Antibacterial activity of cocoa pod husk phenolic extract against *Escherichia coli* for food processing

E M Diniardi¹, B D Argo² and Y Wibisono²

¹ Department of Agricultural Engineering, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia
² Department of Bioprocess Engineering, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

E-mail: Y_Wibisono@ub.ac.id

**Abstract.** Cocoa pod husk (CPH) which is a waste of cocoa plantation contains phenolic compounds which can be used as antibacterial agents. Phenolic compounds in CPH include phenolic acids, flavonoids and flavones. The aim of this study was to analyze the antibacterial activity of CPH extract which was extracted by using Microwave-Assisted Extraction (MAE) method. Extraction using 96% ethanol solvent with a ratio of 1:4, 1:6 and 1:8 (w/v) for 2, 3 and 4 minutes, respectively. The extraction results with the highest total phenolic content were tested for their antibacterial activity using a disk diffusion method at an extract concentration of 5, 7.5, 10 mg/mL and 15 mg/mL with three replications, respectively. The highest total phenolic content of 453 mg GAE/g dry extract was obtained from MAE treatment with a solvent ratio of 1:4 (w/v) for 4 minutes. The results of the antibacterial activity of extracts against *Escherichia coli* showed that inhibitory zones had formed at a concentration of 5 mg/mL. The width of the inhibition zone increases as the concentration of extract increases.

1. Introduction
Cacao pod husk (CPH) which is a cocoa plantation waste, contains phenolic compounds which can be used as antibacterial substances. Phenolic compounds themselves are compounds resulting from plant secondary metabolism containing one or more hydroxy derivatives of the benzene ring. This compound is widely distributed in plants and has a defense function [1]. Phenolic compounds in CPH include phenolic acids (protocatechuic acid, p-hydroxybenzoic acid, and salicylic acid), flavonols (kaempferol) and flavones (linarin) [2]. The presence of these compounds, allows CPH extract to be used as a natural preservative for food products [3].

A promising method for obtaining CPH extract is the microwaved assisted extraction (MAE). MAE is an extraction method that utilizes microwaves to accelerate the selective extraction process by heating solvents quickly and efficiently [4]. With the application of MAE, CPH extract is expected to have a higher total phenol value with shorter processing time.

The purpose of this study was to evaluate the antibacterial activity of extracted CPH from the MAE method. Antibacterial activity was assessed by disk diffusion method with *E. coli* as a model. *E. coli* is a gram-negative bacterium commonly used as an indicator of contamination in assessing water and food hygiene [5].
2. Materials and Method

2.1. Materials

CPH is obtained from local farmers in Malang Regency, ethanol (96%), n-hexane, gallic acid, Folin-Ciocalteu reagent (10%) and sodium carbonate (7.5%).

2.2. Material preparation

CPH was cleaned with running water, followed by thinly chopped with the size of 2 mm. Then, dried using an oven blower at 50 °C until the moisture content was less than 10% wb. The dried CPH was milled and sieved with a 100-mesh sieve, to produce CPH powder.

2.3. Procedure of extraction

Before extracting with ethanol, CPH powder underwent the defatting process using n-hexane [6]. As much as 25 g of CPH powder was added 50 mL hexane and stirred using a magnetic stirrer for 30 minutes then left 15 minutes and decanted. The process of defatting was done twice. The material from the defatting then added ethanol 96% solvent with a variation of the ratio of 1:4; 1:6; and 1:8 (w/v) and included in a microwave (Samsung ME731K, 180 watts of power) with variations in treatment times 2, 3, and 4 minutes, respectively. Maceration was carried out for 24 hours while stirring occasionally. The maceration solution was filtered using Whatman number 1 filter paper in order to obtain the filtrate 1. Maceration was repeated one more time by adding 0.5 volumes of the initial solvent to the residue from the filtration and filtrate 2 was obtained. Filtrate 1 and 2 were inserted into the rotary evaporator (IKA HB 10) at temperature of 40 °C, 65 rpm, until it became thick solution. The concentrated extract was dried using a vacuum oven (temperature 50 °C) and then mashed with mortar. The extracts powder was characterized by FTIR (Fourier Transform Infrared) spectrophotometer (Shimadzu 8400S).

2.4. Measurement of total phenolic content (TPC)

The extraction results of each treatment were measured by the total phenol content using the Folin-Ciocalteu method [7]. As much as 0.5 mL of the extract solution was taken with a concentration of 125 mg/L and 2.5 mL of Folin-Ciocalteu reagent (10%) was added and then vortexed and incubated for 5 minutes. As much as 2 mL of sodium carbonate (7.5%) was added and then re-vortexed and incubated 15 minutes. Furthermore, the absorbance of the solution was measured by a UV-Vis spectrophotometer (Shimadzu UV-1280) at a wavelength of 765 nm. The measurement was repeated three times. Gallic acid was used as a reference standard and the total phenol content was expressed as a gallic acid equivalent (mg GAE/g dry extract), with a calibration curve made between 0-100 mg/L.

2.5. Antibacterial activity test

Antibacterial activity test was carried out using the disk diffusion method with E. coli as a bacterial model [8]. The antibacterial activity of the extract was evaluated based on the thickness of zone of inhibition formed around the paper disc. Before testing, a bacterial solution was prepared. One inoculating loop of bacteria were grown in 5 mL NB (Nutrient Broth) and incubated for 24 hours at 37° C, followed by preparation of NA media (Nutrient Agar). Sterile paper disc (MN 287 ATD) impregnated with an extract solution at an extract concentration of 5, 7.5, 10 and 15 mg/mL, respectively. The bacterial solution was inoculated on the agar plate with sterile swabs and paper discs that had been impregnated with extract placed. Agar plates were then incubated for 24 hours at 37° C. The zone of inhibition formed was then measured using a caliper.

3. Results and Discussion

3.1. Characteristics of CPH extract

Characterization using FTIR was aimed to determine the functional groups of extracts produced based on the intensity of infrared light absorbed by the material. Phenolic compounds generally consisted of
one aromatic ring and a primary OH functional group that is attached directly to the ring. The presence of aromatic rings is characterized by vibrations of the aromatic C-H function groups in the wave number region of more than 3000 cm\(^{-1}\). However, it is sometimes difficult to distinguish the vibrations of C-H alkenes and aromatic rings due to the appearance of the same range of wave numbers. The presence of a C-H vibration can also be observed in the area of 900-690 cm\(^{-1}\). Strong absorption of the area can be used to determine the position of the substituent on the aromatic ring. While the vibration of C=C on the aromatic ring can be seen in the regions 1600 and 1475 cm\(^{-1}\). The OH function group generally experiences vibrations in the area of about 3600 cm\(^{-1}\) with a phenolic O-H functional group generally in the area of 3610 cm\(^{-1}\). However, the presence of ortho-carbonyl in intramolecular hydrogen bonds in phenolic compounds can shift the O-H band to a lower frequency. In addition, alcoholic and phenolic compounds also showed a C-O band vibration absorption in the area of 1260-1000 cm\(^{-1}\) [9].

![Figure 1. FTIR Spectra of CPH Extract](image)

The results of FTIR testing on CPH extract are shown in Figure 1. Absorption peaks formed showed that CPH extract consisted of various compounds. Phenolic compounds in CPH extract are shown by the presence of strong and widespread absorption in the area of 3422.25 cm\(^{-1}\) (O-H stretching) and supported by moderate absorption in the area of 1370.13 cm\(^{-1}\) (O-H bending) which is then strengthened by the absorption at 1285.27 cm\(^{-1}\) (C-O group of phenol) which is a stretch of the aromatic group.

3.2. Total phenolic content (TPC)
The TPC of CPH extract extracted by the MAE method is shown in Figure 2. TPC values ranged from 346 mg GAE/g dw to 453 mg GAE/g dw. The highest TPC was 453 mg GAE/g dw obtained at 1: 4 solvent ratio and 4 minutes of extraction time. Overall, the 4-minute MAE treatment showed a higher TPC value in each solvent and ingredient ratio treatment. This value was much greater than previous studies, namely 49.92 mg GAE/g extract (using 70% ethanol solvent) and 94.2 mg GAE/g extract (using acetone 70% solvent) with 24 hour maceration [10]. This was possible because the use of hexane in the defatting process in this study was able to remove most of the impurities (non-phenol) that can be extracted by ethanol. The result was higher TPC obtained in the extract.

In general, although the risk of damage to the analyte can occur, the amount of extracted analyte will increase by extending the extraction time [11]. This is possible because the longer the exposure to microwaves is given, the more intensive the cell wall will be damaged so that it makes it easier for ethanol to dissolve the phenol in the ingredients. This is in accordance with Izza et al. [12], in general,
the TPC value of Moringa seed extract increases with increasing microwave exposure time. However, in this study, the treatment duration of 2 minutes and 3 minutes of TPC did not show a significant difference (except for ratio of 1:6). The results were due to the cell wall was not damaged enough to release more phenolic compounds, therefore the TPC value was relatively similar between treatment 2 and 3 minutes.

![Figure 2. Total value of Total Phenolic Content CPH](image)

Figure 2 shows that the increase in the ratio of solvents and relative materials did not show a significant difference in the value of TPC. This is possible because at MAE the heating rate is more determined by the solvent dielectric properties and the size of the material. This is in accordance with the study conducted by Rostagno et al. [13] on the extraction of isoflavone from soybeans, with the same mass of solid material the addition of solvent volume did not have a significant effect on the amount of isoflavones extracted with MAE.

3.3. Antibacterial activity

The results of the testing showed that CPH extract had antibacterial activity against *E. coli*. The zone of inhibition had formed at an extract concentration of 5 mg/mL (Figure 3), in accordance to studies conducted by Loppies et al. [3] with maceration method. This showed that microwave exposure did not damage the antibacterial activity of CPH extract. Of the three replications, in general, the zone of inhibition diameter increased with increasing extract concentration (Figure 4).

*E. coli* is a gram negative bacterium. Compared to gram-positive bacteria, gram-negative bacteria have a more complex cell wall structure. It has a bilayer outer membrane consisting of phospholipids in the inner layer and lipopolysaccharides in the outer layer. Therefore it is relatively more difficult for antibacterial materials to enter cells [14]. Especially in old *E. coli* cells because they have more phospholipids [15]. This might explain why the zone of inhibition formed is not perfectly rounded. Because there are more resistant *E. coli* cells with the zone of inhibition narrows at a certain point (Figure 3). However, compounds of flavones and especially flavonol in CPH extract are known to have strong activity against gram-positive bacteria and gram-negative bacteria [16].

Phenolic compounds as antibacterial, work by denaturing proteins that can cause bacterial cell metabolic activity to stop. The cessation of metabolic activity can inhibit growth to result in death in bacterial cells [17]. In low concentrations, phenolic compounds inactivate important enzyme systems in bacterial cells, whereas in high concentrations, phenolic compounds can interfere and penetrate the cell wall which precipitates proteins of the bacterial cell [18].
Figure 3. Antibacterial activity of CPH extract

Figure 4. Diameter of zone of inhibition in different CPH extract concentration, error bars indicate the SDs from three replication

4. Conclusions
FTIR spectra showed that CPH proved to contain phenolic compounds. The highest TPC of CPH extract was 453 mg GAE/g dw obtained at 1:4 solvent ratio and 4 minutes extraction time. The results of the antibacterial activity of extracts against *Escherichia coli* showed that zone of inhibition had formed at a concentration of 5 mg/mL. The width of zone of inhibition increases as the concentration of extract increases.

References
[1] Maddox C E, Laur L M, Tian L 2010 Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa* Curr. Microbiol. 60 53–58.
[2] Karim A A, Azlan A, Ismail A, Hashim P, Gani S S A, Abdullah B H Z, Azilah N 2014 Phenolic composition, antioxidant, anti-wrinkles and tyrosinase inhibitory activities of cocoa pod extract *BMC Complement. Altern. Med.* 14 1–13.
[3] Loppies J E and Yumas M 2014 Extraction of active compounds of the cocoa pod husk and
their utilization as natural preservative for food products J. Plant. Based Ind. 9 59–68.

[4] Vivekananda M, Yogesh M, Hemalatha S 2007 Microwave assisted extraction - an innovative and promising extraction tool for medicinal plant research Pharmacogn. Rev. 1–18.

[5] Odonkor S T, Ampofo J K 2013 Escherichia coli as an indicator of bacteriological quality of water: an overview Microbiol. Res. (Pavia). 4 5-11.

[6] Utami R R, Armunanto R, Rahardjo S, Supriyanto 2016 Effects of cocoa bean (Theobroma cacao L.) fermentation on phenolic content, antioxidant activity and functional group of cocoa bean shell Pakistan J. Nutr. 15 948–953.

[7] Dewi S R, Masyrifa L, Izza N, Al Riza D F, Hendrawan Y, Argo B D, Wibisono Y 2017 Phenol extraction from Moringa oleifera as biofouling reducer agent for mixed matrix Proceeding 76th IASTEM Int. Conf. 18–21.

[8] Doughari J H 2006 Antimicrobial activity of Tamarindus indica Linn Trop. J. Pharm. Res. 5 597–603

[9] Pavia D L, Lampman G M, Kriz G S 2000 Introduction to spectroscopy Thompson Learning Inc.

[10] Rachmawaty, Mu’nisa A, Hasri, Pagarra H, Hartati, Maulana Z 2018 Active compounds extraction of cocoa pod husk (Theobroma cacao L.) and potential as fungicides J. Phys. Conf. Ser. 1028 012013 1-8.

[11] Barbero G F, Palma M, Barroso C G 2006 Determination of capsaicinoids in peppers by microwave-assisted extraction-high-performance liquid chromatography with fluorescence detection Anal. Chim. Acta 578 227–233.

[12] Izza N, Dewi S R, Setyanda A, Sukoyo A, Utoro P, Al Riza D F, Wibisono Y 2018 Microwave-assisted extraction of phenolic compounds from Moringa oleifera seed as antbiofouling agents in membrane processes MATEC Web Conf. 204 03003 1-6.

[13] Rostagno M A, Palma M, Barroso C G 2007 Microwave assisted extraction of soy isoflavonones Anal. Chim. Acta 588 274–282.

[14] Nurnasari E, Wijayanti K S 2019 Antibacterial activities of tobacco leaf essential oil against Escherichia coli and Staphylococcus aureus J. Kefarmasian Indonesia 9 48–56. [In Indonesia]

[15] Srivastava R B, Thompson R E M 1966 studies in the mechanism of action of phenol on Escherichia coli cells Br. Journal Exp. Pathol. XLVII 317–323.

[16] Xie Y, Yang W, Tang F, Chen X, Ren L 2014 Antibacterial activities of flavonoids: structure-activity relationship and mechanism Curr. Med. Chem. 22 132–149.

[17] Sabel A, Bredefeld S, Schlander M, Claus H 2017 Wine phenolic compounds: antimicrobial properties against yeasts, lactic acid and acetic acid bacteria Beverages 3 29-43.

[18] Oliver S P, Gillespie B E, Lewis M J, Ivey S J, Almeida R A, Luther D A, Johnson D L, Lamar K C, Moorehead H D, Dowlen H H 2010 Efficacy of a new premilking teat disinfectant containing a phenolic combination for the prevention of mastitis J. Dairy Sci. 84 1545–1549.