to vitamin C (ascorbic acid; 7.6 μg/mL). The cytotoxic test demonstrated that extracts of 2-day fermented broth exhibited selective cytotoxic activity towards human carcinoma cells, Hep 3B (IC_{50}=150.2 μg/mL), and did not affect normal human lung fibroblasts, MRC-5 ($p<0.05$). The results indicate the potential applications of fermented black soybean milk as functional food, pharmaceutical or cancer therapy formula.

Key words: black soybean, *Rhizopus oligosporous*, antioxidant activity, cytotoxic activity, isoflavone

Introduction

Black soybean (*Glycine max* (L.) Merrill), a variety with a black seed coat, has been widely utilized as food supplement and key ingredient in Chinese herbal medicine for hundreds of years (*1*). The seed coats of black soybeans are darker than the seed coats of other types of soybean because they contain anthocyanins, which is why they show several therapeutic effects (*2*). Black soybeans are rich in dietary fibre and provide 8 human essential amino acids, which can enhance gastrointestinal function and reduce discomfort caused by flatus (*2*).

Isoflavones, which are abundant in soybean, demonstrate several health-enhancing properties, such as the ease of symptoms in postmenopausal women (*3*), reducing the risk of osteoporosis (*4,5*), prevention of cardiovascular diseases (*6,7*) and antimutagenic effects (*8*). Hendrich (*9*) reported that biological activity of isoflavones is mainly from the aglycone forms. It has also been demonstrated that isoflavone aglycones are absorbed faster and in greater amount than their glycosides in the human intestine (*10*).

Besides isoflavones, black soybeans consist of other bioactive components like flavonoids and saponins (*11*), which exhibit biological functions such as free radical scavenging activity, antitumour activity, inhibition of low density lipoprotein (LDL) oxidation, and reduction of DNA damage by cyclo-phosphamide (*12–14*). Our previous study also supported these findings since the maximum antioxidant activity of fermented black soybean did not match the maximum isoflavone content obtained during cultivation, implying the existence of other antioxidants (*15*).

Fermented black soybeans also exhibit antimutagenic and antioxidant activities (*1,8*). Compounds isolated from fermented soybean by filamentous fungi demonstrate several bioactive functions. Esaki *et al.* (*16*) reported

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that 3–hydroxyanthranilic acid (HAA) had higher antioxidant ability than vitamin E at the same concentration. HAA also demonstrated its ability to induce apoptosis of liver cancer cells (*17*). Several antioxidants, such as 6-hydroxydaidzein (6-OHD), 2,3-dihydroxybenzoic acid (2,3-DHBA), 8-hydroxydaidzein (8-OHD), and 8-hydroxygenistein (8-OHG) were later reported (*18–20*). These antioxidants are derivatives of daidzein and genistein from fermented soybean, which is correlated with *Aspergillus oryzae* by secreting extracellular β-glucosidase during fermentation process.

The evidence shown above clearly demonstrates the potential applications of isoflavone and other bioactive components from soybeans in functional food, pharmaceutical and cancer therapy areas. However, the data regarding the antioxidant and cytotoxic activities by a mixture of isoflavones and extracts from fermented soybean broth are rare. As a result, it would be of great advantage for functional food industry if the fermented broth of black soybean could be proven with antioxidant and cytotoxic activities without further isolation or purification. Moreover, although many compounds with cytotoxic effect on cancer cells have been reported, the drawback is that they often exhibit cytotoxicity towards normal cells at the same time (*21*).

To promote the utilization of domestic black soybean as well as to evaluate the antioxidant and cytotoxic activities of fermented black soybeans, a filamentous fungus *Rhizopus oligosporous* NTU5 under optimal cultivation conditions reported in our previous study (*15*) was used for black soybean fermentation in a solid-state bioreactor. The fungus has the Generally Recognized As Safe (GRAS) status. The extracts of fermented black soybean were further fractionated and purified in order to evaluate its antioxidant activity and compared with commonly used antioxidants, vitamins C and E. The cytotoxicity of fermented black soybean extracts was also evaluated against human carcinoma cell lines and normal lung fibroblast cells.

Materials and Methods

Chemicals

Genistein, daidzein, genistin, and daidzin and other chemicals were purchased from Sigma (St. Louis, MO, USA). Liquid chromatography grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). Potato dextrose broth and Bacto agar were purchased from Difco (Detroit, MI, USA). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and trypsin/EDTA were obtained from Gibco (Grand Island, NY, USA). Ethyl acetate, *n*-hexane and thin layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide and all other chemicals were purchased from Sigma (St. Louis, MO, USA). Black soybean (*Glycine max* (L.) Merr. Tainan No. 3) was purchased from Shiuejia Farmers' Cooperative (Tainan, Taiwan). The black soybeans were milled with a grinder (CP-75 S, Ladyship, Kaohsiung, Taiwan) and the powder after passing through a 20-mesh sieve was used for medium preparation.

Microorganism

Filamentous fungus *Rhizopus oligosporous* NTU-5 had previously been isolated in our laboratory. The working cultures were maintained on potato dextrose agar (PDA) slants after growing at 30 °C and subcultured every two weeks.

Preparation of spore suspension

The cultures of *R. oligosporous* were grown on PDA plates for 5 to 7 days at 30 °C. Mycelia were then rinsed with 10 mL of 0.05 % Tween 80 and scraped off with glass 'hockey stick' to detach spores. The solution was then filtered through sterile gauze to remove the mycelia. The filtrate containing spores was adjusted to around 10^6 spores/mL and stored at 4 °C until use. For long term storage, the spore suspensions in a 0.05 % Tween 80 solution were stored at –80 °C.

Fermentation of black soybean with R. oligosporous

Black soybean fermentation was conducted in a 5-litre bioreactor (KMJ-5, Mitsuwa Co, Osaka, Japan) at 30 °C. Seed culture was prepared as follows: 1 mL of the prepared spore suspension was added to 100 mL of medium (containing in g/L of distilled water (pH=6.0)): glucose 2, yeast extract 1, KH_2PO_4 0.2, and $MgSO_4 \cdot 7H_2O$ 0.1 in 500-mL Hinton flasks. Cultivation was carried out on a reciprocal shaker at 125 rpm and 30 °C for 72 h. The seed culture (300 mL) was subsequently inoculated with 6 % (by mass per volume) black soybean milk in the bioreactor (working volume 3 L) for 6-day cultivation under the optimal conditions from our previous study (*15*). A luffa cylindrical fibre (10 cm outside diameter and 15 cm length) was implemented as biofilm support. Fermentation broth was sampled daily and analyzed for its polyphenol and isoflavone content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging rate and pH.

Isoflavone quantification

Extraction of fermented broth was carried out as follows: 10 mL of 80 % EtOH were added to the lyophilized powder of 10 mL of fermentation broth and then incubated in a shaker at 65 °C and 125 rpm for 2 h. After incubation, the mixture was centrifuged at 12 000×*g* for 15 min to remove the insoluble debris. The supernatant was then filtered through a 0.45-μm nylon filter (Millipore, Bedford, MA, USA) for the quantification of isoflavones using high performance liquid chromatography (HPLC) modified from the work of Wang and Murphy (*22*). The HPLC system consisted of a Shimadzu SCL--10Avp system controller, two LC-10ATvp liquid chromatograph pumps and an SPD-M10Avp photodiode array detector (Shimadzu, Nakagyo-ku, Kyoto, Japan). A Chemcosorb 7-ODS-H column (250×4.6 mm; Chemco Scientific Co, Ltd, Osaka, Japan) was used. A linear HPLC gradient was composed of 0.1 % (by volume) glacial acetic acid water solution (solvent A) and 80 % (by volume) acetonitrile in 0.1 % (by volume) glacial acetic acid water solution (solvent B). After injection of 20 μL of the sample, solvent B was increased from 15 to 70 % in 25 min, and

then returned to 15 % in 5 min. The solvent flow rate was 1.6 mL/min. The absorbance of the elution was monitored at 262 nm. Commercial isoflavones purchased from Sigma were used as standard.

Antioxidant activity

DPPH radical scavenging method was used to determine the antioxidant activity of fermented samples. One mL of extraction sample from the fermented broth was added to 2 mL of 400 mM DPPH methanol solution and placed in the dark for 30 min. The absorbance of the mixture was monitored at 517 nm. The DPPH radical scavenging rate (in %) was defined as follows:

$$[1-(A_{517\ nm}(\text{sample}))/(A_{517\ nm}(\text{control}))]\cdot 100 \qquad /1/$$

Polyphenol quantification

Total amount of polyphenols was identified by the modified method of Shetty *et al.* (*23*). After a 6-day cultivation, 2 mL of the culture broth from each day were centrifuged at 12 000×*g* for 15 min at 15 °C. One mL of 95 % EtOH, 5 mL of distilled H_2O, and 0.5 mL of 50 % Folin-Ciocalteu reagent were added to 1 mL of the supernatant. After 5 min of the reaction, 1 mL of 5 % sodium carbonate was added and the culture was left to react for another 1 h in the dark room at room temperature. The absorbance was then measured at 750 nm. Gallic acid was used as standard for quantification of total polyphenols in the fermentation broth.

Preparation and fractionation of ethanol extracts

After a 2-day cultivation, 1 L of the culture broth was centrifuged at 12 000×*g* at 15 °C for 15 min to remove the insoluble debris. The lyophilized supernatant was extracted using 80 % ethanol in a water bath at 65 °C for 2 h. After extraction, the mixture was centrifuged at 12 000×*g* 15 °C for 15 min to remove the insoluble debris. The supernatant was then concentrated using a Rotavapor-R rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) under reduced pressure at 30 °C. The residue was designated as the fermented black soybean extract (FBE). The FBE (7.53 g) was then resuspended in *n*-hexane. After initial analysis, the FBE in *n*-hexane was subjected to separation on a silica gel column (40×5 cm i.d.; Merck, Darmstadt, Germany) with 50 g of silica gel by loading in *n*-hexane and eluted using a combination of *n*-hexane/ethyl acetate/methanol at different ratios. After silica gel column chromatography, each fraction was determined by its polyphenol content and DPPH scavenging effect. Eight main fractions were obtained and the solvent was removed under reduced pressure at 40 °C. The most antioxidant fraction was further separated by HPLC system (Shimadzu LC-10A) using a MetaChem Polaris C18-A column (5 μm particles, 250×10 mm i.d., Varian Inc, Palo Alto, CA, USA) and eluted with 80 % methanol in H_2O. Different peaks were collected separately for further analysis.

Cell line and culture conditions

MRC-5, a human lung fibroblast cell line, was obtained from the Bioresources Collection and Research Center (Hsinchu, Taiwan). Hep 3B, a human hepatitis B virus-positive hepatocellular carcinoma cell line, and Hep G2, a human hepatoblastoma cell line, were gifts from Dr B.H. Chiang (Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan). HeLa, a human cervical epithelioid carcinoma cell line, and CL1-1, a human lung adenocarcinoma cell line, were a gift from Dr F.H. Chang (Institute of Biochemistry and Molecular Biology, National Taiwan University, Taipei, Taiwan). All cells were cultured in 10 % FBS in DMEM with 100 U/mL of penicillin and 100 μg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5 % CO_2.

Cytotoxicity of fermented black soybean milk

Cytotoxicity towards human cell lines was measured using the MTT assay, which is based on the ability of the cells to convert tetrazolium salt into purple formazan (*24*). Briefly, cells were subcultured in a 96-well plate with 4000 cells per well in 100 μL of the medium. After 48 h of incubation, the medium in each well was discarded and replaced with 200 μL of fresh medium containing the test samples dissolved in 0.4 % DMSO. DMSO alone was used as control. After 2 h of incubation, the medium was discarded and 55 μL of MTT solution (0.5 mg/mL) were added to each well. The plates were then incubated for another 4 h at 37 °C. The supernatant was removed and 100 μL of DMSO were added to each well to dissolve the formazan. The absorbance was measured at 570 nm using a MRX II microplate reader (DYNEX, Chantilly, VA, USA). The inhibition of cell viability was expressed as a percentage of the proliferation of the control cells.

Data analysis

All experiments were performed in triplicates and expressed as mean value±standard deviation. The significant difference of the results was evaluated using the Tukey's honestly significant difference (HSD) multiple comparison module of the MINITAB Statistical Software package (release 13.30; State College, PA, USA).

Results

Black soybean fermentation

The 6-day cultivation process was conducted in a 5-litre reactor with luffa cylindrical fibre as solid support. Total amount of polyphenols, the concentration of four major isoflavones, and DPPH radical scavenging activity are summarized in Table 1. The results demonstrated that fermented black soybean reached its maximum antioxidant activity on the second day (75 %), which, however, did not match either the amount of polyphenol or isoflavone aglycones. Total amount of polyphenol and isoflavone aglycones increased and reached maximum on the fifth and sixth day, respectively. To further investigate this finding, fermented black soybeans obtained from the second day were chosen for further study.

Fractionation of fermented black soybean

After concentrating the black soybean extracts, 7.53 g of FBE were fractionated by eight different ratios of

Table 1. Chemical composition and scavenging activity of black soybean milk extracts depending on the time of fermentation with *Rhizopus oligosporous*

t/day	*c*(glycoside)/μM		*c*(aglycone)/μM		Scavenging effect	*γ*(polyphenol)
	daidzin	genistin	daidzein	genistein	%	mg/mL
0	(77.3±1.6)^{a}	(74.0±2.3)^{a}	(19.3±0.8)^{a}	(18.9±0.6)^{a}	(52.0±0.5)^{a}	(235.5±12.6)^{a}
1	(16.6±0.7)^{b}	(8.6±0.9)^{b}	(20.8±1.2)^{a}	(18.5±0.9)^{a}	(60.0±0.3)^{b}	(260.8±8.3)^{a}
2	n.d.	(4.2±0.5)^{c}	(32.3±0.8)^{b}	(19.2±0.8)^{ab}	(75.0±0.3)^{c}	(317.4±10.9)^{b}
3	n.d.	n.d.	(31.1±0.8)^{b}	(19.2±0.8)^{ab}	(72.0±0.4)^{d}	(325.9±11.2)^{b}
4	n.d.	n.d.	(31.5±0.7)^{b}	(21.5±1.2)^{b}	(68.0±0.6)^{e}	(348.4±9.6)^{c}
5	n.d.	n.d.	(42.9±1.3)^{c}	(23.3±1.3)^{b}	(66.0±0.5)^{e}	(350.1±12.5)^{c}
6	n.d.	n.d.	(57.8±1.5)^{d}	(27.8±1.3)^{c}	(62.0±0.3)^{b}	(322.3±16.7)^{b}

Cultivation conditions are as follows: temperature 30 °C, medium black soybean powder 6 % (*m*/*V*), aeration at 0.1 vvm, agitation at 100 rpm with 10 % inoculum; values (in the same column) marked with a different letter are significantly different ($p<0.05$; $N=3$); n.d.=not detected

Table 2. Chemical composition and scavenging activity of fractionated fermented black soybean extracts by silica gel chromatography

Fraction	*m*(FBE)	Yield	*w*(polyphenols)	DPPH radical scavenging effect
	mg	%	μg/mg	%
1	2.9±1.2	0.0	(2.7±0.6)^{a}	(1.4±0.1)^{a}
2	98.1±6.5	1.3±0.0	(18.4±1.3)^{b}	(1.7±0.3)^{a}
3	269.4±12.6	3.6±0.5	(21.8±2.3)^{b}	(3.4±0.6)^{a}
4	65.7±3.8	0.9±0.1	(14.9±1.7)^{c}	(30.1±2.5)^{b}
5	431.3±20.5	5.7±0.6	(36.1±3.4)^{d}	(74.7±3.2)^{c}
6	1596.9±102.4	21.1±0.7	(17.5±1.6)^{b}	(37.4±1.1)^{d}
7	2441.4±202.5	32.3±0.5	(15.4±0.8)e	(37.2±1.6)^{d}
8	1884.2±132.7	24.9±0.9	(17.3±1.5)^{b}	(52.3±0.8)^{e}
Total	6790.2±452.8	89.9±3.3	144.1±13.2	

Values (in the same column) marked with a different letter are significantly different ($p<0.05$; $N=3$); solvent composition: fraction 1: *n*-hexane, fraction 2: *n*-hexane/ethyl acetate (2:1), fraction 3: *n*-hexane/ethyl acetate (1:2), fraction 4: ethyl acetate, fraction 5: ethyl acetate/methanol (2:1), fraction 6: ethyl acetate/methanol (1:2), fraction 7: methanol, fraction 8: 80 % methanol; total phenolic content in FBE was determined as μg/mg of gallic acid equivalent

n-hexane/ethyl acetate/methanol solvents. Elutes were harvested, condensed and measured for their dry mass. Total polyphenol and DPPH radical scavenging effect were determined and summarized in Table 2. Fraction 5 (FBE5) yielded the highest polyphenol content as well as the highest DPPH radical scavenging effect. Therefore, FBE5 was chosen for further separation using HPLC method.

The HPLC results of FBE5 demonstrated that four compounds, namely FBE5-A, FBE5-B, FBE5-C and FBE5-D, were successfully separated. FBE5-C and FBE5-D were later identified as daidzein and genistein using LC/MS (data not shown). DPPH radical scavenging effect analysis was conducted for comparison of FBE5-A, which exhibited the strongest antioxidant activity among four compounds, with other commercial antioxidants, and the results are summarized in Table 3. The results demonstrate that IC_{50} of FBE5-A is 7.5 μg/mL, which is stronger than the commonly used antioxidant, vitamin E (α-tocopherol; 17.4 μg/mL) and similar to vitamin C (ascorbic acid; 7.6 μg/mL). Pure FBE5-A was obtained (Fig. 1a) and an attempt to identify the structure of FBE5-A was carried out. However, due to the limitation of instruments in our laboratory and the low yield of the product, only UV spectrometric data were obtained. UV λ_{max} were 208 and 262 nm (Fig. 1b).

Table 3. Comparison of IC_{50} of DPPH radical scavenging effect among different samples

Sample	*γ*(IC_{50})/(μg/mL)
ascorbic acid	(7.6±0.2)^{a}
α-tocopherol	(17.4±0.6)^{b}
FBE	(1323.6±2.5)^{c}
FBE5	(17.8±0.5)^{b}
FBE5-A	(7.5±0.3)^{a}

Values (in the same column) marked with a different letter are significantly different ($p<0.05$; $N=3$); the IC_{50} value was defined as the concentration of each sample causing 50 % reduction of the DPPH free radical

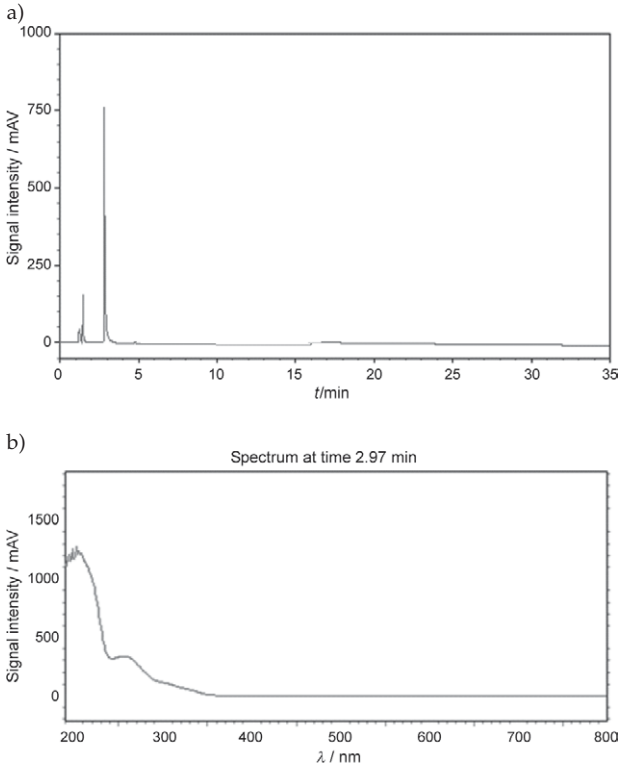

Fig. 1. HPLC results of: a) the unknown compound, FBE5-A, and b) its UV spectrum

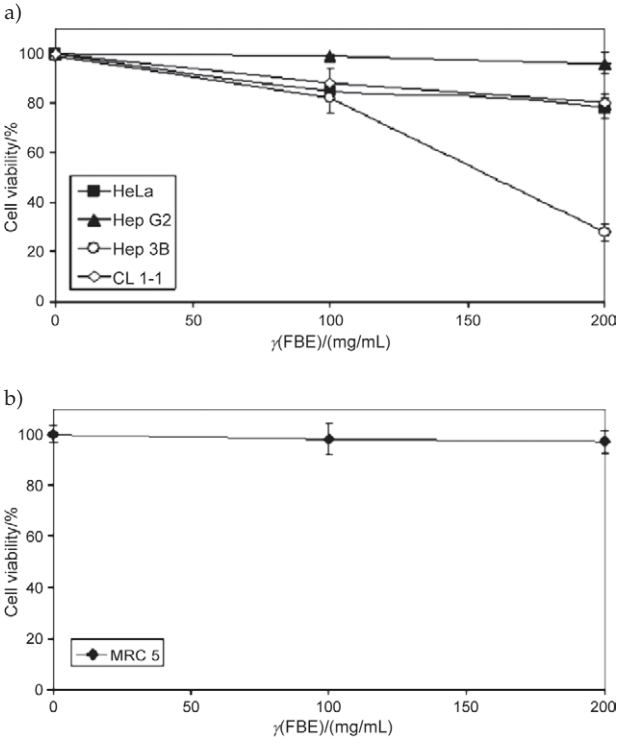

Fig. 2. Effect of fermented black soybean extracts (FBE) on cell viability of: a) carcinoma cell lines HeLA, Hep G2, Hep 3B and CL 1-1, and b) MRC-5 (N=5)

Evaluation of cytotoxicity

FBE was evaluated for its cytotoxicity using cell viability assay. Four human carcinoma cell lines (HeLa, Hep G2, Hep 3B, and CL 1–1) were tested and normal human lung fibroblast cell line, MRC 5, was used as control. The results demonstrated that FBE exhibited selective cytotoxic activity towards human carcinoma cells Hep 3B (IC_{50}=150.2 μg/mL) and no significant effect on the other three carcinoma cell lines (Fig. 2a). On the other hand, FBE did not affect the viability of normal human lung fibroblast MRC-5 cells (Fig. 2b).

Discussion

Fermented black soybeans have long been used not only as a food or dessert, but also as daily supplement in China and Japan (*25*). There are many reports mainly focused on the conversion efficiency of the deglycosylation of isoflavone glycosides by different microorganisms since isoflavone aglycones exhibit higher gastrointestinal absorption (*26*). Besides, other bioactive functions, such as antioxidant activity, cytotoxicity towards carcinoma cells of fermented soybeans have been investigated (*8,15,18*). Moreover, black soybeans exhibit high potential applications as food, dessert and condiments due to their physical nature, such as higher polyphenol content and lower content of flatulence-causing raffinose and stachyose compared to common soybeans (*25,27*). Therefore, further studies on the fermented black soybeans are needed.

This study may provide insight into the effects of fermented black soybean towards its antioxidant and cytotoxic activities. DPPH radical scavenging effect of black soybean fermented with *R. oligosporous* did not correspond to total polyphenol or isoflavone aglycone content. Extracts of the fermented broth reached their highest antioxidant activity on the second day, although polyphenol and isoflavone aglycones were still accumulating. This inconsistency was also reported by Lee *et al*. (*1*) when performing solid-state fermentation using *Aspergillus awamori*. The reason is probably the accumulation of specific phenolic compounds or the generation of new isoflavone derivatives during fermentation since *R. oligosporous* has been reported to possess β-glucosidase and other enzymes which can convert two major native isoflavones, daidzin and genistin, into their aglycones or derivatives (*28*).

From the results of fractionation of fermented black soybean broth, an unknown compound, FBE5-A, was isolated, and DPPH radical scavenging test showed that it had strong antioxidant activity (Table 3), which is higher than vitamin E and similar to vitamin C. FBE5-A is not one of the twelve major isoflavones determined by HPLC analysis (data not shown) and could be a new derivative of polyphenols produced during cultivation. Since oxygen-free radicals and other reactive oxygen molecules can cause deterioration of cell components, such as membrane proteins, enzymes, nucleic acid, and lipids (*29*), it is worthwhile to further identify FBE5-A and evaluate its antioxidant activity.

Hung *et al*. (*8*) reported that fermented black soybeans exhibited antimutagenic effect towards 4-NQO and B[a]P, which are carcinogenic compounds produced by *Salmonella typhimurium*. Black soybean was also reported to effectively reduce the incidence of DNA damage caused by cyclophosphamide (*14*). Instead of prevention,

an efficient formula for cancer therapy is also crucial. Fermented black soybean by *R. oligosporous* exhibited selective cytotoxicity towards human hepatocellular carcinoma cell, Hep 3B, and did not affect normal human lung fibroblast cells (MRC-5). The relatively high cytotoxic activity observed in the fermented black soybean extracts is generally associated with the higher content of total phenolic compounds, anthocyanin and isoflavone aglycones (*1*). However, it could also be the result of the formation of other antimutagenic metabolites during cultivation (*30*) and could vary under different fermentation conditions. The main difference between Hep 3B and other strains is the presence of hepatitis B virus (*31*), which may cause the malfunction of the tumour suppressor gene, *p53*. The addition of FBE may restore the action of *p53* and trigger apoptosis to remove the infected cells. However, the exact mechanism of cell death is worth investigating further.

Conclusion

It was found in this study that fermented black soybean milk showed antioxidant activity, which does not correspond to either total polyphenol or isoflavone content. Further fractionation of the extract demonstrated that an unknown compound, FBE5-A, was isolated and it exhibited strong DPPH radical scavenging effect, which makes it an antioxidant candidate. The cell viability test also suggested that fermented black soybean milk possessed selective cytotoxicity towards carcinoma cells but not towards healthy ones. Further study on the identification of FBE5-A is needed. The mechanism of cell death triggered by FBE is another issue for cancer therapy studies.

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References

1. I.H. Lee, Y.H. Hung, C.C. Chou, Total phenolic and anthocyanin contents, as well as antioxidant activity, of black bean koji fermented by *Aspergillus awamori* under different culture conditions, *Food Chem. 104* (2007) 936–942.
2. M.G. Choung, I.Y. Baek, S.T. Kang, W.Y. Han, D.C. Shin, H.P. Moon, K.H. Kang, Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.), *J. Agric. Food Chem. 49* (2001) 5848–5851.
3. C. Nagata, N. Takatsuka, N. Kawakami, H. Shimizu, Soy product intake and hot flashes in Japanese women: Results from a community-based prospective study, *Am. J. Epidemiol. 153* (2001) 790–793.
4. A. Cotter, K.D. Cashman, Genistein appears to prevent early postmenopausal bone loss as effectively as hormone replacement therapy, *Nutr. Rev. 61* (2003) 346–351.
5. C.M. Weaver, J.M.K. Cheong, Soy isoflavones and bone health: The relationship is still unclear, *J. Nutr. 135* (2005) 1243–1247.
6. M.S. Anthony, T.B. Clarkson, C.L. Hughes Jr., T.M. Morgan, G.L. Burke, Soybean isoflavones improve cardiovascular risk factors without affecting the reproductive system of peripubertal rhesus monkeys, *J. Nutr. 126* (1996) 43–50.
7. D. Goodman-Gruen, D. Kritz-Silverstein, Usual dietary isoflavone intake is associated with cardiovascular disease risk factors in postmenopausal women, *J. Nutr. 131* (2001) 1202–1206.
8. Y.H. Hung, H.Y. Huang, C.C. Chou, Mutagenic and antimutagenic effects of methanol extracts of unfermented and fermented black soybean, *Int. J. Food Microbiol. 118* (2007) 62–68.
9. S. Hendrich, Bioavailability of isoflavones, *J. Chromatogr. B, 777* (2002) 203–210.
10. K.D.R. Setchell, N.M. Brown, L. Zimmer-Nechemias, W.T. Brashear, B.E. Wolfe, A.S. Kirschner, J.E. Heubi, Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability, *Am. J. Clin. Nutr. 76* (2002) 447–453.
11. T.H. Kao, W.M. Wu, C.F. Hung, W.B. Wu, B.H. Chen, Anti-inflammatory effects of isoflavone powder produced from soybean cake, *J. Agric. Food Chem. 55* (2007) 11068–11079.
12. H.F. Liao, C.J. Chou, S.H. Wu, K.H. Khoo, C.F. Chen, S.Y. Wang, Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U973 cells, *Anti-Cancer Drugs, 12* (2001) 841–846.
13. M. Yamai, K. Tsumura, M. Kimura, S. Fukuda, T. Murakami, Y. Kimura, Antiviral activity of a hot water extract of black soybean against a human respiratory illness virus, *Biosci. Biotechnol. Biochem. 67* (2003) 1071–1079.
14. L.R. Ribeiro, D.M.F. Salvadori, Dietary components may prevent mutation-related diseases in humans, *Mutat. Res. 544* (2003) 195–201.
15. K.C. Cheng, J.T. Lin, J.Y. Wu, W.H. Liu, Isoflavone conversion of black soybean by immobilized *Rhizopus* spp., *Food Biotechnol. 24* (2010) 312–331.
16. H. Esaki, O.H. Onozaki, S. Kawakishi, T. Osawa, New antioxidant isolated from tempeh, *J. Agric. Food Chem. 44* (1996) 696–700.
17. M. Matsuo, N. Nakamura, Y. Shidoji, Y. Muto, H. Esaki, T. Osawa, Antioxidative mechanism and apoptosis induction by 3-hydroxyanthranilic acid, an antioxidant in Indonesian food tempeh, in the human hepatoma-derived cell line, HuH-7, *J. Nutr. Sci. Vitaminol.* (Tokyo), *43* (1997) 249–259.
18. W. Wang, N.A. Bringe, M.A. Berhow, E. Gonzales de Mejia, β-Conglycinins among sources of bioactives in hydrolysates that inhibited leukemia cells *in vitro*, *J. Agric. Food Chem. 56* (2008) 4012–4020.
19. H. Esaki, H. Onozaki, Y. Morimitsu, S. Kawakishi, T. Osawa, Potent antioxidative isoflavones isolated from soybeans fermented with *Aspergillus saitoi*, *Biosci. Biotechnol. Biochem. 62* (1998) 740–746.
20. H. Esaki, S. Kawakishi, Y. Morimitsu, T. Osawa, New potent antioxidative *o*-dihydroxyisoflavones in fermented Japanese soybean products, *Biosci. Biotechnol. Biochem. 63* (1999) 1637–1639.
21. C. Hadjur, M.J. Richard, M.O. Parat, A. Favier, P. Jardon, Photodynamically induced cytotoxicity of hypericin dye on human fibroblast cell line MRC5, *J. Photochem. Photobiol. B, 27* (1995) 139–146.
22. H. Wang, P.A. Murphy, Isoflavone content in commercial soybean foods, *J. Agric. Food Chem. 42* (1994) 1666–1673.
23. K. Shetty, R.A. Korus, D.L. Crawford, Growth kinetics and phenolics production in *Glycine max* cell suspension cultures, *Appl. Biochem. Biotechnol. 20/21* (1989) 825–843.
24. T. Mosmann, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays, *J. Immunol. Methods, 65* (1983) 55–63.

25. Y.C. Su, Traditional fermented food in Taiwan, *Proceedings of the Oriental Fermented Foods*, Food Industry Research and Development Institute, Taipei, Taiwan (1980) p. 15 (in Chinese).

26. Y.B. Choi, K.S. Kim, J.S. Rhee, Hydrolysis of soybean isoflavone glucosides by lactic acid bacteria, *Biotechnol. Lett. 24* (2002) 2113–2116.

27. S.T. Li: *Ben Tsao Gong Mu (Chinese Botanical Encyclopedia)*, Great Taipei Publishing Co, Taipei, Taiwan (1990) p. 350 (in Chinese).

28. T. Toda, T. Uesugi, K. Hirai, H. Nukaya, K. Tsuji, H. Ishida, New 6-O-acyl isoflavone glycosides from soybeans fermented with *Bacillus subtilis* (natto). I. 6-O-succinylated isoflavone glycosides and their preventive effects on bone loss in ovariectomized rats fed a calcium-deficient diet, *Biol. Pharm. Bull. 22* (1999) 1193–1201.

29. B. Halliwell, M.A. Murcia, S. Chirico, O.I. Aruoma, Free radicals and antioxidants in food an *in vivo*: What they do and how they work, *Crit. Rev. Food Sci. Nutr. 35* (1995) 7–20.

30. K.Y. Park, K.O. Jung, S.H. Rhee, Y.H. Choi, Antimutagenic effects of doenjang (Korean fermented soypaste) and its active compounds, *Mutat. Res. 523* (2003) 43–53.

31. B.B. Knowles, C.C. Howe, D.P. Aden, Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen, *Science, 209* (1980) 497–499.