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Antioxidative and Antimicrobial Activities of the Extracts from the Seed Coat of Bambarra Groundnut (*Voandzeia subterranea*)

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Abstract

Antioxidative and antimicrobial activities of extracts from Bambarra groundnut seed coat, prepared using water, ethanol and acetone as the media at various temperatures (30, 60, 90ºC) were investigated. Extraction yields and phenolic contents of Bambarra groundnut seed coat extracts (BGSEs) varied from 5.21 to 13.23% and from 169 to 569 mgGAE/g dry extract, respectively. BGSEs showed varying antioxidative activities including DPPH, ABTS, hydroxyl and superoxide anion radical scavenging activities, ferric reducing antioxidant power and metal chelating activity, depending on extraction conditions ($p<0.05$). Antioxidative activities of BGSE prepared using ethanol at 60ºC ($BGSE_{E60}$) were stable in a wide pH range and heat treatment. $BGSE_{E60}$ exhibited antioxidative activities in lecithin liposome system and steamed chicken mince as evidenced by the retarded formation of conjugated diene, peroxide value and thiobarbituric reactive substances (TBARS) ($p<0.05$). Additionally, the $BGSE_{E60}$ (2.5-30 mg/ml) possessed antimicrobial activities against bacteria (*Staphylococcus aureus, Escherichia coli, Bacillus cereus*), yeast (*Candida albicans*) and mold (*Aspergillus niger*), in a dose-dependent manner. Scanning and transmission electron microscopic studies also revealed the damages of microbial cells after being treated with $BGSE_{E60}$. No cytotoxicity of $BGSE_{E60}$ against the primate cell line (Vero) was observed. Therefore, extraction yields, total phenolic contents and antioxidative activities of BGSEs were governed by extracting solvents and temperatures used. Additionally, BGSEs could be used as natural antioxidant and antimicrobial agent without cytotoxicity.

Key word: antioxidative activity, antimicrobial activity, phenolic compound, extract, Bambarra groundnut

1 Introduction

Nuts play a key role in the diets of human beings throughout the world. The total per capita consumption of nuts markedly increased over the past two decades due to increased attention to beans as functional foods.[1] Nut by-products are generated during production and consumption and basically recognized as market value residues. Bambarra groundnut, an indigenous groundnut of southern part of Thailand, has been used in the local area as food. Before consumption, seed coat was removed and regarded as waste. Many seed coats have been reported to possess a plenty of phenolic compounds.[2-4] Additionally, those seed coats are inexpensive source of nutraceuticals and functional ingredients. However, seed coat of Bambarra groundnut has not been fully exploited.

Extraction processes have been used to extract phenolic compounds from the under-utilized wastes, which can be used as nutraceuticals and functional ingredients in food industry.[5] Solvents such as methanol, ethanol, butanol, acetone, chloroform and water have been commonly used for extraction of phenolics from plants.[6] The extracts from seed coats of peanut,[7] hazelnut, lentils,[4] cashew nut[3] and legumes[2] have been reported to be rich in phenolic compounds and contain numerous types of phenolic compounds, which play an important protective role against oxidative damage.[7] Additionally, polyphenol-rich nuts correlate with a wide range of physiological properties, including antioxidative[8-9] and antimicrobial activities.[10]

Although, synthetic agents are efficient and have been widely used to preserve food products by delaying deterioration, the strict regulation based on awareness of health risks leads to the searching for natural antioxidants and antimicrobial agent as alternatives. Nevertheless, there is no information regarding the antioxidative and antimicrobial activities of the extracts from Bambarra groundnut seed coat. Therefore, the objective of this study was

to prepare the extracts from Bambarra groundnut seed coat with various conditions and to study their antioxidative and antimicrobial activities.

2 Materials and methods

2.1 Chemicals

2,2-diphenyl-1-picryhydrazyl (DPPH) (PubChem CID:2735032), 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (PubChem CID:6871216), 2,4,6-tripyridyl-s-triazine (TPTZ) (PubChem CID:77258), 2-deoxy-D-ribose (PubChem CID:5460005), nitro-blue tetrazolium (NBT) (PubChem CID:9281), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (PubChem CID:40634) and α-tocopherol (PubChem CID:14985) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

2.2 Sample preparation

Bambarra groundnut was purchased from a supplier in Phatthalung province and transported to a laboratory, Thaksin University, Phatthalung. The groundnut was peeled manually and dried at 60°C for 24 h. Thereafter, the groundnut seed coat was separated and ground using a grinder and sieved through a 60-mesh sieve. Ground seed coat was subjected to determination of moisture, protein, fat, ash and fiber contents.[11]

2.3 Extraction of seed coat

The ground seed coat was suspended in water, 99.9% ethanol or 99.5% acetone at a ratio of 1:10 (w/v), homogenized for 1 min by homogenizer (IKA T18, Ultra-Turrax, Becthai, Bangkok, Thailand). The homogenate was incubated at 30, 60 and 90°C in a temperature controlled water bath (Memmert, WNB22, Becthai, Bangkok,Thailand) for 60 min. The

mixtures were filtered through Whatman #1 filter paper. The solvents were evaporated under vacuum in a rotary evaporator (Buchi, Rotavapor R-210, Bangkok,Thailand) at 40°C and kept in amber bottle at -20°C until analyses.

2.4 Determination of extraction yields

Extraction yield (%) of BGSEs was determined and defined as weight of extract, compared with the weight of seed coat. Extraction yield was calculated according to the following equation:

Extraction yield (%) = (weight of dry extract/ weight of dry seed coat) x100

2.5 Determination of total phenolic contents

The total phenolic content of BGSEs was investigated with a slight modification.[12] One ml of appropriately diluted extracts was added with 9 ml of deionized water. Thereafter, 1 ml of Folin & Ciocalteu's phenol reagent was added to the mixture and vortexed. After 5 min, 10 ml of 7% Na_2CO_3 solution was added and mixed well. Five ml of deionized water were added. After incubation for 90 min at 25°C, the absorbance was read at 750 nm. Gallic acid (20-100 mg/l) was used as standard. Total phenolic content of BGSEs was expressed as mg gallic acid equivalent (GAE)/g dry extract.

2.6 Determination of antioxidative activities

2.6.1 DPPH radical scavenging activity

DPPH radical scavenging activity was measured with a slight modification.[13] BGSEs were diluted with deionized water to obtain a concentration of 0.001 mg dry extract/ml. To 2 ml of sample solutions, 0.5 ml of 0.2 mM DPPH solution was added and mixed vigorously.

After incubating for 30 min, the absorbance of the resulting solutions was measured at 517 nm using a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Kyoto, Japan).

2.6.2 ABTS radical scavenging activity

BGSEs were diluted with deionized water to obtain a concentration of 0.05 mg dry extract/ml. To 100 µl of sample solutions, 3 ml of $ABTS^{\bullet+}$ were added and mixed well. After incubating for 6 min, the absorbance of the resulting solutions was measured at 734 nm.[14]

2.6.3 Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was assayed using the 2-deoxyribose oxidation method.[15] The reaction mixture consisted of 0.1 ml of 2.8 mM 2-deoxyribose, 0.2 ml of the mixture between 100 µmol $FeCl_3 \cdot 6H_2O$ and 104 µmol EDTA (1:1), 0.1 ml of 1 mM H_2O_2, 0.1 ml of 1 mM L-ascorbic acid and 0.5 ml of BGSEs (0.00001 mg dry extract/ml). The reaction mixture was incubated at 37°C for 1 h. One ml of 0.5% thiobarbituric acid (w/v) in 10% trichloroacetic acid (w/v) was added into the reaction mixture and mixed well. The mixture was boiled for 15 min, cooled in ice for 5 min and the absorbance was read at 532 nm.

2.6.4 Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was measured.[16,17] Superoxide anion was generated in a non-enzymatic system. One ml of each solution prepared in phosphate buffer (0.1 M, pH 7.4) including 150 µM NBT, 60 µM Phenazine methosulphate (PMS), and 468 µM Dihydro-nicotin-amidadenin-dinucleotide (NADH) were added to 1 ml of BGSE (0.00001 mg dry extract/ml), respectively. The reaction mixture was incubated at room temperature (25-27°C) for 5 min. The absorbance of the reaction mixture was measured spectrophotometrically at 560 nm. Superoxide anion derived from dissolved oxygen by PMS-NADH coupling reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is

measured at 560 nm. The inhibition of the blue NBT formation and decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion.[17]

2.6.5 Ferric reducing antioxidant power (FRAP)

FRAP assay was determined.[18] Acetate buffer (0.3 M, pH 3.6) was prepared by dissolving 3.1 g $C_2H_3O_2Na{\cdot}3H_2O$ and 16 ml of acetic acid in 1 l of deionized water. TPTZ solution was prepared by dissolving 10 mmol TPTZ in 1 l of 40 mM HCl solution. Ferric solution (20 mM) was prepared using $FeCl_3{\cdot}6H_2O$. The FRAP reagent was freshly prepared by mixing acetate buffer, TPTZ and ferric solutions at a ratio of 10:1:1. Five hundred µl FRAP reagent was mixed with 480 µl of deionized water and was incubated at 37°C in a water bath for 10 min, followed by adding 20 µl of each BGSE with a concentration of 0.01 mg dry extract/ml. After 10 min, the absorbance was read at 593 nm.

2.6.6 Metal chelating activity

The metal chelating activity on Fe^{2+} was investigated.[19] One ml of BGSE (0.02 mg dry extract/ml) was mixed with 3.7 ml of deionized water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) for 20 min at room temperature. The absorbance was read at 562 nm.

Trolox (0-0.2 mg/ml) was used as standard for all assays, except for metal chelating activity, in which EDTA (0-0.06 mg/ml) was used. The activities were expressed as Trolox equivalent (TE)/g or EDTA equivalent (EE)/g. The control blank of each reaction was conducted in the same manner except that deionized water was used instead of BGSE. Sample blank was also prepared in the same manner of each reaction except that deionized water was used instead of working reagent.

The BGSE rendering the highest antioxidative activities was selected for further study.

168 **2.7 Thermal and pH stability measurement**

169 $BGSE_{E60}$ with the highest antioxidative activities was dissolved in deionized water to obtain
170 the concentration of 0.001, 0.05, 0.01 and 0.02 mg dry extract/ml for determining DPPH and
171 ABTS radical scavenging activities, FRAP and metal chelating activity, respectively.

172 **2.7.1 Thermal stability**

173 The sample solutions were placed in a temperature-controlled water bath at 20, 40, 60,
174 80 and 100°C for 30 min. At the time designated, the samples were cooled rapidly in iced
175 water. Residual activities were determined.

176 **2.7.2 pH stability**

177 The sample solutions (5 ml) were prepared using 100 mM acetate buffer (pH 2 or 4)
178 and with 100 mM Tris HCl buffer (pH 6, 8 or 10). The solutions were then allowed to stand
179 for 30 min at room temperature, followed by adjusting to pH 7.0 with 1 and 6 M HCl or 1 and
180 6 M NaOH. The final volume of all solutions was brought up to 20 ml using deionized water
181 as previously described.[20] Residual activities of $BGSE_{E60}$ were examined.

182 Residual antioxidative activities were reported, relative to that found at 20ºC and pH 6.

183

184 **2.8 Preventive effect of selected seed coat extract in lecithin liposome system and steamed**
185 **chicken mince**

186 **2.8.1 Lecithin liposome system**

187 Lecithin was suspended in deionized water at a concentration of 8 mg/ml using a
188 homogenizer for 15 min. $BGSE_{E60}$ was added to the 30 ml of lecithin liposome system to
189 obtain the final concentrations of 6, 30 and 60 mg/l. After addition of the $BGSE_{E60}$, the
190 liposome suspension was homogenized again for 2 min. Thereafter, 10 µl of 30 mM cupric

acetate was added. The mixtures were shaken at a speed of 120 rpm at 37°C in the dark using a shaker. The control (without the $BGSE_{E60}$) and the system containing 6 mg/l Trolox or α-tocopherol were also prepared. Liposome oxidation was monitored every 6 h for 36 h by monitoring conjugated dienes[21] and thiobarbituric acid reactive substance (TBARS).[22,23]

2.8.2 Steamed chicken mince

Chicken meat was ground using a grinder and mixed with $BGSE_{E60}$ to obtain the final concentrations of 6, 30 and 60 mg/kg. The chicken mince was cooked by steaming at 100°C for 15 min. The samples were placed in polyethylene bag and kept at 4°C for 15 days. The chicken mince without $BGSE_{E60}$ (control) and containing 6 mg/kg Trolox or α-tocopherol were also prepared. Oxidation was monitored every 3 day by determining peroxide value (PV) and TBARS.[22-23]

2.9 Determination of antimicrobial activities

2.9.1 Preparation of microorganisms and inocula

Staphylococcus aureus (*S. aureus*) TISTR118, *Escherichia coli* (*E. coli*) TISTR780, *Bacillus cereus* (*B. cereus*) TISTR687 and *Aspergillus niger* (*A. niger*) ATCC6275 were obtained from the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand. *Candida albicans* (*C. albicans*) PSSCMI7010 was gifted from the Department of Microbiology, Prince of Songkla University, Hat Yai, Thailand. Bacteria were kept on NA slants, whereas yeast and mold were kept on PDA slants at 4°C until use. To activate microorganisms before culturing, the inocula were prepared. One loopful of each bacterium (*S. aureus*, *E. coli*, *B. cereus*) cultured on NA slants for 15 h and yeast (*C. albicans)* cultured on PDA slant for 24 h was inoculated in NB and PDB for bacteria and yeast, respectively. The

culture broths were then incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeast. Thereafter, 1.5 ml of culture broths were inoculated in 50 ml NB and PDB for bacteria and yeast, respectively and incubated at 37°C for 15 h for bacteria and 25°C for 24 h for yeast to obtain late log phase inocula. The inocula were adjusted to obtain $5x10^5$ cell/ml using hemacytometer. For mold, *A. niger* was sub-cultured onto PDA slant and incubated at 25°C for 48 h. This process was conducted twice to produce spores. Spore suspension with the concentration of $5x10^5$ spore/ml measured by direct count using hemacytometer was prepared.

2.9.2 Inhibition zone measurements

To measure the inhibition zone of $BGSE_{E60}$ against bacteria, yeast and mold, the agar diffusion method was conducted. Five µl of $BGSE_{E60}$ at the concentrations of 2.5, 5, 10 and 30 mg/ml was dropped on 5 mm diameter paper disc. Inocula ($5x10^5$ cell/ml)(0.1 ml) was aseptically added to a 20 ml sterilized media (Mueller-Hinton Agar, MHA) at 50°C in a temperature controlled water bath. The seeded agar media were immediately mixed and poured in petri dish and left for solidification. Each paper disc added with $BGSE_{E60}$ at different levels was placed on the surface of agar and the plates were left at room temperature for 30 min to allow diffusion of $BGSE_{E60}$ into media. Control (sterilized water) and penicillin or amphotericin B (2.5 mg/ml) were also used. Plates were incubated at 37°C for bacteria and 30°C for yeast and mold, until visible growths of tested microorganisms were evident (6 h for bacteria and 12 h for yeast and mold). Inhibition zones in mm around disc (including disc diameter) were measured. The antimicrobial activity was expressed as the diameter of inhibition zones produced by $BGSE_{E60}$ against tested microorganisms, in comparison with penicillin for bacteria and amphotericin B for yeast and mold.

238 **2.9.3 Scanning and transmission electron microscopies**

239 One ml of suspension for each microorganism ($1X10^8$ cells/ml) was centrifuged at
240 3000 x g (Mikro 200, Hettich Zentrifugen, Germany) for 20 min at 25°C. The supernatant was
241 discarded, and 1 ml of $BGSE_{E60}$ (5 mg/ml) was then added to the pellet, mixed well and
242 incubated for 12 h at 25°C. The cell suspensions in $BGSE_{E60}$ were centrifuged as previously
243 described. The cell pellet was resuspended in 1 ml of 0.85% NaCl solution, mixed and
244 centrifuged under the same conditions. The washed cells were fixed with glutaraldehyde,
245 coated with gold and viewed under scanning electron microscope (SEM) (Quanta400, FEI,
246 Czech Republic) and transmission electron microscope (TEM) (JEM-2010, JEOL, Tokyo,
247 Japan).

248

249 **2.10 Cytotoxicity determination**

250 Cytotoxicity of $BGSE_{E60}$ against primate cell line (Vero) was investigated by green fluorescent
251 protein (GFP) detection method[24]. Ellipticine was used as the positive control, while
252 0.5%DMSO was used as negative control. Test concentration was 50 µg/ml. The GFP-
253 expressing Vero cell line was generated in-house by stably transfecting the African green
254 monkey kidney cell line (Vero, ATCC CCL-81), with pEGFP-N1 plasmid (Clontech). The cell
255 line was maintained in minimal essential medium supplemented with 10% heat-inactivated
256 fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate
257 and 0.8 mg/ml geneticin, at 37°C in a humidified incubator with 5% CO_2. The assay was
258 carried out by adding 45 µl of cell suspension ($3.3x10^4$ cells/ml) to each well of 384-well
259 plates containing 5 µl of test compounds previously diluted in 0.5% DMSO, and then
260 incubated for 4 days in an incubator at 37°C with 5% CO_2. Fluorescence signals were
261 measured by SpectraMax M5 microplate reader (Molecular Devices, USA) in the bottom

reading mode with excitation and emission wavelengths of 485 and 535 nm. Fluorescence signal at day 4 was subtracted with background fluorescence at day 0. The percentage of cytotoxicity was calculated by the following equation, where FUT and FUC represent the fluorescence units of cells treated with test compound and untreated cells, respectively:

$$\% \text{ cytotoxicity} = [1-(FUT / FUC)] \times 100$$

2.11 Statistical analysis

One-way ANOVA was used and mean comparison was performed by Duncan's multiple range test.[25] Statistical analysis was carried out using SPSS statistic program (Version 11.0) for Window (SPSS Inc. Chicago, IL).

3 Results and discussion

3.1 Chemical compositions of Bambarra groundnut seed coat

Bambarra groundnut seed coat contained 6.14% moisture, 11.56% protein, 1.16% fat, 4.68% ash, 58.26% carbohydrate and 24.34% fiber (dry basis). Thus, carbohydrate was the main constituent, followed by fiber in seed coat. Chemical compositions of little millet seed coat have been reported to be 6.26% protein, 20.51% ash, 2.04% fat and 13.08% fiber.[26]

3.2 Effect of extracting solvents and temperatures on extraction yields, total phenolic contents and antioxidative activities of BGSEs

Extraction yields, total phenolic contents and antioxidative activities of BGSEs prepared using different extracting solvents including water (W), ethanol (E) and acetone (A) at various temperatures (30, 60, 90°C) ($BGSE_{W30}$, $BGSE_{W60}$, $BGSE_{W90}$, $BGSE_{E30}$, $BGSE_{E60}$, $BGSE_{E90}$, $BGSE_{A30}$ $BGSE_{A60}$, $BGSE_{A90}$) are depicted in Table 1.

3.2.1 Extraction yields

Extraction yields of BGSEs varied from 5.21 to 13.23%. $BGSE_A$ and $BGSE_E$ showed the highest yield at 30 and 60°C, respectively, while $BGSE_W$ exhibited the highest yield at 90°C ($p<0.05$). $BGSE_A$ and $BGSE_E$ had the lowest yield at 90°C ($p<0.05$). The boiling point at atmospheric pressure of water, ethanol and acetone is 100, 79 and 56°C, respectively.[27] The temperature close to boiling point could facilitate the extraction of phenolic or bioactive compounds from Bambarra groundnut seed coat due to the turbulence of solvent, which could contact target compounds more effectively. Conversely, at the temperature above boiling point of the solvents, the extraction yields were quite low, possibly caused by the evaporation of the solvents to some extent.

The high extraction yield at high temperatures of water could be due to the fact that higher temperatures increased solubility and mass transfer rate as well as decreased the viscosity and surface tension of the solvent, thus enabling the solvent to reach the sample matrix, and consequently improving the extraction rate.[28] The result suggested that suitable temperature for individual solvent could enhance extraction yield.

The extraction yields of ethanolic extracts from cashew nut[3] and dry *Anacardium excelsum*[29] seed coats have been reported to be 45% and 44.6%, respectively. The extraction yield of dry *Anacardium excelsum* seed coats was varied, depending on solvents polarities.[29] Type of solvent and suitable duration of the extraction played an important role on extraction yield of basil and oregano.[30] Additionally, the extraction yield of algae depended on the type of solvents with varying polarities, pH, extraction time and temperature and the chemical compositions of samples.[6]

3.2.2 Total phenolic contents

Total phenolic contents of BGSEs were different, depending on extracting solvents and temperatures ($p<0.05$). As the extracting temperatures increased, total phenolic contents in all samples generally increased ($p<0.05$). $BGSE_{E90}$ and $BGSE_{W90}$ showed higher total phenolic content than did $BGSE_{W60}$ and $BGSE_{W30}$, respectively ($p<0.05$). At the same extracting temperature, $BGSE_A$ exhibited the highest total phenolic content. $BGSE_{E90}$ showed higher total phenolic content than did $BGSE_{A90}$ ($p<0.05$). The high temperature could favor the extraction of phenolic compounds from Bambarra groundnut seed coat. Thus, types of extracting solvents and temperatures influenced total phenolic content of BGSEs.

Total phenolic contents of the extracts from seed coats of cashew nut,[3] lentil[4] and little millet[26] were 243, 53.14 and 2.12 mg/g dry extract, respectively. Recoveries of phenolic compounds from plants are mainly dependent upon the type of solvent used,[1,6] solvent polarity[6] and method of extraction.[1] Ethanol and water extracts of spices and herbs had higher total phenolic contents than hexane extract.[31] However, no correlation was found between extraction yield and total phenolic content.[6]

BGSEs with higher amounts of phenolic compounds possessed higher antioxidative activities. Correlations were also established between phenolic contents and antioxidant properties in the extracts from soybean seed coat,[8] nuts including almonds, Brazil nuts, cashews, hazelnuts, macadamia nuts, pecans, pine nuts, pistachios, walnuts, peanuts,[9] basil, oregano,[30] *Pongamia pinnata* seeds[1] and alga.[6]

3.2.3 Antioxidative activities

Different antioxidative activities of BGSEs were observed, depending on extracting temperatures and solvents ($p<0.05$) (Table 1). There was the correlation between the phenolic contents and antioxidant properties.

3.2.3.1 DPPH radical scavenging activities

$BGSE_{A60}$ showed the strongest DPPH radical scavenging activity, when compared with others ($p<0.05$). At the same extracting temperature, $BGSE_A$ generally exhibited the highest DPPH radical scavenging activity, following by $BGSE_E$ and $BGSE_W$, respectively ($p<0.05$). At 90°C, $BGSE_{E90}$ showed higher activity than did $BGSE_{A90}$ ($p<0.05$). These activities were related to total phenolic contents in BGSEs. As the extracting temperature increased, activity of $BGSE_E$ increased ($p<0.05$). Nevertheless, $BGSE_A$ revealed the highest activity at 60°C that was related to its phenolic content. However, no difference was observed in $BGSE_W$, when different temperatures were used for extraction ($p>0.05$). *Moringa oleifera* seeds coat extract using water showed maximum activity at 70ºC, among extraction temperature ranging from 30-100ºC.[28]

Different DPPH radical scavenging activities in various extracts were more likely due to various compounds, which could donate H-atom to DPPH differently. The interaction of phenolic compounds with DPPH depended on their structural conformation, including the number of available hydroxyl groups and structural features such as O-H bound dissociation energy, resonance delocalization of the antioxidant and steric-hindrance derived from bulky groups substituting hydrogen in the antioxidant compound.[1] DPPH radical scavenging activities of BGSEs therefore confirmed their hydrogen donating capacity to radical to become stable molecule.[13] Basil extracts were also able to scavenge DPPH radical.[30]

Efficiency of the active constituents of phenolic compound depends on the method of extraction.[28] Changes on solvent polarity alter its ability to dissolve a selected group of phenolics and antioxidant compounds. Each extract might have different classes of phenolic compounds which have varying antioxidant potentials. However, solvent polarity does not change drastically the total amounts of phenolic groups, but the phenolic profiles.[6] The

358 extracting solvent significantly affected total phenolic contents and antioxidant activities of
359 several extracts from algae (*Stypocaulon scoparium*).[6]

360 **3.2.3.2 ABTS radical scavenging activity**

361 $BGSE_{A60}$ also exhibited the highest ABTS radical scavenging activity among all
362 BGSEs. At the same temperature, $BGSE_A$ showed the highest activity, followed by $BGSE_E$
363 and $BGSE_W$, respectively ($p<0.05$). At 90°C, $BGSE_{E90}$ had the strongest activity ($p<0.05$). As
364 the extracting temperature increased, activities of $BGSE_E$ and $BGSE_W$ generally increased
365 ($p<0.05$). Nevertheless, $BGSE_{A60}$ exhibited the strongest ABTS radical scavenging activity
366 among all BGSEs. ABTS radical scavenging activities of BGSEs were also related to their
367 total phenolic contents ($p<0.05$).

368 $BGSE_{E60}$ and $BGSE_{A60}$ possessed the strong scavenging activity against ABTS
369 radical that might be due to their ability to donate electron to ABTS radical. The stable
370 nitrogen-centered free radical $ABTS^{\bullet+}$ is frequently used for the estimation of free radical
371 scavenging ability of active compounds by quenching and discoloring $ABTS^{\bullet+}$ synthetic free
372 radical.[14]

373 **3.2.3.3 Hydroxyl radical scavenging activity**

374 No monitored difference was observed in the extracts as influenced by extracting
375 solvent and temperature. At 60°C, it was noted that $BGSE_{A60}$ showed higher activity than did
376 $BGSE_{W60}$ ($p<0.05$). Additionally, $BGSE_{W90}$ exhibited stronger activity than did $BGSE_{W30}$ and
377 $BGSE_{W60}$ ($p<0.05$).

378 Hydroxyl radical scavenging ability was evaluated as the inhibition rate of 2-
379 deoxyribose oxidation by hydroxyl radicals, the most reactive form of activated oxygen
380 formed by the Fenton reaction.[15] Due to its extreme reactivity, hydroxyl radicals react

immediately with biomolecules and can cause oxidative damage to DNA, phospholipids, lipids and proteins[30] and degrade to fragments (malondialdehyde) which generates a pink chromogen on heating with TBA at low pH.[15]

3.2.3.4 Superoxide anion radical scavenging activity

BGSEs showed strong superoxide anion radical scavenging activity compared with Trolox. No difference in activity was observed when extracting temperature of 30 and 60°C were used. $BGSE_{E90}$ and $BGSE_{A90}$ showed lower activity than did $BGSE_{W90}$ ($p<0.05$), while $BGSE_E$ and $BGSE_A$ exhibited the strongest activity at 60°C ($p<0.05$).

Superoxide radicals, a biologically oxygen molecule with one unpaired electron, is a weak oxidant. However, it gives rise to the generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen. Both of which contribute to the oxidative stress.[1] In the reaction with H_2O_2, superoxide anion radical produces hydroxyl ion (Fenton reaction), while the reaction with nitrogen (I) oxide formed peroxynitrite anion ($ONOO^-$) which may have greater toxicity.[30]

3.2.3.5 Ferric reducing antioxidant power

FRAP assay of BGSEs was estimated from their ability to reduce Fe^{3+}-TPTZ complex to the ferrous form (Fe^{2+}) and reflect antioxidant power involving single electron transfer reaction.[18] As the extracting temperature increased, FRAP of $BGSE_W$ and $BGSE_E$ increased. Among all extracts, $BGSE_{A60}$ showed the highest FRAP ($p<0.05$). At the same extracting temperature, $BGSE_A$ generally showed the stronger FRAP than did $BGSE_E$ and $BGSE_W$ ($p<0.05$).

3.2.3.6 Metal chelating activity

As the extracting temperature increased, metal chelating activity of $BGSE_W$ decreased ($p<0.05$). Additionally, $BGSE_E$ and $BGSE_A$ showed the highest activity, when

405 extracted at 30°C ($p<0.05$). At the same extracting temperature, $BGSE_W$ exhibited the
406 strongest activity, following by $BGSE_E$ and $BGSE_A$, respectively ($p<0.05$). Phenolic
407 compounds containing in BGSEs were capable of complexing with transition metal ions. As a
408 result, those ions could not participate in metal-catalyzed initiation and hydroperoxide
409 decomposition reactions.[19] However, BGSEs had relatively lower metal chelation than did
410 EDTA. Cashew nut seed coat extract also showed lower Fe^{2+} chelating than did EDTA.[3]

411 $BGSE_{E60}$ was selected for further study, since it showed potent antioxidative
412 activities. Additionally, it was extracted with edible and safe solvent.

413

414 **3.3 Thermal and pH stabilities of antioxidative activities of $BGSE_{E60}$**

415 **3.3.1 Thermal stability**

416 Antioxidative activities of $BGSE_{E60}$ still remained after heating from 20°C up to 80°C
417 for 30 min and slightly increased when heated at 100°C ($p<0.05$) (Fig. 1a). DPPH, ABTS
418 radical scavenging and metal chelating activities slightly decreased as the temperature
419 increased and then slightly increased at 100°C ($p<0.05$). However, FRAP slightly increased as
420 the temperature increased ($p<0.05$). Phenolic like compounds possessing antioxidative
421 activities could be generated while heating.[7] Phenolic compound was more likely stable to
422 heat. Additionally, phenolic like compounds were generated, when peanut seed coat was
423 roasted.[7] However, chlorogenic acid was partly lost during heating.[32]

424 Thus, $BGSE_{E60}$ could be used as natural antioxidants in thermal processed foods, since
425 antioxidative activities still remain after subjected to heating.

426

427

3.3.2 pH stability

Antioxidative activities of $BGSE_{E60}$ were stable over a pH range of 4-8 (Fig. 1b). At the very acidic (pH 2) and alkaline pH (pH 10), metal chelating activity of $BGSE_{E60}$ tended to increase, while ABTS radical scavenging activity decreased ($p<0.05$). DPPH radical scavenging activity of $BGSE_{E60}$ increased at pH 2 and deceased at pH 10. FRAP was still stable at the pH ranging from 2 to 8, but decreased drastically at the pH of 10 ($p<0.05$). The loss in antioxidative activities of $BGSE_{E60}$ at very acidic and alkaline pHs might be due to the degradation of active compounds responsible for ABTS radical scavenging activity and FRAP to some extent. Generally, phenolic compounds in $BGSE_{E60}$ were not dramatically affected by charge modification designated by pH changes. Certain phenolic compounds were rarely influenced by pH, whereas some compounds were affected considerably by pH.[33]

The results suggested that phenolic compounds in $BGSE_{E60}$ responsible for ABTS radical scavenging activity and FRAP might undergo the conformation changes at very alkaline and acidic pHs, leading to the loss in their ability in scavenging ABTS radical and reducing ferric ion. Thus, it was likely that compounds possessing different antioxidative activities might be different in term of composition. Some compounds with ABTS radical scavenging activity and FRAP might undergo the changes caused by repulsion at very acidic or alkaline pHs. Thus, $BGSE_{E60}$ could be supplemented in foods with the wide pH ranges (4-8), in which $BGSE_{E60}$ still functioned as natural antioxidant. The polyphenols present in the extracts isolated from cocoa, green tea and strawberries were varied depending on pHs. The flavan-3-ols of cocoa were more stable at pH 6.5 than 7.4. Green tea predominant catechins were very vulnerable at pH 6.5 and 7.4.[33] Phenolic components of olive oil show high antioxidant capacity in the pH range of 3.5–7.4.[34] Heat, pH and storage time diminished

451 phenolic compounds and the stability of phenolic compounds strongly depended not only on
452 the structure of the phenolic compound and storage time but also on the pH of the systems.[32]
453
454 **3.4 Antioxidative activities of $BGSE_{E60}$ in lecithin liposome system and steamed chicken**
455 **mince**
456 **3.4.1 Lecithin liposome system**
457 Liposome system is used as substrate for examining the lipid peroxidation, since the
458 highly unsaturated fatty acids membrane lipids are particularly susceptible to oxidation by free
459 radicals.[30] $BGSE_{E60}$ at different concentrations were able to delay the formation of conjugated
460 diene (Fig. 2a) and TBARS (Fig. 2b) in lecithin liposome system when compared with control
461 ($p<0.05$). However, at the same level (6 mg/l), Trolox showed stronger antioxidative activity
462 than did α-tocopherol and $BGSE_{E60}$ ($p<0.05$). Based on TBARS, the systems containing
463 $BGSE_{E60}$, α-tocopherol and Trolox possessed the longer induction period than did control. The
464 longer induction time expressed the oxidative stability of lecithin liposome system.[20,21]
465 As indicated by conjugated diene and TBARS, $BGSE_{E60}$ at the concentration of 60
466 mg/l showed the stronger antioxidative activity than the lower concentrations (30 and 6
467 mg/ml) ($p<0.05$). The amount of conjugated diene in all samples significantly increased at the
468 early stage ($p<0.05$). The decrease of conjugated diene in the control was found after 12 h.
469 $BGSE_{E60}$ could retard the early stage of lipid oxidation that conjugated diene or hydroperoxide
470 was formed and also inhibited the propagation of the oxidation process, in which
471 hydroperoxide was degraded to TBARS, the secondary products.[21]
472 Generally, α-tocopherol, a lipid soluble chain-breaking antioxidant, could inhibit
473 oxidation in phospholipid bilayer of liposome by retarding free radical-mediated lipid

peroxidation.[30] However, Trolox was more effective in liposome system than did α-tocopherol in the present study. According to the polar paradox, less polar antioxidants (α-tocopherol) are concentrated at oil-water interfaces, while more polar antioxidants (Trolox) are mainly present in the aqueous phase. The presence of the aqueous phase often decreases the activity of non-polar antioxidants because hydrogen-bonded complexes formed with water are ineffective in scavenging radicals by hydrogen donation.[30] Lipids in emulsions exist as lipid dispersions in an aqueous matrix that may contain a variety of water-soluble components including transition metals used to initiate lipid oxidation.[34] Additionally, α-tocopherol with lower polarity might be able to access to the reaction sites and engage in the process of defense from the lipid peroxidation to a lower extent, compared to more polar Trolox. According to ethanolic extraction, $BGSE_{E60}$ contained polar and non-polar phenolics (data not shown) and could be dissolved both in polar and non-polar substance. Thus, phenols in $BGSE_{E60}$ may also chelate transition metal ions and reduce metal-induced oxidative reactions. The polarity of phenolic components affected their ability to inhibit lipid peroxidation in liposome system.[30] Spice extracts possessed high total phenolic contents and antioxidative activity by showing strongly inhibitory of TBARS formation in liposome system.[35] Five extracts from Et_2O, $CHCl_3$, EtOAc, *n*-BuOH, and H_2O of basil and oregano exhibited protective effects against lipid peroxidation in liposomes.[30] Aqueous leaf extract from *Aloe vera* exhibited inhibitory capacity against Fe^{3+}/ascorbic acid induced phosphatidylcholine liposome oxidation and its antioxidant activity was related to the presence of phenolic compounds.[36] Phenolic constituents of lentil seed coats can counteract lipid peroxidation and can be utilized as potent antioxidants.[4]

497 **3.4.2 Steamed chicken mince**

498 $BGSE_{E60}$ exhibited antioxidative activity in steamed chicken mince as evidenced by
499 the retarded formation of PV (Fig. 3a) and TBARS (Fig. 3b) ($p<0.05$). As the storage time
500 increased, PV and TBARS in all samples increased ($p<0.05$). However, the steamed chicken
501 mince added with $BGSE_{E60}$ at different concentrations and α-tocopherol showed the slower
502 increase in PV and TBARS than did the control throughout the storage time ($p<0.05$).
503 Additionally, Trolox possessed the strongest antioxidative activity ($p<0.05$). $BGSE_{E60}$ also
504 showed antioxidative activity in a concentration dependent manner in steamed chicken mince.
505 Thermal stability of $BGSE_{E60}$ was also confirmed by this result. Cooked pork patties
506 containing clove, rosemary, and cassia bark extracts also had markedly reduced TBARS
507 formation and off-flavour scores during storage. Additionally, the extract identified to have the
508 greatest total phenolic contents, were strongly inhibitory of TBARS formation.[35]

509

510 **3.5 Antimicrobial activities of $BGSE_{E60}$**

511 **3.5.1 Inhibition zone measurements**

512 $BGSE_{E60}$ at different concentrations showed antimicrobial activities in dose-dependent
513 manner (Table 2). As the concentrations of $BGSE_{E60}$ increased, the stronger antimicrobial
514 activities against microorganisms were observed ($p<0.05$) as indicated by the wider clear
515 zones. When compared with penicillin at the same concentration (2.5 mg/ml), no difference
516 was observed for *S. aureus*. However, penicillin exhibited stronger antibacterial activities
517 against *E. coli and B. cereus* than did $BGSE_{E60}$ ($p<0.05$). Additionally, $BGSE_{E60}$ expressed
518 antifungal activity against *C. albicans* and *A. niger* only at the high dose. *C. albicans* was
519 susceptible to amphotericin B as evidenced by the wide clear zone. However, no antifungal

activity of amphotericin B against *A. niger* was observed ($p<0.05$). Although fungi such as yeast and mold are heterotrophic organisms with eukaryotic cell,[37] the cell structure and shape of yeast and mold are different. Therefore, the adsorption effect and inhibition activity of amphotericin B against *C. albicans* and *A. niger* might be different. *A. niger* was resistant to amphotericin B. Drug resistance of fungi appears to be due to the restricted penetration of drugs inside the exopolymeric matrix, which can bind or restrict the diffusion of the antifungals.[38] The extract from seed coat of *Moringa oleifera,* medicinal species, exhibited antimicrobial activity against human pathogens,[28] while the extract from cashew nut seed coat showed antifungal activity.[10]

The antimicrobial activity of $BGSE_{E60}$ correlated well with the concentration of the extract. Higher phenolic content was plausibly responsible for higher antimicrobial activity of $BGSE_{E60}$. The antimicrobial activities of the extracts are primarily attributable to their phenolic compounds.[29,31,39,40] The relationships between phenolic compound levels and antimicrobial activities were also reported in the extracts from *Anacardium excelsum* seed and seed coat,[29] spice and herb,[31] *Ginkgo biloba*[39] and cashew nut seed coat.[10] Antimicrobial activities were also varied depending on the kind of phenols,[29] type[31] and polarity of extracting solvents,[29,10] type of the extracts[40] and type of microorganisms.[10]

$BGSE_{E60}$ was more effective in inhibition of gram positive cocci (*S. aureus*) and bacilli (*B. cereus*) than gram negative bacteria (*E. coli*) tested. Similar trend was observed for free and bound phenolic extracts of *Moringa oleifera* seed,[40] ethanolic extract from cashew nut[29] and spice and herb extracts.[31] Gram positive bacteria were more sensitive to natural antimicrobials than gram negative organisms.[41] This might be due to the differences in cell structures and the complexity of gram negative cell wall. The outer peptidoglycan layer of gram positive bacteria is an ineffective permeability barrier, whereas the porins present in

544 gram negative bacteria restrict entry of solutes and make them resistant to antibacterial
545 compounds.[41] Gram-negative bacteria also possess an outer membrane surrounding cell wall,
546 which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering.[37]
547 $BGSE_{E60}$ could be an efficient antimicrobial agent acting on both prokaryotic (*S.*
548 *aureus, E. coli, B. cereus*) and eukaryotic cells (*C. albicans, A. niger*). Typically, antimicrobial
549 agent can penetrate through cytoplasmic membrane,[31] change permeability and destroy
550 cytoplasmic membrane, leading to leakage of cytoplasm and/or cytoplasm coagulation and
551 deformed the shape accompanied by cell lysis. Those changes resulted in the death of
552 microorganisms.[42] Antimicrobial agent can also inhibit mycelial growth and spore germination
553 of fungi.[10] The hydrophilicity and hydrophobicity of $BGSE_{E60}$ possibly were important
554 characteristics which enabled them to accumulate in cell membranes, thus disturbing the
555 structures and causing change of permeability. Thereafter, the leakage of intracellular
556 constituents, impairment of microbial enzyme systems and the death of cell could occur.[42]
557 Therefore, antimicrobial activities of $BGSE_{E60}$ were varied depending on the concentration of
558 the extract and types of microorganisms tested.

559 **3.5.2 Scanning and transmission electron microscopic studies**

560 After 12 h of exposure to the $BGSE_{E60}$, the remarkable changes in morphology and
561 ultrastructure of microbial cells visualized from SEM (Fig. 4) and TEM (Fig. 5) were found,
562 suggesting the extensive cell damage. Shrinkage (Fig. 4a, j, n), deformation (Fig. 4a, e, h, k,
563 m) and rupture (Fig. 4c, h, i) of microbial cells were observed from SEM photomicrographs.
564 The cells showed the notable alterations in cell membrane and cell wall (Fig. 4a, i, n) and the
565 size of cell decreased (Fig. 4a, f, h). The cells were damaged and hollow with leaking cell
566 contents (Fig. 4a d, g, h, k, m) through the pores (Fig. 4b, f, i, m), resulting in cell death. The
567 cell surfaces were coarse (Fig. 4c, k, l), and the cell walls were markedly damaged (Fig. 4i, o).

Additionally, *A. niger* mycelium was damaged (Fig. 4m, n) and spore was collapsed (Fig. 4o). TEM internal slice images of microbial cells revealed the irregular shapes (Fig. 5a, d, g, j, m), broken cell wall and infiltrated cell contents (Fig. 5b, c, e, f, h, i, k, n, o). The cytoplasmic volume decreased and the cell membrane invaginated with notable structural disorganization within the cell cytoplasm (Fig. 5i, l). Additionally, some interface between the cell wall and cell membrane disappeared (Fig. 5b, e, h, l, n). That might be due to the lysis or separation of cell wall and membrane.[43] Previous findings from SEM and TEM studies also suggested that potential bioactive compound of the extract from *Cassia spectabilis*[44] and lemon grass essential oils[38] had distinct influence on microbial growth and structural development.

Both SEM and TEM analyses suggested that the potential mechanism underlying the inhibitory effect of $BGSE_{E60}$ could mainly involve the damages of cell surface, cell wall, cell membrane and cytoplasm. $BGSE_{E60}$ might interact with the cell surface, change the microbial cell wall and membrane permeability, thereby restraining cell growth.[45]

$BGSE_{E60}$ components might have the capability to alter cell permeability by entering between the fatty acyl chains making up membrane lipid bilayers and disrupt the lipid packing. Since the cytoplasmic membrane serves a vital role in cell wall synthesis and turnover, perturbing it may affect cell wall integrity and autolysin regulation.[43] $BGSE_{E60}$ might also affect the regulation and function of the membrane bound enzymes, thus altering the synthesis of many cell wall polysaccharide components and affecting the cell growth and morphogenesis.[38]

3.6 Cytotoxicity of $BGSE_{E60}$ against primate cell line (Vero)

No cytotoxicity of $BGSE_{E60}$ against primate cell line (Vero) was observed from green fluorescent protein (GFP) detection assay (Table 2). Many extracts from plants possessed

antimicrobial activities. However, some of them were found to exhibit cytotoxicity. The extract from *Balanites aegyptiaca*, a medicinal plant, showed high hemolytic activity on both human and rabbit red blood cells and acute toxicity. [46] Thus, $BGSE_{E60}$ could be applied as a potential antimicrobial agent without cytotoxicity.

4 Conclusions

Extraction yields, total phenolic contents and antioxidative activities of BGSEs were governed by extracting solvents and temperatures used. Antioxidative activities of $BGSE_{E60}$ were stable in a wide pH range and heat treatment. $BGSE_{E60}$ could retard lipid oxidation in lecithin liposome system and steamed chicken mince. $BGSE_{E60}$ also possessed antimicrobial activities against bacteria, yeast and mold without cytotoxicity. Therefore, BGSEs could be used as natural antioxidant and antimicrobial agent.

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Table 1 Extraction yields, phenolic contents and antioxidative activities of Bambarra groundnut seed coat extracts with different extracting temperatures and solvents

BGSE	Extraction yield (%)	Phenolic content (mgGAE/ g dry extract)	Antioxidative activity					
			Radical scavenging activity (g Trolox Equivalent/g dry extract)					Metal Chelating (g EDTA Equivalent/g dry extract)
			DPPH	ABTS	Hydroxyl	Superoxide anion	FRAP	
$BGSE_{W30}$*	5.21 ± 0.02^{cC}	169.23 ± 3.44^{cC}	0.56 ± 0.06^{cA}	0.30 ± 0.02^{cC}	0.56 ± 0.03^{aB}	2.98 ± 0.27^{aA}	0.24 ± 0.02^{cC}	0.89 ± 0.07^{aA}
$BGSE_{E30}$	10.58 ± 0.01^{bB}	411.61 ± 0.39^{bC}	0.65 ± 0.05^{bB}	0.90 ± 0.02^{bB}	0.54 ± 0.05^{aA}	2.56 ± 0.10^{aB}	0.55 ± 0.02^{bB}	0.30 ± 0.02^{bA}
$BGSE_{A30}$	13.23 ± 0.01^{aA}	521.83 ± 17.51^{aB}	0.80 ± 0.01^{aB}	1.03 ± 0.05^{aB}	0.57 ± 0.05^{aA}	2.44 ± 0.81^{aAB}	0.62 ± 0.03^{aB}	0.23 ± 0.01^{bA}
$BGSE_{W60}$	6.20 ± 0.01^{cB}	186.40 ± 3.27^{cB}	0.55 ± 0.01^{cA}	0.53 ± 0.02^{cB}	0.46 ± 0.05^{bC}	2.62 ± 0.55^{aA}	0.30 ± 0.02^{cB}	0.57 ± 0.01^{aB}
$BGSE_{E60}$	12.01 ± 0.06^{aA}	483.97 ± 4.54^{bB}	0.94 ± 0.02^{bA}	1.25 ± 0.02^{bA}	0.51 ± 0.07^{abA}	3.21 ± 0.18^{aA}	0.68 ± 0.02^{bA}	0.22 ± 0.01^{bC}
$BGSE_{A60}$	10.53 ± 0.03^{bB}	569.15 ± 13.82^{aA}	1.22 ± 0.08^{aA}	1.56 ± 0.07^{aA}	0.60 ± 0.05^{aA}	3.04 ± 0.31^{aA}	0.86 ± 0.03^{aA}	0.19 ± 0.02^{cC}
$BGSE_{W90}$	11.23 ± 0.07^{aA}	314.92 ± 2.02^{cA}	0.57 ± 0.06^{cA}	0.71 ± 0.03^{cA}	0.65 ± 0.05^{aA}	2.68 ± 0.47^{aA}	0.43 ± 0.04^{bA}	0.29 ± 0.02^{aC}
$BGSE_{E90}$	6.16 ± 0.11^{cC}	567.18 ± 16.61^{aA}	0.98 ± 0.03^{aA}	1.23 ± 0.03^{aA}	0.62 ± 0.05^{aA}	1.67 ± 0.27^{bC}	0.66 ± 0.06^{aA}	0.27 ± 0.00^{bB}
$BGSE_{A90}$	7.32 ± 0.06^{bC}	544.81 ± 7.45^{bAB}	0.80 ± 0.06^{bB}	1.09 ± 0.02^{bB}	0.65 ± 0.03^{aA}	1.49 ± 0.27^{bB}	0.64 ± 0.02^{aB}	0.21 ± 0.01^{cB}

Mean±SD from triplicate determinations.

^{abc} Different lowercase superscripts in the same column within the same temperature indicate significant difference between solvents ($P<0.05$)

^{ABC} Different uppercase superscripts in the same column within the same solvent indicate significant difference between temperatures ($P<0.05$)

*BGSE: Bambarra groundnut seed coat extract; W: water, E: ethanol, A: acetone; 30:30ºC, 60:60ºC, 90:90ºC

Table 2 Antimicrobial activities against microorganisms and cytotoxicity against primate cell line (Vero) of Bambarra groundnut seed coat extract ($BGSE_{E60}$)[$]

Antimicrobial activities against microorganisms	Inhibition zone (mm)				
	Concentration of $BGSE_{E60}$ (mg /ml)				Penicillin/Amphotericin B
	2.5	5	10	30	2.5 mg/ml
S.aureus	6.00±0.00^{bA}	6.12±0.08^{b}	6.12±0.02^{b}	6.23±0.02^{a}	6.07±0.03^{A}
E.coli	5.83±0.03^{cB}	5.97±0.02^{b}	6.00±0.00^{b}	6.12±0.02^{a}	6.00±0.00^{A}
B.cereus	5.97±0.02^{dB}	6.12±0.02^{c}	6.20±0.00^{b}	6.25±0.00^{a}	6.03±0.02^{A}
C. albicans	-	-	5.90±0.00^{b}	5.95±0.00^{a}	16.67±1.53
A. niger	-	-	5.81±0.02^{b}	5.90±0.00^{a}	-
Cytotoxicity against primate cell line (Vero)					non-cytotoxic

[$]The extract was prepared using ethanol at 60 ºC for 1 h

*Mean±SD from triplicate determinations.

$^{a-d}$ Different lowercase superscripts in the same row indicate significant difference ($P<0.05$)

^{AB} Different uppercase superscripts in the same row indicate significant difference between $BGSE_{E60}$ and penicillin at the same concentration ($P<0.05$)

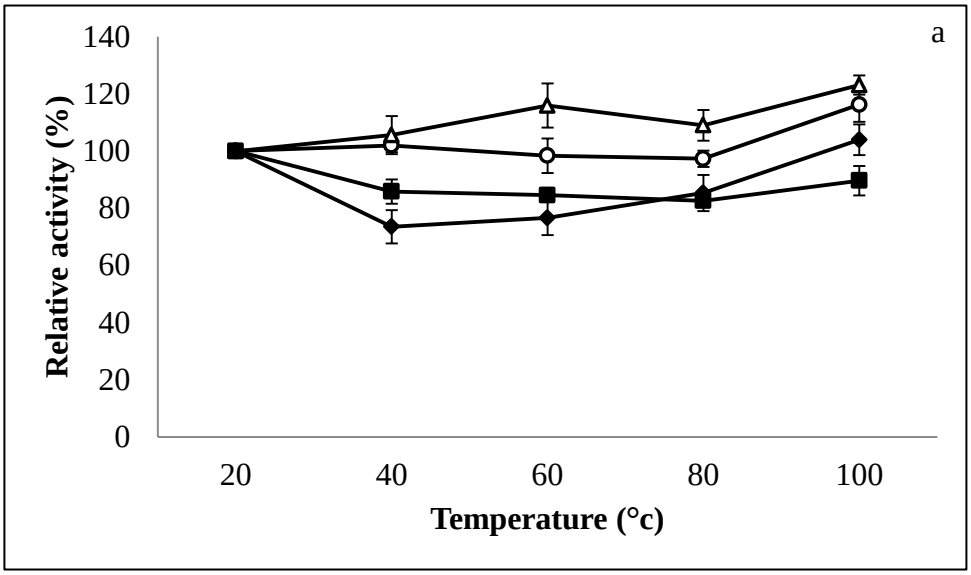
Figure 1 Thermal (a) and pH stabilities (b) of $BGSE_{E60}$ as measured by DPPH (○) and ABTS radical scavenging activities (■), FRAP (∆) and metal chelating activity (♦). Bars represent standard deviation (n=3).

Figure 2 The formation of conjugated diene (a) and TBARS (b) in lecithin liposome systems containing $BGSE_{E60}$ at the concentrations of 6 (●), 30 (■) and 60 mg/l (▲), compared with Trolox (∆), α-tocopherol (□) at the concentration of 6 mg/l and the control (○). Bars represent standard deviation (n=3).

Figure 3 The formation of PV (a) and TBARS (b) in steamed chicken mince containing $BGSE_{E60}$ at the concentrations of 6 (●), 30 (■) and 60 mg/kg (▲), compared with Trolox (∆), α-tocopherol (□) at the concentration of 6 mg/kg and the control (○). Bars represent standard deviation (n=3).

Figure 4 Scanning electron microscopic photomicrographs of *S. aureus* (a-c), *E. coli* (d-f), *B. cereus* (g-i), *C. albicans* (j-l) and *A. niger* (m-o) treated with $BGSE_{E60}$.

Figure 5 Transmission electron microscopic photomicrographs of *S. aureus* (a-c), *E. coli* (d-f), *B. cereus* (g-i), *C. albicans* (j-l) and *A. niger* (m-o) treated with $BGSE_{E60}$.



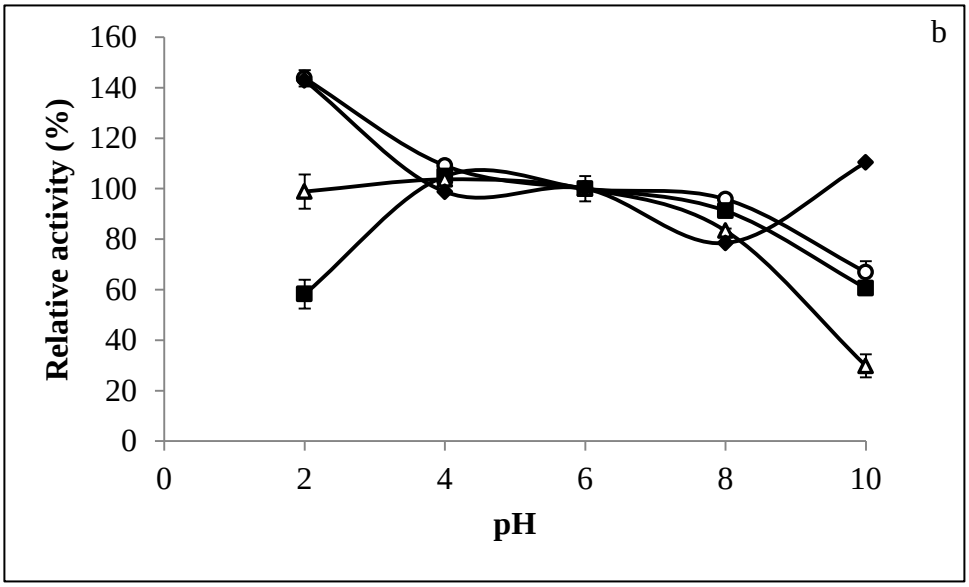


Fig. 1

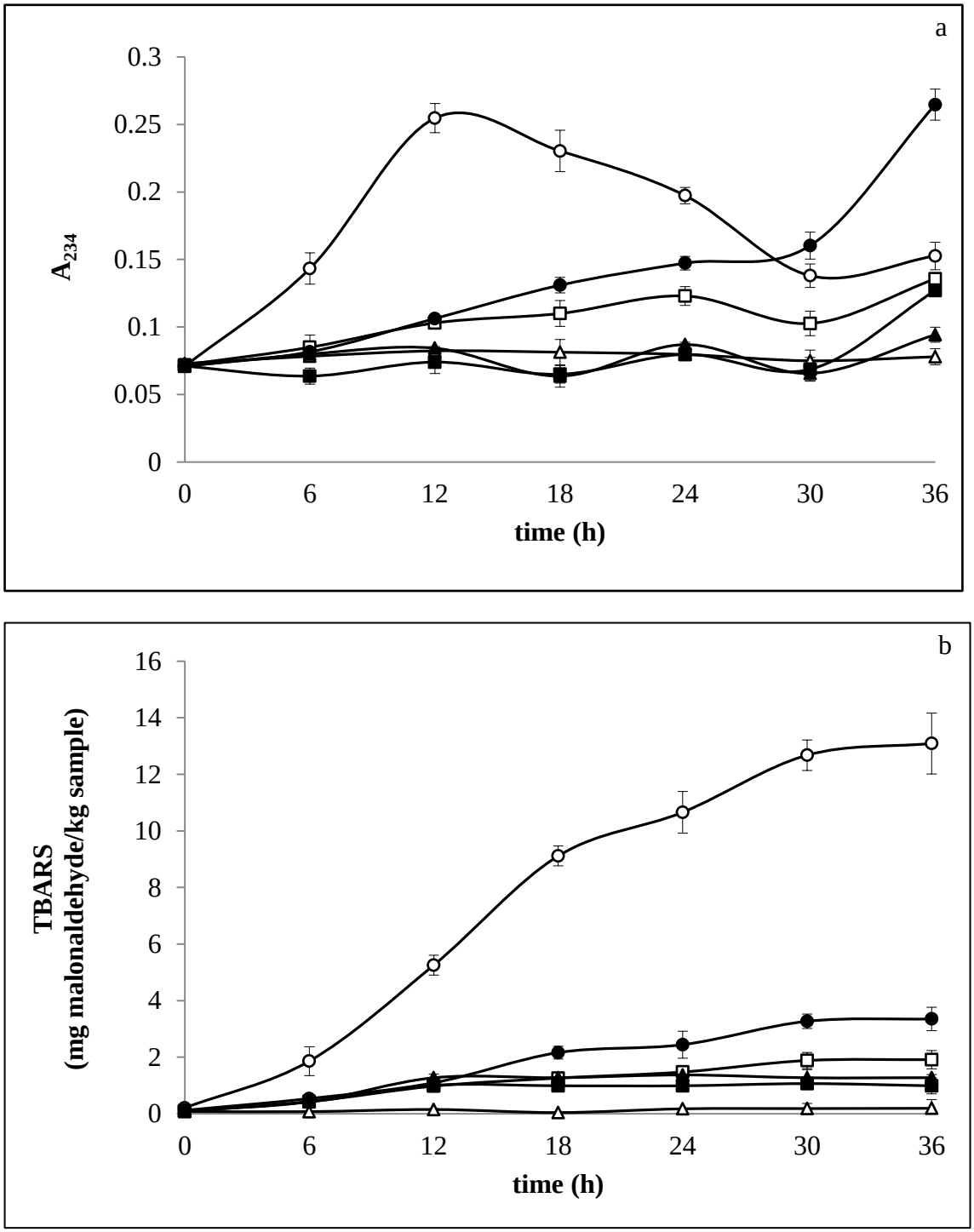
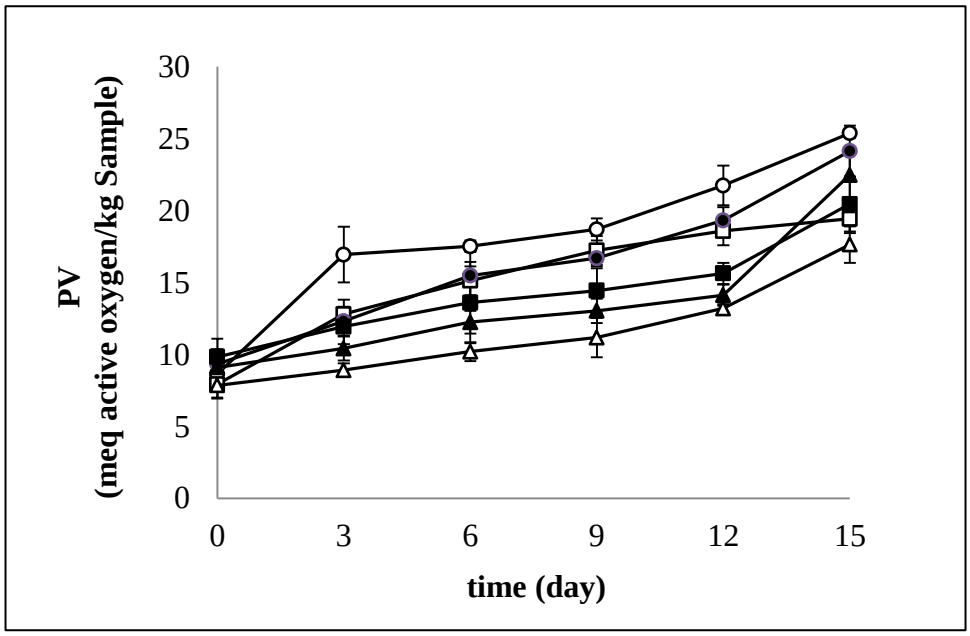


Fig 2



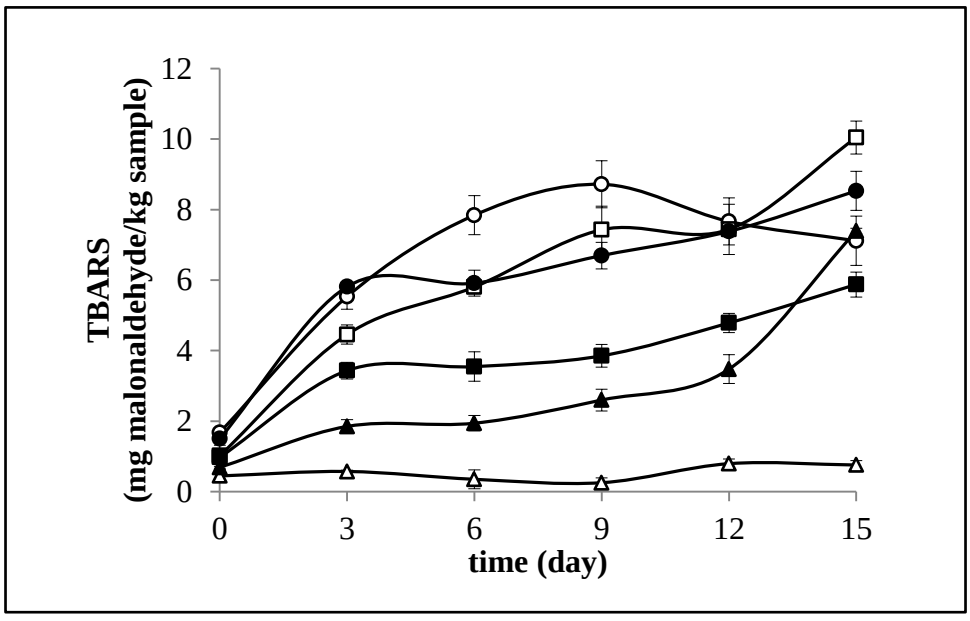


Fig. 3

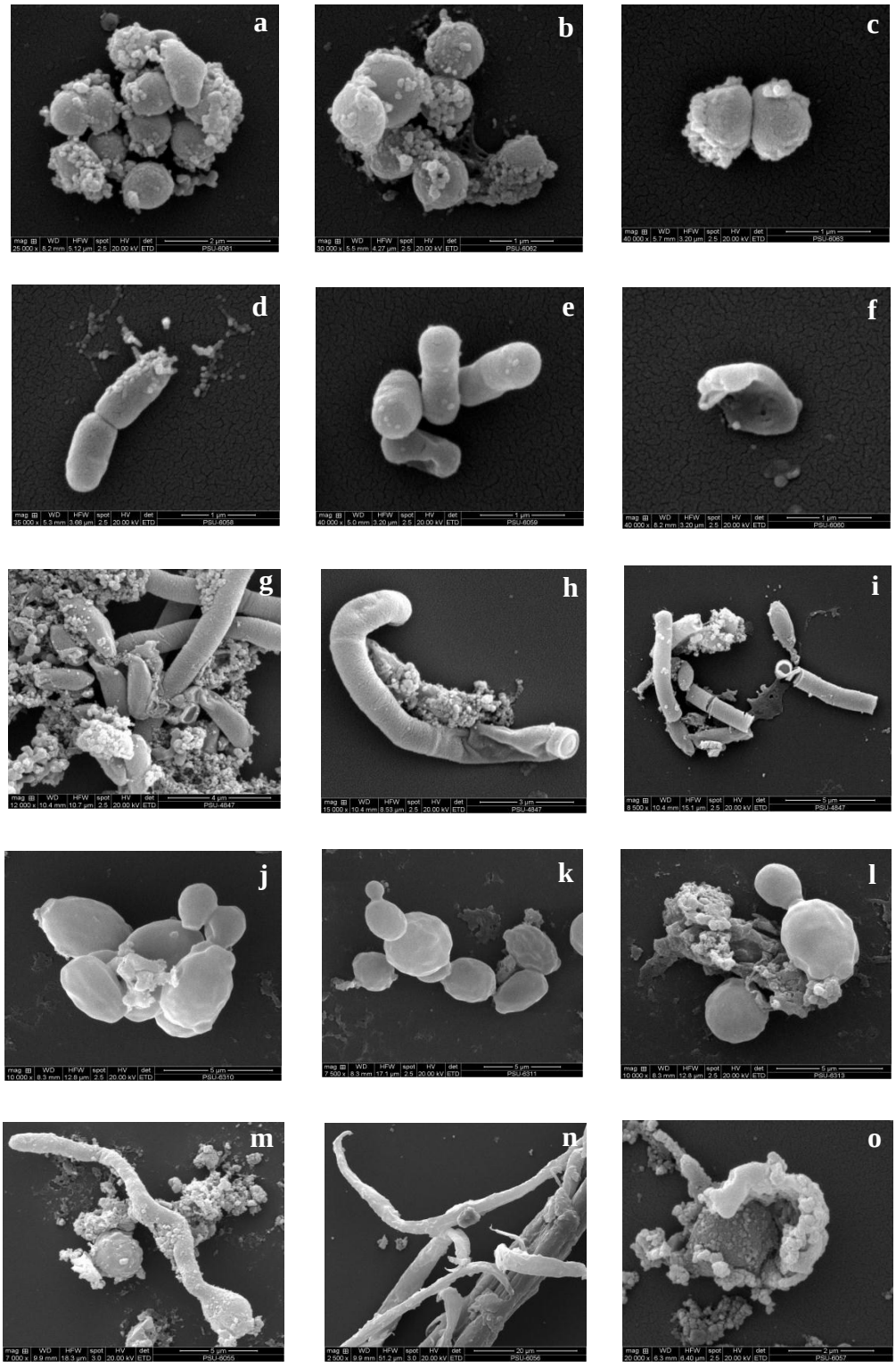


Fig. 4

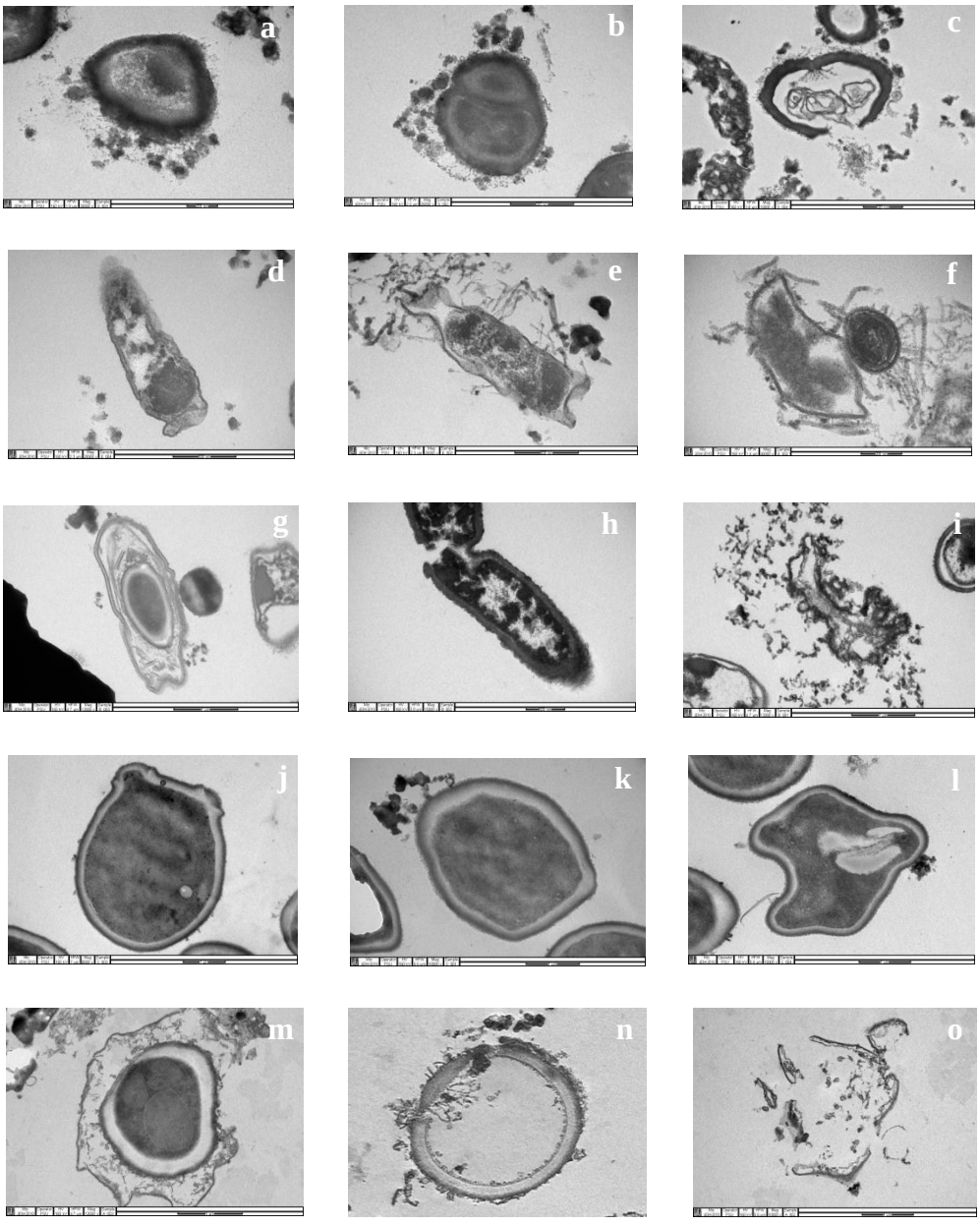


Fig. 5