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Understanding the heat stability and solubility of cocoa bean shell extract as antioxidant and antibacterial functional ingredients

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Abstract

Cocoa bean shell is one of the main waste products with the proportion of 12 - 20% of the bean mass, and considered as a potential functional ingredient due to its antioxidant and antimicrobial properties. In the present work, the stability of the bioactive compounds during heat treatment and the effect of solvent polarity on the solubility of the cocoa bean shell extract were explored. Results showed that heating at 60°C for 1 h did not affect the antioxidant and antibacterial properties of the cocoa bean shell extracted with ethanol. The estimated half time (time required to reduce the antioxidant capacity by half) ranged from 4.7 to 3.1 h when the ethanolic extract was heated at temperature 60 to 100°C, respectively. Bioactive compounds that had antioxidant property in the cocoa bean shells were mostly water-soluble, with only a little amount that was acetone-soluble. However, all extracts from four different solvents (water, ethanol, methanol, and acetone) had similar antibacterial activities against *Escherichia coli, Bacillus cereus,* and *Staphylococcus aureus.*

Introduction

Cocoa bean shell is one of the main waste products in the chocolate production line with the proportion of 12 - 20% of the bean mass (Rojo-Poveda *et al.,* 2019). This waste is considered as a potential material for functional food because of its antioxidant and antimicrobial properties, as well as its high fibre content (Rojo-Poveda *et al.,* 2019). The cocoa bean shell extract contained antioxidants, such as catechins, epicatechins, theobromine, and caffeine (Grillo *et al.,* 2019). Residue of terpenes and sesquiterpenes have also been found in the extract of cocoa bean shells that may be responsible for its antimicrobial properties (Nsor-Atindana *et al.,* 2012b; Gabbay Alves *et al.,* 2019).

Attempts to use cocoa bean shell extract in food products have been reported in literature, even though the numbers are still limited. Rojo-Poveda *et al.* (2019) used the extract in beverages, and found that the product had a significant antioxidant property. The polyphenol-rich cocoa bean shell extract has also been found to be able to improve the cooking oil stability (Manzano *et al.,* 2017). Another recent study by Jozinović *et al.* (2019) also reported a proportional increase in antioxidant activity of extruded snack products with the addition of cocoa bean shells.

When the cocoa bean shells are used as a food ingredient, it will be exposed to various food processing conditions, including heat exposure. The stability of bioactive compounds in cocoa bean shells may be affected during cooking, and could be lost if the heating process is severe. Thus, it is important to understand the stability of the cocoa bean shell extract to the heat exposure. However, to the best of our knowledge, there is no study reported on the stability of the antioxidant and antimicrobial properties of the cocoa bean shell extract under the heating treatment. Moreover, since the food products have a wide range of polarity, such as oils and beverages, the solubility of the cocoa bean extract in the food matrix is also important to be considered.

Therefore, the present work aimed to understand the heat stability and solubility of cocoa bean shell extract as antioxidant and antibacterial ingredients. Chemical composition of the cocoa bean shells was also studied in order to explore the potential of the residue of this material after the bioactive compounds were extracted.

Materials and methods

Materials

Cocoa bean shells were kindly provided by PT Kalla Kakao Industri. The shells were packed in zip lock plastic bags and stored in a freezer. Since cocoa bean shells are considered a waste, they are usually dumped in an open air after production, thus exposed to dust. The samples that we received were also dusty, and to prevent the dust to contaminate and interfere with the

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antibacterial analysis, the cocoa bean shells were washed with water and dried in an oven at 60°C until the moisture content was less than 12% before they were powdered. Low temperature was chosen for the drying in order to avoid the degradation of the active compounds in the cocoa bean shells. Dried samples were then crushed and sieved (80 mesh pore size) to obtain uniform powder.

Cultures of bacteria namely *Escherichia coli, Staphylococcus aureus,* and *Bacillus cereus* were purchased from the Biotechnology Laboratory, Faculty of Mathematics and Natural Science, Universitas Halu Oleo. Bacterial resuscitation was periodically performed to retain their viability.

Proximate analysis of cocoa bean shells

Protein, lipid, carbohydrate, cellulose, hemicellulose, lignin, ash, and water contents of cocoa bean shell powder were analysed. Protein content was analysed with the biuret method (Robinson and Hodgen, 1940). Lipid was determined using Soxhlet apparatus (AOAC, 1995). Cellulose (Datta, 1981), hemicellulose (Datta, 1981), lignin (Datta, 1981), ash (AOAC, 1995), and water (AOAC, 1995) contents were analysed gravimetrically. Total carbohydrate (non-lignocellulosic) was calculated by the difference of protein, lipid, carbohydrate, cellulose, hemicellulose, lignin, ash, and water (AOAC, 1995).

Extraction of bioactive compounds

Ten grams of cocoa bean shell powder was separately macerated with 40 mL solvent (ethanol, methanol, distilled water, acetone) for 6 h on a shaker. After that, the mixture was filtered, and the filtrate was kept in a dark bottle, while the residue was mixed with another 40 mL solvent. The mixture was put on a shaker for another 6 h, and filtered afterwards. The filtrate from the second filter was mixed with the filtrate from the first filter.

Ethanolic extract was sampled as a model to study the stability of the active compounds to heat exposure. Extraction with ethanol was the most convenient since it was easier to be evaporated than water, and was the most common solvent used to extract cocoa bean shells reported in the literature. The extract was treated with three different temperatures, which were 60, 80, and 100°C. Periods of exposure were 1, 2, 3, 4, and 5 h.

Evaluation of antioxidant stability and capacity

Antioxidant stability and capacity were evaluated using a 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (Shimamura *et al.,* 2014). The percent inhibition was determined every hour of heat exposure, and value was plotted against time. Arrhenius equation was used to predict the antioxidant stability of the extract. Inhibition concentration at 50% (IC_{50}) was also determined for all extracts to evaluate the effectiveness of the solvent to extract the bioactive compounds.

Evaluation of antibacterial stability and capacity

Antibacterial properties of extracts were evaluated against *E. coli, S. aureus,* and *B. cereus* on nutrient agar (NA) plates through disk diffusion method. A loopful of the bacterial culture was taken and diluted in 100 µL sterile water. The diluted culture (20 µL) was then aseptically spread on NA plates. A paper disk (Ø 7 mm) made of a sterile filter paper was dipped in the extract, and let dry in a fume cupboard. Dipping and drying processes were repeated three times. After that, the disks were places on the inoculated NA plates. The plates were then incubated in 37°C for 24 h. Diameter of the clear/inhibition zone was measured and recorded.

Statistical analysis

Antimicrobial activity data was statistically analysed using one-way analysis of variance (ANOVA) and Tukey's post hoc test ($\alpha = 95\%$) with Minitab Pro 16.2.0.0.

Results and discussion

Chemical composition of the cocoa bean shells

The chemical composition of cocoa bean shells is presented in Table 1. Moisture content of the shells was approximately 3.35%, which was relatively low for a powder. For comparison, moisture contents of the shell sample reported by Emiola *et al.* (2011) and Martínez *et al.* (2012) were approximately 7.8 and 5.8%, respectively. The sample was very dry, which could be caused by the roasting treatment that was applied to the shells during cocoa processing in the factory.

Table 1. Chemical composition of cocoa bean shells.

Component	Content (mean ± SD)
Water	3.35 ± 0.25 g/100 g
Ash	8.10 ± 0.79% (d.m.)
Lipid	7.07 ± 1.30% (d.m.)
Protein	14.51 ± 1.09% (d.m.)
Lignin	14.55 ± 1.62% (d.m.)
Cellulose	21.84 ± 1.38% (d.m.)
Hemicellulose	6.92 ± 0.76% (d.m.)
Carbohydrate (non-lignocellulosic)	27.00 ± 0.58% (d.m.)

The ash and protein contents of the cocoa bean shells in the present work are similar to the results of Emiola *et al.* (2011) and Martínez *et al.* (2012), which were in the range of 7 - 8% for ash, and 14 - 15% for protein. However, the lipid content of the cocoa bean shells was much higher (7%) than the sample of Martínez *et al.* (2012), which was only approximately 2%, although it was lower than the value reported in Emiola *et al.* (2011) (10%). Variation in the lipid content could be due to the species variation, or the different in the purity level of the shells. During the separation of cocoa beans and the shells, some fraction of the beans can still be attached on the layer of the shells. Broken bean can also contaminate the shells. Since cocoa beans are rich in lipid (Aremu *et al.,* 1995), shells that contain some fraction of the beans would have higher lipid content than the pure shells.

Lignocellulosic materials in cocoa bean shells was approximately 43%, with cellulose as the main fraction (21.84%), followed by lignin (14.55%) (Table 1). Lignocellulosic material is a potential biomass for many applications including for high fibre functional foods. Many studies have reported that cocoa bean shells are rich in fibre (Martínez *et al.,* 2012; Nsor-Atindana *et al.,* 2012a). However, data on the amount of lignocellulosic material of cocoa bean shells are scarcely available from the literature; thus, it was difficult to conclude whether our sample was rich in cellulose or not as compared to other varieties. Even so, Bentil (2012) reported in his dissertation that his cocoa bean shell contained mostly lignin (45%), and low amount of cellulose (11%) and hemicellulose (8%). This once again shows variation in the cocoa bean shell chemical composition.

Antioxidant and antibacterial properties

Temperature effects

Antioxidant stability

The stability of the antioxidant properties of the cocoa bean shells extracted with ethanol was evaluated at three different temperatures (60, 80, and 100°C). Figure 1A shows the change in the percent of DPPH inhibition during the heating treatment. The antioxidant capacity of the extract is equivalent to DPPH inhibition value. The antioxidant properties of the extract were relatively stable during heating at 60°C for 3 h. However, beyond 3 h, the antioxidant capacity plummeted. On the other hand, heating at 80°C or higher caused a gradual decrease in the antioxidant properties of the extract. Heating for 5 h at 60, 80, and 100°C reduced the DPPH inhibition from 50% to approximately 30, 20, and 10%, respectively.

Data from Figure 1A was used to predict the half time ($T_{1/2}$) of the antioxidant capacity of the extract when it was treated at temperature 60 - 100°C. Arrhenius equation was used for this purpose. The model that was best fitted to the data was zero order model (Figure 1B). Based on the model in Figure 1B, a linear line was generated by plotting the ln k with 1/T (Figure 1C).

Based on Figure 1C, the line equation was y = -1284.5x + 5.5229 (Eq.1) with R^2 = 0.979. The Arrhenius equation is $\ln k = \ln A - \frac{E}{RT}$ (Eq. 2), where k is specific constant at a specific temperature, A is Arrhenius constant (minute^{-1}), E is activation energy (cal/mol), R is gas constant (8.3144 cal/mol °K), and T is temperature (°K).

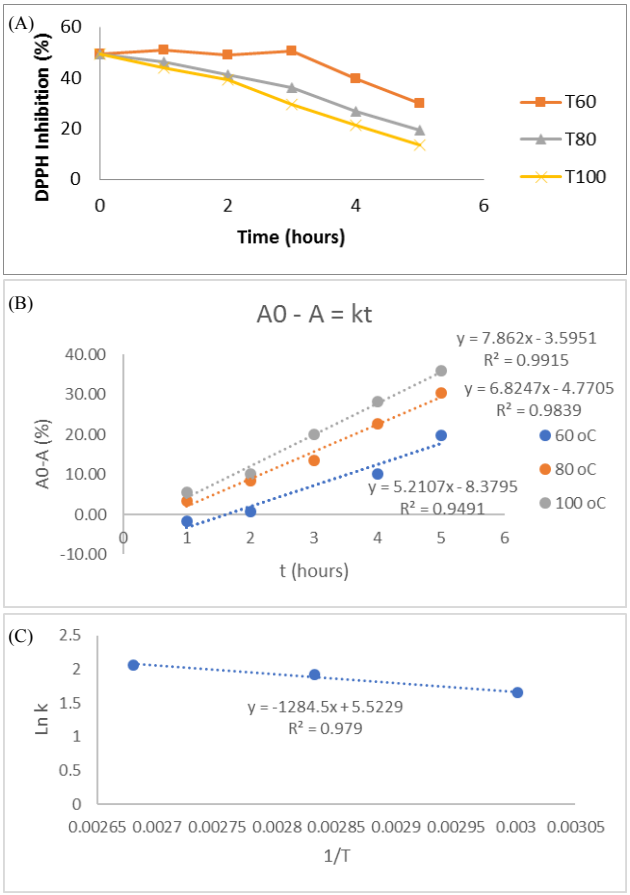

Figure 1. Antioxidant stability of cocoa bean extract: (A) Percent inhibition of DPPH radical by cocoa bean extract after exposure to high temperature, (B) Zero order model of antioxidant capacity, where A0 is %inhibition at the beginning (before heat treatment), and A is %inhibition at certain time, and (C) A linear line of Ln k against 1/T.

Based on Eq. 1, E/R was 1284.5 °K^{-1}, and ln A was 5.5229. Since R was 8.3144 cal/mol °K, E was then 10.68 KJ/g.mol. As the value of A, E, and R are known, the value of k for a specific T could be determined. When k is known, the $T_{1/2}$ can be calculated using the initial model equation (A0-A = kt). Table 2 lists the estimation $T_{1/2}$ for temperature 60 - 100°C. It

can be concluded that the time required for reducing the antioxidant capacity of the cocoa bean shells into half at temperature 60 - 100°C was approximately 5 to 3 h. This half time should be considered when the extract is used as a source of antioxidant in food during processing.

Table 2. Estimated time required to reduce the antioxidant capacity of cocoa bean extract due to heat exposure.

Temperature (°C)	Ln *k*	*k*	Half time ($T_{1/2}$) (h)*
60	1.667279	5.297735	4.67
70	1.779639	5.927716	4.17
80	1.885635	6.590541	3.75
90	1.985794	7.284830	3.40
100	2.080585	8.009150	3.09

*With assumption that the initial DPPH inhibition was 49.49%.

Antibacterial stability

The effect of temperature to the antibacterial properties of the ethanolic extract was also evaluated against *E. coli, B. cereus,* and *S. aureus* (Table 3). In this case, the extract was only heated for 1 h, and the control was the extract that was kept at room temperature for 1 h. The antibacterial capacity of the control was comparable with ethanolic extract of cocoa bean shells reported by Nsor-Atindana *et al.* (2012b).

Table 3. Antibacterial activity of cocoa bean extracts after heat treatment for 1 h.

Samples	Inhibition zone (mm)		
	Escherichia coli	*Bacillus cereus*	*Staphylococcus aureus*
Control	10.8 ± 0.8^{a}	7.0 ± 0.0^{a}	11.8 ± 1.6^{a}
T 60°C	9.3 ± 1.2^{ab}	8.0 ± 0.5^{a}	9.5 ± 0.9^{ab}
T 80°C	8.3 ± 1.0^{b}	10.3 ± 2.4^{a}	8.8 ± 0.6^{b}
T 100°C	7.3 ± 0.3^{b}	9.7 ± 1.4^{a}	7.7 ± 0.6^{b}

Data are means ± standard deviation. Means in the same column that share the same superscript letter are not significantly different ($p > 0.05$).

With regards to the effect of the heat exposure, antibacterial activity against *B. cereus* was not significantly affected by the heat treatment up to 100°C. However, the antibacterial activity significantly ($p < 0.05$) decreased against *E. coli* and *S. aureus* when the extract was heated at 80 and 100°C. Based on the antioxidant and antibacterial data, it can be concluded that the heat treatment at 60°C for 1 h was considered safe as the heating did not influence both the antibacterial and antioxidant properties of the cocoa bean extract. However, a careful consideration should be taken when the cocoa bean extract is exposed to a processing at temperature higher than 60°C. It is also worth noting that this experiment was done to a pure extract of cocoa bean shells. The effect of food matrix may have a protective effect on the reduction of the cocoa bean shell functional properties, although the opposite effect is also possible.

Effects of the types of solvent

As mentioned above, food products have a wide range of polarity that affects the solubility of ingredients in the food matrix. Therefore, in the present work, cocoa bean shells were extracted with four different solvents which had different polarity, namely water, ethanol, methanol, and acetone. The antioxidant and antibacterial properties of each extract was evaluated.

Antioxidant capacity

The antioxidant capacity of cocoa bean shell extracts from four types of solvent with different polarity in the present work was expressed as IC_{50} and presented in Figure 2A. The lowest IC_{50}, or the highest antioxidant capacity, was found when the cocoa bean shells were extracted with water. On the other hand, extract that was soluble in acetone had the lowest antioxidant capacity. The main compounds in cocoa bean shells that are responsible for its antioxidant properties are polyphenols and theobromine (Okiyama *et al.,* 2017). As polyphenols consist of thousands of chemical compounds, their degree of polarity also vary (Bravo, 1998). The results obtained in the present work indicated that the cocoa bean shells mainly contained water-soluble polyphenols, and only a little amount of less polar polyphenols. However, this finding may be sample-dependent since Nsor-Atindana *et al.* (2012b) found contrast results, where their acetone-based extract contained the highest amount of polyphenols, while the water-based extract had the lowest. The cocoa variety and the history of how the cocoa bean was processed could be the reason of the variation on the polyphenol profile in the cocoa bean shells. Chemical structure and composition of cocoa bean as well as their antioxidant properties change during fermentation, drying, and roasting (de Brito *et al.,* 2001; Summa *et al.,* 2006; Kendari *et al.,* 2012).

Antibacterial capacity

The effect of solvent types on the antibacterial activity of the cocoa bean shell extract was evaluated against *E. coli, B. cereus,* and *S. aureus*. Figure

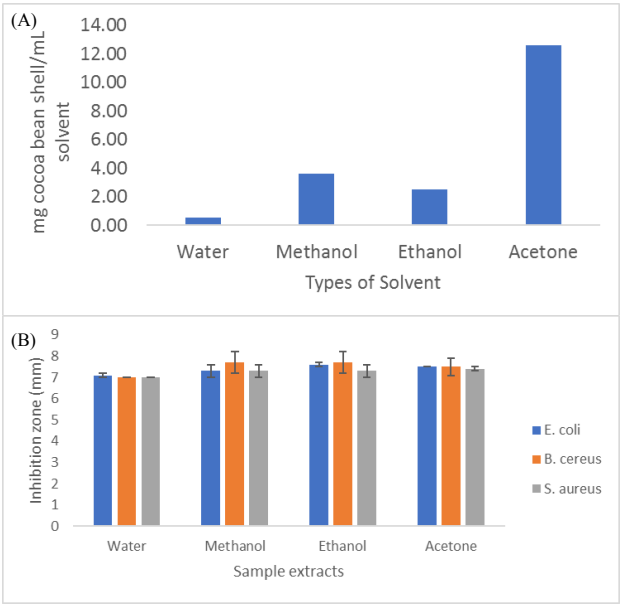

Figure 2. Effect of solvents on functional properties of cocoa bean shells: (A) Antioxidant, and (B) Antibacterial.

2B shows the radius of the inhibition zone. In general, the antibacterial activity of the extract was moderate, as the inhibition zone was slightly wider than the diameter of the paper disk. There were also no great differences in the diameter of inhibition zone between extracts with different types of solvent. Considering that the antioxidant capacity of the extracts was highly affected by the solvent types, while the effect was absence in antibacterial capacity, this could show that not all the bioactive compounds that had antioxidant property also had an antibacterial activity. In addition, the antibacterial compounds may have amphiphilic property since they were found in all solvents with different polarities.

Conclusion

Antioxidant and antibacterial stability of cocoa bean shells were significantly affected by heat treatment. However, heating the cocoa bean shell extract for 1 h at 60°C was considered safe in term of antioxidant and antibacterial capacity. The estimated half time (time required to reduce the antioxidant capacity by half) ranged from 4.7 to 3.1 h when the extract was heated at temperature 60 to 100°C, respectively. Bioactive compounds that had antioxidant property in this cocoa bean shells were mostly water-soluble and only a little amount that was acetone-soluble. However, all extracts from four different solvents (water, ethanol, methanol, and acetone) had similar antibacterial activities against *E. coli, B. cereus,* and *S. aureus.*

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