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Content of Total Phenolics and Anthocyanins and the Antimutagenicity of *Aspergillus awamori*-Fermented Black Soybean after Heat Treatment

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ABSTRACT

The *Aspergillus awamoir*-fermented black soybean, possessing enhanced functional properties, was previously suggested to be a potentially functional ingredient in the formation of healthy foods. In this study, the effect of heat treatments (40-100°C for 30 min) on the changes of total phenolics and anthocyanin contents, mutagenicity and the antimutagenicity of fermented black soybeans against 4-nitroquinoline-*N*-oxide, a direct mutagen, and benzo[a]pyrene, an indirect mutagen, on *Salmonella* Typhimurium TA100 and TA98 were examined.

Results revealed that the heated-fermented black soybean showed no mutagenicity. Heating the fermented black soybean at 80 or 100°C for 30 min resulted in a reduced antimutagenicity with the methanol extract. Reduction in antimutagenicity varied with the test strains of *S.* Typhimurium and the type of mutagens examined. However, the fermented black soybean still possessed antimutagenicity after exposure to 100°C for 30 min. Contents of total phenolics and anthocyanins reduced significantly ($p < 0.05$) as the heating temperature was raised to 40 and 80°C, respectively. Reduction in antimutagenicity of the fermented black soybeans after heating might be due to the lower anthocyanin content.

Key words: Fermented black soybean, anthocyanin, total phenolics, antimutagenicity, heat treatment

INTRODUCTION

Similar to normal soybeans, black soybeans, *Glycine max* (L.) Merr, is considered a nutritionally rich foodstuff. Compounds possessing biological activity such as anthocyanin, saponin, vitamin E and isoflavone can be found in black soybeans[1-4]. The functional properties of black soybeans have been examined by various investigators. Riberio and Saloadori[5] reported that black soybeans effectively reduce the incidence of DNA damage by cyclophosphamide. Takahashi *et al.*[6] observed that black soybeans inhibited low density lipoprotein oxidation. Moreover, Rodriguez-Bűrger *et al.*[7] suggested that combining rice with the *Rhizopus azygosporus*-fermented black soybeans is a viable method to develop a nutritious weaning food. In our laboratory, we have previously noted that content of aglycone, the bioactive isoflavone, was increased through fermentation with fungi[8]. Furthermore, fermentation was found to enhance certain functional properties such as the antioxidative and antimutagenic activities of black soybeans[9,10]. For these reasons, it is clear that fermented black soybeans are potentially useful as a dietary adjunct or ingredient in the formation of healthy food.

In the food industry, heating is commonly used to enhance the preservation of food products. Alternation in the functional properties, e.g. antimutagenicity, of food materials as a result of heating has been observed by various investigators[11-14]. Furthermore, the heating of food has also been reported to result in the induction or enhancement of mutagenic activity[15,16]. To assess the functional properties that contribute to health in fermented black soybeans, this study examined the antimutagenicity of fermented black soybeans across various heat treatments. Moreover, the effect of heat treatment on the total phenolics and anthocyanin content of fermented black soybeans was also examined.

MATERIALS AND METHODS

I. *Materials*

Black soybeans were purchased from the local market. The test strains of *S.* Typhimurium including TA98 and TA100 were obtained from the Bioresourc-

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es Collection and Research Center (BCRC), Hsinchu, Taiwan. Professor Yu, Graduate Institute of Food Science and Technology, National Taiwan University provided *A. awamori* used to prepare fermented black soybeans.

4-Nitroquinoline-*N*-oxide (4-NQO) and benzo[a]pyrene (B[a]P) were obtained from Sigma-Aldrich Co. (St. Louis, MI, USA). Both mutagens were dissolved in dimethylsulfoxide (DMSO, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at concentrations of 0.5 and 20 μg/mL for 4-NQO and B[a]P, respectively. Rat liver-S9 homogenate treated with Aroclor 1254 was purchased from MP Biomedicals, Inc. (Solon, Ohio, USA). S9 mix (S9 fraction of liver homogenate with cofactors) was prepared according to the method of Maron and Ames[17] and used for metabolic activation of B[a]P.

II. *Preparation of Heat Treated-fermented Black Soybean and Methanol Extract*

The fermented black soybeans were first prepared according to the procedures described by Lee and Chou[8]. Briefly, a solid fermentation of the steamed black soybeans with *A. awamori* was performed at 30°C and 95% RH for 3 days. The fermented black soybeans were then lyophilized in a freeze-drier (Free Dry System/Freezone® 4.5; Labconco Co., Kansas, Missouri, USA) and were ground to 30-mesh powder screen using a grinder (Model HF-365, Shivn Feng Enterprise Co. Ltd., Taipei, Taiwan). The powder was first heated in an electric oven at 40, 60, 80 or 100°C for 30 min, and then extracted with methanol (1:10, w/v) by refluxing at 25°C for 24 h with gentle shaking. The extracts, after filtering through Whatman No.1 filter paper, were vacuum concentrated and freeze dried.

III. *Mutagenicity and Antimutagenicity Assay*

Detailed procedures for the assay of mutagenicity and antimutagenicity were described in our previous paper[9]. Essentially, mutagenicity was assayed using *S*. Typhimurium TA98 or TA100 in the absence or presence of S9 mix. The antimutagenicity against 4-NQO and B[a]P was assayed by incubating with 0.625-0.5 mg extract of the treated- or untreated sample per plate, using *S*. Typhimurium TA98 or TA100. S9 mixture was also added when B[a]P was tested as the mutagen. After incubating at 37°C for 48 h, the His^+ revertant colonies were counted. In the preliminary study, the doses of the tested samples of fermented black soybean extract were found to show no toxicity against *S*. Typhimurium.

Each assay was performed in triplicate, and antimutagenic activity was expressed as a percentage of mutagenic inhibition using the formula:

$$\text{Inhibition (\%)} = 1-[(A-E)/(B-D)]\times 100;$$

where A and B are the numbers of mutagen-induced revertants in the presence and absence of sample, respectively. E and D are the number of spontaneous revertants observed with sample and control, respectively.

IV. *Measurements of Total Phenolics and Anthocyanins*

The content of total phenolics, expressed in mg of gallic acid/g dried fermented black soybeans, was determined according to that described in the paper of Lee and Chou[8]. The method described by Abdel-Aal and Hucl[18] was followed to determine the content of total anthocyanin, which was expressed as cyaniding 3-glucoside equivalents (mg/g dried fermented black soybeans).

V. *Statistical Analysis*

The mean values and the standard deviation were calculated from the data obtained from three separate experiments. Means were compared using Duncan's multiple range test method in SAS, version 8 (SAS Institute, Gary, NC, USA).

RESULTS AND DISCUSSION

I. *The Effect of Heat Treatment on the Mutagenicity of Fermented Black Soybean Extract*

Heating food may introduce mutagenic and carcinogenic compounds such as heterocyclic aromatic amines[15]. Surono and Hosono[16] also reported that heating increased the mutagenicity of Terasi, a traditional fermented product of Indonesia. Therefore, mutagenicity of the fermented black soybean subjected to various heat treatments was evaluated. As shown in Table 1, the revertants in presence of the heated fermented black soybean extract (0.625-5.0 mg/plate) for *S*. Typhimurium TA98 with or without the S9 mixture were close to those for the negative control (spontaneous revertants in absence of heated fermented black soybean extract) and were less than twice that of spontaneous revertants. This indicated that no mutagenic factor formed in the fermented black soybeans after the various heat treatments. Similar phenomenon was also noted with *S*. Typhimurium TA100 (data not shown). Therefore, extracts of the heated fermented black soybean showed no mutagenic effect on both strains of *S*. Typhimurium.

II. *The Effect of Heat Treatment on the Antimutagenicity of Fermented Black Soybean Extract*

Variation in the effect of heat treatment on the antimutagenic activity of antimutagens has been studied. Vis *et al*.[14] reported that heat-denaturated ovalbumin showed a strong increase in antimutagenicity against MNNG compared to undenaturated ovalbumin. An enhanced antimutagenicity against MNNG was also

Table 1. Mutagenicity of the extracts of fermented black soybean after heating at different temperatures for 30 min in S. Typhimurium TA98

Extracts (mg/plate)	Unheated	Heated (°C)			
		40	60	80	100
	Revertants (CFU/plate)	Revertants (CFU/plate)	Revertants (CFU/plate)	Revertants (CFU/plate)	Revertants (CFU/plate)
……–S9……					
Control	25 ± 5[a]	25 ± 5	25 ± 5	25 ± 5	25 ± 5
5	25 ± 3	23 ± 1	25 ± 3	28 ± 7	31 ± 2
2.5	30 ± 3	29 ± 5	30 ± 3	29 ± 3	29 ± 6
1.25	28 ± 7	28 ± 5	28 ± 7	32 ± 7	29 ± 8
0.625	24 ± 3	28 ± 3	24 ± 3	28 ± 5	32 ± 4
……+S9……					
Control	34 ± 2	34 ± 2	34 ± 2	34 ± 2	34 ± 2
5	55 ± 8	56 ± 5	54 ± 6	54 ± 6	54 ± 6
2.5	50 ± 9	52 ± 3	53 ± 4	53 ± 4	48 ± 6
1.25	52 ± 6	56 ± 4	48 ± 3	48 ± 3	44 ± 8
0.625	53 ± 3	46 ± 4	47 ± 3	47 ± 1	45 ± 1

[a]Results are presented as means ± SD from three separate experiments.

noted with the aqueous and methanol extracts of soybean after heating at 225°C for 12 min by Oshite *et al*.[13] On the other hand, Hosono *et al*.[11] reported that heating the cultured milk fermented by *Lactobacillus bulgaricus* or *Streptococcus thermophilus* at 55°C for 10 min resulted in a decreased antimutagenicity against 4-NQO and 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide. A similar phenomenon of reduced antimutagenicity was also observed by Hsieh *et al*.[19] on lactic fermented soymilk after exposure to 55°C for 10 min.

Table 2 shows the antimutagenicity of the unheated- and heated-fermented black soybean extracts at a dose of 0.625-5.0 mg/plate against 4-NQO and B[a]P in *S*. Typhimurium TA98. Regardless of heat treatment, the antimutagenic effect of the fermented black soybeans extract increased as the dosage increased. At the same dosage level, the antimutagenicity exerted by the extract of the fermented black soybean heated at 40 or 60°C for 30 min showed a profound reduction compared with the extract of unheated fermented black soybean. For example, at the highest dosage of 5.0 mg extract/plate examined, the extract of unheated fermented black soybean exhibited an antimutagenicity of 82.46 and 85.25% against 4-NQO and B[a]P respectively in *S*. Typhimurium TA98. In comparison, significantly lower ($P < 0.05$) antimutagenicity of only 50.87 and 59.01% was noted with the extract of the 80°C heated-fermented black soybean.

Using *S*. Typhimurium TA100 as the test strain, the effect of heat treatment on the antimutagenicity of fermented black soybean against 4-NQO and B[a]P (Table 3) was found similar to that noted with *S*. Typhimurium TA98 (Table 2). Compared with the unheated fermented black soybean extract, a substantial reduction in the antimutagenicity was observed with the extracts of fermented black soybeans heated at 80°C or higher. The parameter of IC_{50}, the efficient concentration of test samples that inhibited 50%, was obtained by polynomial adjustment of a second grade equation analysis of data shown in Tables 2 and 3 to further elucidate the effect of heat treatment on the antimutagenicity of the fermented black soybean extract. As shown in Table 4, the IC_{50} for the antimutagenicity of the fermented black soybean extract against 4-NQO and B[a]P in both test strains of *S*. Typhimurium was relatively stable at a temperature up to 60°C. However, significantly ($P < 0.05$) increased IC_{50} or reduced antimutagenicity was noted with the extract of fermented black soybean heated at 80℃ or higher for 30 min when compared with that of the control (unheated-fermented black soybean). For example, the extract of the unheated-fermented black soybean showed an IC_{50} of 1.17 and 1.68 mg/plate against the mutagenesis induced by 4-NQO and B[a]P, respectively in *S*. Typhimurium TA98. On the other hand, the extract of the 80°C heated-fermented black soybean showed significantly larger IC_{50} of 4.93 and 3.69 mg/plate, for 4NQO and B[a]P, respectively. These results demonstrated that the antimutagenic factors present in the fermented black soybean were thermolabile at 100°C. Nevertheless, extracts of the fermented black soybean still possess considerable antimutagenicity after heating at 100°C for 30 min.

Table 2. Antimutagenicity effect exerted by extracts of unheated- and heated-fermented black soybeans against 4NQO or B[a]P in S. Typhimurium TA98

Extracts (mg/plate)	Unheated		Heated (°C)							
			40		60		80		100	
	Revertants (CFU/plate)	Inhibition[a] (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
……4NQO……										
Control	57 ± 6[b]		57 ± 6		57 ± 6		57 ± 6		57 ± 6	
5	D10 ± 1b[c]	A82.46a	D15 ± 1b	A73.69b	D12 ± 3b	A78.95b	C28 ± 1a	A50.87c	C26 ± 4a	A54.39c
2.5	C19 ± 1b	B66.67a	C22 ± 3b	B61.41b	C22 ± 3b	B61.41b	B41 ± 5a	B28.07c	B41 ± 1a	B28.07c
1.25	B26 ± 2b	C54.39a	B30 ± 1b	C47.37b	B28 ± 3b	C50.88ab	B42 ± 4a	C26.31c	A45 ± 3a	C21.05d
0.625	A35 ± 2b	D38.60a	A34 ± 1b	D40.35a	A 38 ± 1b	D33.33b	A50 ± 5a	D12.28d	A46 ± 6a	D19.29c
……B[a]P……										
Control	61 ± 5		61 ± 5		61 ± 5		61 ± 5		61 ± 5	
5	C9 ± 2a	A85.25ab	D10 ± 1a	A83.60b	B8 ± 1a	A86.88a	B25 ± 6a	A59.01c	B28 ± 1a	A54.09d
2.5	B24 ± 4b	B60.65b	C22 ± 1ab	B63.93a	B24 ± 1ab	B60.65b	AB37 ± 3a	B39.34c	AB37 ± 1a	B39.34c
1.25	A35 ± 1a	C42.62b	BC27 ± 2a	C55.73a	A37 ± 3a	C39.34b	A47 ± 1a	C22.95d	A44 ± 6a	C27.86c
0.625	A38 ± 3a	D37.70a	AB44 ± 3a	D27.86b	A42 ± 5a	D31.15b	A50 ± 1a	D18.03c	A53 ± 1a	D13.11d

[a] Result are presented as means ± SD from three separate experiments.

[b] Number of revertants reported is total His^+ revertants minus spontaneous His^+ revertants per plate. Spontaneous revertants were those observed with sample or control. Statistical differences were calculated by Duncan's multiple range test. Value in the same row (a, b, c, d) and column (A, B, C, D) with same letters are not significantly different ($p > 0.05$).

[c] Inhibition (%) = [1–number of induced revertants in the presence of sample / number of induced revertants in the absence of extract of sample]×100.

III. *The Effect of Heating on the Content of Total Phenolic Compounds in Fermented Black Soybean*

Phenolic compounds are commonly found in plants. Dry beans contain many polyphenolic phytochemicals that are known to possess antioxidants, as well as antimutagenic and anticarcinogenic properties[1]. Different from soybeans, black soybeans contain anthocyanin which is a kind of flavonoid. Anthocyanins are found to significantly suppress the growth of cultured tumor cells and have been shown to have greater inhibitory effect than other flavonoids[20]. Previously, we noted that fermentation of black soybeans with fungi resulted in higher content of total extractable phenolics and anthocyanins[10].

The effect of heat treatment on the total phenolics in fermented black soybeans is shown in Figure 1. Heat treatment, regardless of heating temperature, resulted in significantly decreased ($P < 0.05$) total phenolics content in the fermented black soybean, while the total phenolics content in the various heated fermented black soybean were similar.

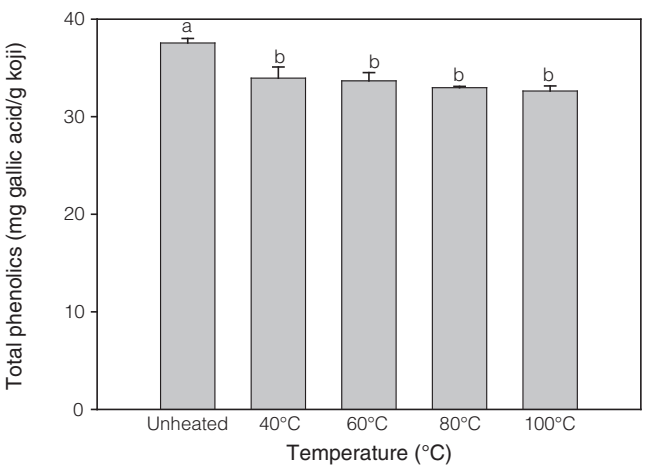

Figure 1. Total phenolic content of fermented black soybeans after heating at different temperatures for 30 min. Means (bar values) with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

Despite the association of phenolic compounds with antimutagenic activity, the extent of reduced mutagenicity of the fermented black soybean (Tables 2-4) did not correspond precisely with the degree of reduced total phenolics (Figure 1) caused by the heat treatments examined. For example, the total phenolics content of the vari-

Table 3. Antimutagenicity effect exerted by extracts of unheated- and heated-fermented black soybeans against 4NQO or B[a]P in S. Typhimurium TA100

Extracts (mg/plate)	Unheated		Heated (°C)							
			40		60		80		100	
	Revertants (CFU/plate)	Inhibition[a] (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)	Revertants (CFU/plate)	Inhibition (%)
4NQO										
Control	311 ± 11[b]		311 ± 11				311 ± 11		311 ± 11	
5	C70 ± 1b[c]	A77.49a	D83 ± 7b	A73.31b		A70.73b	D173 ± 10a	A44.37c	D183 ± 1a	A41.15c
2.5	B117 ± 11b	B62.37a	C131 ± 10b	B57.87b		B53.05b	C276 ± 9a	B11.25d	C231 ± 1a	B25.72c
1.25	B166 ± 8b	C46.62a	B177 ± 7b	C43.09ab		C39.54b	B261 ± 1a	C16.07c	B265 ± 2a	C14.79c
0.625	A182 ± 6b	C41.47a	A205 ± 5b	D34.08b		D29.58c	A280 ± 2a	D9.96d	A285 ± 1a	D8.36d
B[a]P										
Control	177 ± 2		177 ± 2		177 ± 2		177 ± 2		177 ± 2	
5	D46 ± 2a	A74.01a	C45 ± 3b	A74.57a	D44 ± 1b	A75.14a	C99 ± 6a	A44.06c	D84 ± 2a	A52.54b
2.5	C69 ± 3c	B61.01a	B69 ± 2bc	B61.01a	C73 ± 10ab	B58.75b	C115 ± 3ab	B35.02d	C109 ± 1a	B38.41c
1.25	B115 ± 4a	C35.03ab	A109 ± 2a	C38.41a	A122 ± 8a	C31.07b	B160 ± 2a	C 9.60d	B144 ± 3a	C18.64c
0.625	A131 ± 1a	D25.98a	A128 ± 1a	D27.68a	A145 ± 9a	D18.07b	A169 ± 2a	C4.51c	A174 ± 2a	D 1.69d

[a] Result are presented as means ± SD from three separate experiments.
[b] Number of revertants reported is total His^+ revertants minus spontaneous His^+ revertants per plate. Spontaneous revertants were those observed with sample or control. Statistical differences were calculated by Duncan's multiple range test. Value in the same row (a, b, c, d) and column (A, B, C, D) with same letters are not significantly different ($p > 0.05$).
[c] Inhibition (%) = [1–number of induced revertants in the presence of sample / number of induced revertants in the absence of extract of sample]×100.

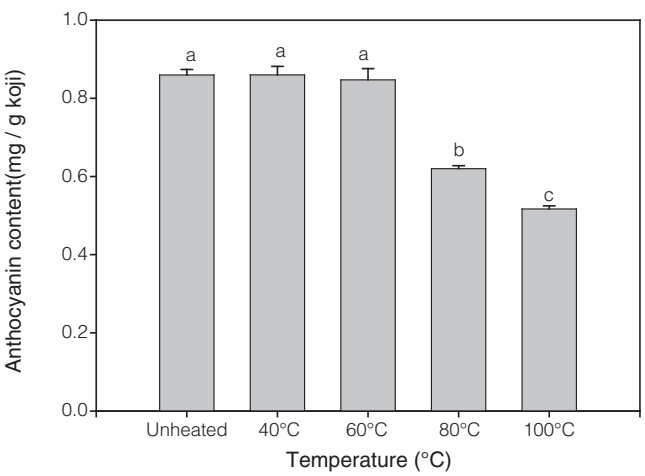

Figure 2. Anthocyanin content of black soybean koji after heating at different temperatures for 30 min. Means (bar values) with different letters are significantly different by Duncan's multiple range test ($p < 0.05$).

ous heated fermented black soybeans showed no significant difference regardless of heating temperature (P > 0.05) (Figure 2), while the antimutagenic effect exerted by the 80 or 100°C-heated fermented black soybeans was significantly less than other heated- and unheated fermented black soybeans. This suggested that antimutagenic factors other than phenolic compounds present in the fermented black bean were inactivated during heating at 80 and 100°C and thus lead to the significantly lower level of antimutagenicity observed. On the other hand, various types of phenolic compounds present in the test sample might also affect the observed antimutagenicity. These findings merit further investigation.

IV. *The Effect of Heating on the Anthocyanin Content in Fermented Black Soybean*

Figure 1 shows the content of anthocyanins in the fermented black soybean after heating at various temperatures for 30 min. The total level of anthocyanin in the 40 or 60°C heated-fermented black beans was similar to that in the unheated fermented black soybean while a significantly lower (P < 0.05) anthocyanin content was noted in the fermented black soybean after heating at 80°C or higher. In addition, it was noted that as the heating temperature increased from 80 to 100°C, the anthocyanin content further decreased. These results

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Table 4. Half-inhibition (IC_{50}) of the antimutagenicity of the heated-fermented black soybean extracts against 4-NQO or B[a]P in *S.* Typhimurium TA98 and TA100

Treatment temperature (°C)	IC_{50} (mg/plate)[a]			
	TA 98		TA 100	
	4-NQO	B[a]P	4-NQO	B[a]P
Control	1.17 ± 0.47B[b]	1.68 ± 0.14B	1.39 ± 0.24B	1.78 ± 0.14B
40	1.78 ± 0.61B	1.40 ± 0.52B	1.80 ± 0.10B	1.80 ± 0.26B
60	1.84 ± 0.52B	1.57 ± 0.62B	2.17 ± 0.29B	1.95 ± 0.31B
80	4.93 ± 0.67A	3.69 ± 0.42A	5.71 ± 0.90A	3.79 ± 1.00A
100	4.67 ± 0.72A	3.67 ± 0.90A	5.82 ± 0.56A	3.89 ± 0.72A

[a] IC_{50}, obtained by polynomial adjustment of a second grade equation analysis, is the efficient concentration of the test samples that inhibit 50% mutagenic activity.

[b] Result are presented as means ± SD from three separate experiments. Means with different letters in the same column are significantly different ($p < 0.05$).

indicated that anthocyanin in fermented black soybean is thermolabile at 80°C or higher. Reduction in the anthocyanin content as observed in the present study is overall comparable with reports of Aparicio-Fernández *et al.*[1] and Abdel-Aal and Hucl[18]. The former reported a significant reduction in the content of total anthocyanin in the common bean after typical home cooking (100°C for 2.5 h). The latter observed that the degradation of anthocyanin in blue wheat slurries is greater at 95°C followed by 80 and 65°C. Delgado-Vargas *et al.*[21] suggested that heating opened the structure of anthocyanin to form chalcones, which were degraded further to form brown products. This process possibly led to a similar reduction in the total anthocyanin content in the heated fermented black soybean that was observed in the present study. Finally, it was noted that the trend in the reduction of anthocyanin content caused by heat treatment is, in general, similar to that observed on antimutagenicity (Table 4)

CONCLUSIONS

In conclusion, this study demonstrated potential reduction in both antimutagenicity and content of total phenolics and anthocyanins of fermented black soybeans following heat treatment. The reduction of antimutagenicity is generally related to the reduced anthocyanin content in the heated fermented black soybean. Note that components of the fermented black soybean showed no mutagenicity, but still possessed antimutagenicity after exposure to 100°C for 30 min. These findings are valuable when fermented black soybean is further processed and utilized as an ingredient for the formulation of healthy foods.

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