DETERMINATION OF ANTIOXIDANT AND ANTIMICROBIAL BIOACTIVITY FROM PROTEIN HYDROLISATE OF COCOA POD HUSK (CPH)

Hannim Haffeza Haris[1*] and Mohamad Yusof Maskat[2]
[1] Malaysia Cocoa Board, Cocoa Innovation and Technology Centre,71800 Nilai, Negeri Sembilan.
[2] Department of Food Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia
**Corresponding author: hannim@koko.gov.my*

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ABSTRACT - *In cocoa industry, the contents of cocoa beans have high demands, but the cocoa pod husks (CPH), waste of cocoa industry, were considered as are a material that not provide any economic returns and is regarded as an undesirable waste. As concern, this study will apply the cocoa pod husk to become as a high-value resource and will provide advantages in terms of nutrition and health for the consumers. This study was to investigate the bioactive protein of cocoa pod husk from clone of PBC 159 as a source for antioxidant and antimicrobial. Antioxidant activity was assayed by 3 different methods; DPPH, ABTS and FRAP. Antimicrobial screening and determination of MIC's and MBC's against tested microorganisms were undertaken using agar diffusion method and broth dilution methods, respectively. Results of antioxidative activities from 3 methods utilized in this study (DPPH, FRAP, ABTS) demonstrated clone PBC 159 potential for a natural antioxidant source. According to the results obtained, the protein extract of clone PBC159 showed significant DPPH activity with the IC_{50} value of 0.4557mg/ml, while IC_{50} of ascorbic acid as standard was 0.2865mg/ml. The protein extract of cocoa pod husk for PBC159 demonstrated the highest identified activity against Bacillus amyloliquefaciens. The MIC and MBC of the bacteria was 0.390 and 0.780 respectively. From this finding, shows the potential of cocoa pod husk protein in Antioxidant and Antimicrobial activity. In this finding also show the valuable contribution for obtaining cocoa pod husk (CPH), from an abundant, inexpensive, renewable, and sustainable source, to be apply as a high-value resource and will be provided advantages in terms of nutrition and health for the consumers.*

***Key words**:* Antioxidant, antimicrobial, bioactive protein, cocoa pod husk, protein extract

INTRODUCTION

Theobroma cacao L. (Sterculiaceae) or cocoa is an economically important crop and grown worldwide with the purpose to gain cocoa beans for chocolate production. In Malaysia, it is an important comodity for the country due to its high economic value (Daud et al., 2013). Cocoa beans were harvested and fermented into multiple different products from cocoa butter to chocolate itself. According to Makinde et al.(2019), in the year 2017 to 2018, cocoa production was estimated at 4.49 million worldwide. However, along with its great economic importance, cocoa production generates substantial quantities of waste. Within the year, with the large quantity, an estimated 70% of waste was produced (Makinde et al., 2019).

Agriculture waste is defined as remnants, the aftermath of an agriculture activity (Nagendran, 2011; Obi et al., 2016). Cocoa pod husk (CPH), is considered as an agriculture waste as it is the by-product from the fruit. When cocoa beans are harvested from the fruit, the remaining part of the fruit left is the pod itself (Yusof et al., 2016). According to Laconi & Jayanegara (2015), the cocoa pod husk held up to 75% of the fruit weight. Meanwhile, according to Kilama et al.(2019), the cocoa pod husk held about 81 to 90% of the fruit weight, concluding that only 10% of the fruit is commercialized. This situation represents a serious disposal problem (Figueira *et al*., 1993). Normally, the pods are left to rot on the cocoa plantation, which can cause environmental problems. Besides producing foul odours, rotting cocoa pod husks can propagate diseases, such as black pod rot, when left on the cocoa plantations (Donkoh *et al*., 1991; Barazarte *et al*., 2008). The poorly decomposed cocoa pod husk also caused major problems to the drainage system and can possibly be the cause of water pollution (Kilama et al., 2019).

In Malaysia and other cocoa producing countries, processing this cocoa waste could provide economic advantages and decrease some of the environmental problems. Cocoa pod husks is one example of a naturally available agricultural waste, which have the potential for applications in biotechnological aspect because it is non-toxic,

abundant, easily available, totally re-generable, non-exotic, cheap and able to support the rapid growth (Dhanasekaran et al., 2011).

Because cocoa pod husks are readily available, so it could be used to recover value-added compounds such as bioactive protein. According to Adamafio *et al*. (2004), 15% of the chemical compositions are made up from proteins, which meant that the cocoa pods husk can be a high potential source for obtaining bioactive protein.

Recently, interest has been emerging to produce and characterize bioactive protein from plant sources due to their availability, safety and nutritional aspects. Proteins extracted from several plants by products are widely used as nutraceuticals and functional food ingredients for health promotion and disease risk reduction (Zarei *et al*., 2014).

Bioactive protein are defined as specific protein fragments that have a positive impact on the functioning or conditions of living beings, thereby improving their health (Korhonen and Pihlanto, 2006). The beneficial effects are attributed to different properties found in protein such as antimicrobial (Reddy *et al*., 2004; Rajanbabu and Chen, 2011), antioxidant (Sarmadi and Ismail, 2010), antihrombotic (Wang and Ng, 1999), antihypertensive (Erdmann *et al*., 2008) and immunomodulatory activities (Gauthier *et al*., 2006).

Biologically active protein or protein hydrolysates, once liberated as independent entities, can act as potential metabolism modulators and regulatory compounds with hormone-like activities. Upon oral administration, bioactive protein may affect the major body systems depending on their amino acid sequence. Many protein hydrolysates are known to reveal multifunctional properties (Meisel and FitzGerald, 2003).

Several synthetic antioxidants have been widely used in the food, pharmaceutical and cosmetic industries (Di Bernardini *et al*., 2011). However the use of these synthetic antioxidants, as radical scavengers possesses a potential health risk to the human body. Therefore, natural antioxidants are in high demand as dietary supplements to reduce the risk of aging, inflammation and cardiovascular diseases consequently improving the human health. To date, no previous publication has reported the antibacterial activity of cocoa pod husk protein hydrolysate (CPHPH), therefore this study will determine of antioxidant and antimicrobial properties of CPHPH.

MATERIALS AND METHODS

Chemicals

2,2-diphenyl-1-picryhydrazyl (DPPH) (D9132) tripyridyltriazine solvent (TPTZ) (T1253), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (238133) and 2,2'-azino-bis (3-ethlbenzthiazoline-6-sulfonic acid) (ABTS) were purchase from Sigma Aldrich, USA.

Microbial Cultures

In vitro antimicrobial studies were carried out against 4 bacterial strains isolated from human material: *Bacillus amyloliquefaciens, Enterobacter cloacea, Pseudomonas aeruginosa* and *Serratia marcescens*, and one yeast species, *Candida albican.* The strains were isolated in the laboratory of the National Genome Institute (NGI).

Sampling and preparation of CPH powder

Cocoa pod husk (CPH) has been collected from Cocoa Research and Development Centre Jengka. The clone used in this study is PBC 159. Sample was dried using freeze dryer. Then dried CPH was ground to 100µm particle size. The powder was then kept in air tight container prior to use.

Protein Extraction from Cocoa Pod Husk (CPH)

CPH powder was defatted using Soxhlet apparatus with petroleum ether for 8 hours. After defatted the CPH powder were then being dried for overnight in a ventilator in 20°C. Protein obtained from the extraction was then mixed together with NaOH (0.03 N), at 1:30 ratio, and being extracted in memmert water bath shaker at 150 rpm for 2 hours. After the filtration process, the pH of the supernatant will be adjusted to pH 4.0 using HCl. and the precipitation collected by the centrifuge at 10,000 × g for 10 min will be stored at -80°C for further analysis.

Preparation of Cocoa Pod Husk Protein Hydrolysate (CPHPH)

CPHPH was prepared using ezymatic hydrolysis, using Alkalase, Pepsin and Tripsin, separately. The enzymatic hydrolisis was carried out under optimal

temperature and pH conditions to ensure the enzyme functioning well. For enzymatic hydrolysis, all factors were controlled according to the experimental design for 6 hours. Hydrolysislysis was stopped by adding 70% methanol. Then, the suspensions were stirred at room temperature for 1 hour and centrifuged at 20,000xg for 30 minutes. The supernatants were collected, and the methanol was removed using rotary evaporator. Finally, the aqueous solutions were stored at -80°C.

Determination of Protein Content

The protein contents of hydrolysate were measured by the methods of Kjeldahl (AOAC, 2000)

Antioxidant Assay

i) DDPH Radical Scavenging Activity

Determination of 2,2-diphenyl-1-picryhydrazyl (DPPH) radical trapping capacity was carried out based on the method proposed by Yu (2008) with slightly modifications. The radical solution of DPPH (0.15nM in ethanol) was used to react with the sample, in the ratio of DPPH to the sample was 1: 1. Incubation time was 20 minutes at room temperature. The absorption readings at 517nm wavelength were read using UV 2450, Shidmazu, Japan. EC_{50} (Effective Concentration at 50%) is the concentration of a sample capable of trapping 50% DPPH radicals (Yu, 2008). To calculate the EC_{50} of a sample, the sample concentration is provided in the range (0.03125 - 1mg / ml). The percentage of DPPH trapped was calculated based on the equation below. Ascorbic acid was used as a positive control.

$$\text{Percentage of DPPH trap (\%)} = \frac{WI - W2}{W1} \times 100\%$$

Where W1 = absorbance of control; W2 = Absorbance of the sample

ii) ABTS Radical Cation Decolorization Assay

Scavenging activity of ABTS radical was conducted according to Yu (2008) method. To convert ABTS to ABTS- +, ABTS solution (5mM) is prepared in advance. Then, 5g of manganese oxide (MnO) was added to the ABTS solution. The purposed of this process is to oxidize ABTS to become a radical form. This ABTS- + solution were filtered first using Whatman No.1 filter paper. Before using it, ABTS- + solution was diluted with phosphate buffer solution (PBS) with pH of 7.4, to give an absorbance reading of ABTS- + solution 0.7±0.05 at 734nm. All samples were diluted appropriately to give absorbance values 20-80% of the blank. Then, 50 microliters of the diluted sample were mixed with 1.9mL of diluted ABTS-+ solution. The mixture was kept for 6 minutes at room temperature and the absorbance was immediately recorded at 734nm. Trolox solution (0.5mM) was used as a reference standard. From the stock solution, a series of Trolox concentrations were prepared in the range of 1-120 µM to plot standard curves using PBS solution. The radical trapping capacity of ABTS- + samples is shown in units of TEmol TEAC / g (Trolox Equivalent Antioxidant Capacity).

iii) Ferric Reducing Antioxidant Power Assay FRAP

Ferric reducing antioxidant power assay was performed using the method of Wong *et.al* (2006). The appropriate amount of extract was added to 1 ml of FRAP reagent, which contained 300 mM sodium acetate buffer (pH 3.6), 10mM 2,4,6-tri(2-pyridyl)-s-triazine(TPTZ) solution and 20Mm $FeCl_3$ x $6H_2O$ (10:1:1), and subsequently diluted with water to the total volume of 4ml. The reaction mixture was incubated in a water bath at 37°C for 30 minutes. The increase in absorbance was measured with a spectrophotometer at a wavelength of 593nm (UV 2450, Shidmazu, Japan). Trolox is used as a positive control.

Antimicrobial Assay

i) Evaluation of Antibacterial Activity (Disk Diffusion Method)

The concentration of the cell suspension was adjusted to the McFarland standard no 2, and 50µl of each microorganism suspension was spread on a Muller Hinton Agar (MHA) plate. Filter paper disks of 5mm diameter impregnated with different concentration of sample, 25µl, 50µl, and 100µl, were placed onto the surface of agar, with the methanol solvent as a positive control. All plate was incubated at 37°C for 24h. The clear zone of the inhibition was observed and measured in mm.

iii) Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration(MBC)

Determination of the Minimum Inhibitory Concentration (MIC), i.e., the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, and Minimum Bactericidal Concentration (MBC), i.e., is the lowest concentration of an antibacterial agent required to kill a particular bacterium. In this study, an overnight culture of tested strains was used for the preparation of suspension (0.5 McFarland standard turbidity).

A serial dilution of cocoa pod husk protein (CPHP) were prepared at different concentration(3.125, 6.250, 12.5, 25, 50 and 100µg/ml) in 96 microwell plates (Flat Blottom; polystyrene, Eppendor). The plates were then added with 100ml of each bacterium and yeast separated under aseptic technique. For the growth of bacteria and yeast they were incubated at 37°C for 24h and 25°C for 48h respectively. MIC was defined as the lowest concentration of the CPHP extract at which the microorganisms showed no visible growth. In order to determine MBC, broth was taken from each well and inoculated on Mueller Hinton agar (MHA) and Sabouraud Dextrose Agar (SDA) for bacterial and yeast respectively. Both of them were incubated for 24-48h. The MBC is defined as the lowest concentration of the extract at which 99.9% of inoculated microorganism was killed.

RESULTS AND DISCUSSIONS

Antioxidant Assay

In this study, several antioxidant determination tests were conducted to determine the antioxidant properties of cocoa pod husk protein (PCPH). Table 3.1 shows the radical trapping capacity of DPPH by PCPH based on several clones. DPPH is one of the stable free radicals and exists as a free electron at the room temperature (Ng Khar Ling, 2013). Therefore, DPPH is often used to determine the antioxidant activity of a component.

Table 1: Percentage of inhibition of DPPH and IC_{50} for protein cocoa pod husk (CPHP) at different concentrations compared with ascorbic acid.

Concentration	Ability to scavenge DPPH			
	Ascorbic acid	KKM 22	PBC 159	PBC 123
1	111.21	6.65	124.31	-6.57
0.5	109.83	10.21	31.86	-28.99
0.25	84.00	-52.40	25.75	-37.25
0.125	20.93	-38.11	14.05	-44.07
0.0625	3.79	-69.03	4.62	-32.51
0.03125	0.00	-49.83	10.57	-46.69
IC50	**0.2865**	**NOEL**	**0.4557**	**NOEL**

Note: IC_{50} = concentration of sample that cause 50% DPPH radical scavenged NOEL= No Observed Effect Level

The antioxidant activity of cocoa pod husk protein (CPHP) was measured by the ability to scavenge DPPH free radicals compared to ascorbic acid. The scavenging effect of both plant extracts and the standard on the DPPH radical were expressed as half maximal inhibitory concentration (IC_{50}) values; the results are reported in Table 1. Lower IC_{50} value reflects higher DPPH radical scavenging activity. According to the results obtained, the protein extract of clone PBC159 showed significant DPPH activity with the IC_{50} value of 0.4557mg/ml, while IC_{50} of ascorbic acid as standard was 0.2865mg/ml.

In the DPPH test, DPPH free radicals are trapped by proton -donating components (Xie et al., 2008). Based on Figure 3.1, which is a comparison between cocoa pod husk protein from PBC 159 clone with the ascorbic acid, shows ascorbic acid which is a positive control, has a better DPPH free radical trapping capacity when compared to cocoa pod husk protein.

However, the trapping capacity of PCPH is improved as the concentration of PCPH increases. At a concentration of 500 µg/ml, PCPH is capable of trapping as much as 30% of DPPH free radicals. And after that there was a sharp increase in the free radical trapping of DPPH up to a concentration of 1000 µg/ml. Where at this stage, the rate of DPPH free radical trapping for PBC clone 159 exceeds ascorbic acid by 122%.

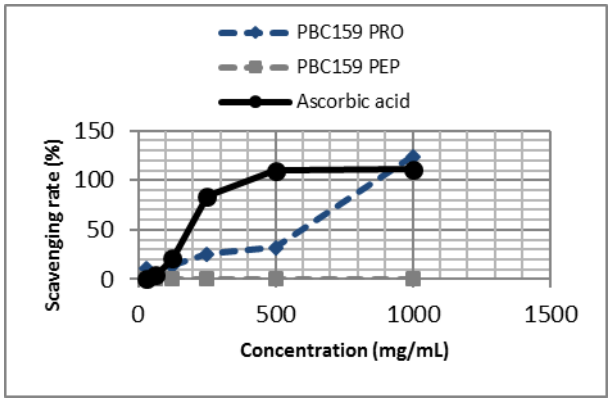

Figure 1: The ability to scavenge DPPH free radicals comparing between clone PBC 159 and ascorbic acid.

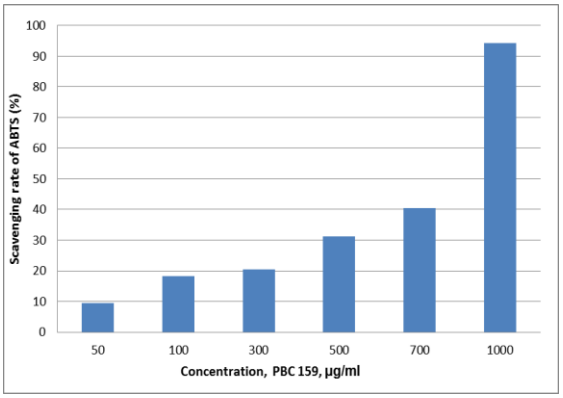

Figure 2: The ability to scavenge ABTS free radicals for clone PBC 159 in different concentrations

Apart from DPPH radicals, ABTS free radicals can also be used to determine the radical trapping capacity of an antioxidant substance. The ABT radical cation is formed by a chemical reaction and this radical has a greenish blue nitrogen atom. When ABTS free radicals are trapped, the color of the solution will fade and become clearer. Figure 3.4 shows the trapping capacity of ABTS free radicals by PCPH for PBC 159 clone. It is found that the trapping capacity increases as PCPH concentration increases, where at 1000 µg/ml it is found that PCPH for PBC 159 clone is able to trap more than 80% of ABTS free radicals, as good as Ascorbic acid.

Apart from free radical trapping, PCPH for PBC 159 clones was also able to show the ability to degrade oxidized components. Based on Figure 3.5 shows the strength of PCPH reduction from PBC 159 and Trolox clones in FRAP test. From the results obtained showed an increase in the pattern, as the sample concentration increased and while at at the end of the experiment it slightly decreased. Although Trolox is a good antioxidant, PCPH from PBC clone 159 is also can lower the iron (III) ions better at a concentration of 200 µg/ml. While at a concentration of 500 µg/ml, the ability of both was the same and then at a concentration of 1000 ug/ml it was found that the PCPH of PBC clone 159 decreased slightly compared to Trolox.

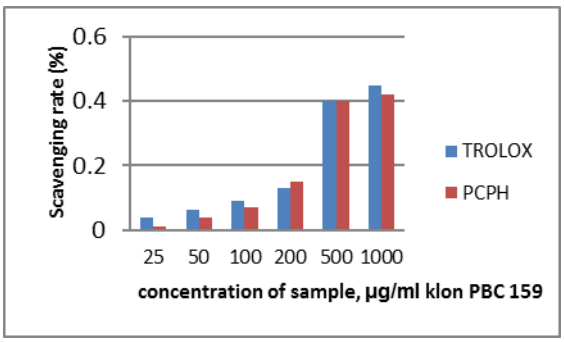

Figure 3: The ability to scavenge Ferric free radicals for clone PBC 159 in different concentrations.

Antimicrobial Assay

Based on preliminary studies in the determination of minimum inhibitory concentration (MIC), 3 clones showing the highest protein values were tested for this analysis. Extracts at concentrations of 100mg/ml, 50mg/ml, and 25mg/ml were pipetted onto discs containing bacteria and yeast.

Table 2: Cocoa pod husk protein (CPHP) extract and inhibitory zone against selected microorganisms.

	Inhibition zone (mm)								
	KKM22 (µg/ml)			PBC159 (µg/ml)			PBC123 (µg/ml)		
	25	50	100	25	50	100	25	50	100
Bacillus amyloliquefaciens	0	0	0	12	15	20	0	0	0
Enterobacter cloacea	0	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	11	14	17	0	0	0
Serratia marcescens	0	0	0	8	10	12	0	0	0
Candida albican.	0	0	0	10	12	15	0	0	0

Table 3: Effect of inhibition and determination of rough MIC value for cocoa pod husk protein extract through agar diffusion method.

Pathogen	Concentration of extract (mg/ml) PBC159 (µg/ml)					
	100	50	25	12.5	6.250	3.125
Bacillus amyloliquefaciens	+	+	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+	+	+
Serratia marcescens	+	+	+	+	-	-
Candida albican.	+	+	+	+	+	-

Note:
+ : have an inhibition zone
- : no inhibition zone

To our knowledge, no previous publications have reported the antibacterial activity against cocoa pod husk protein extract (CPHP). Therefore, antibacterial activity of CPHP against the tested bacteria strain was assessed by the presence and absence of inhibition zones using disk diffusion method. This antibacterial study revealed that only CPHP from PBC159 clone gave the positive results and the range of the inhibition is between 8 to 12mm in 25µg/ml of concentrations.

The protein extract of cocoa pod husk for PBC159 demonstrated the highest identified activity against *Bacillus amyloliquefaciens*. The MIC and MBC the bacteria was 0.390 and 0.780 respectively. From the result showed that the cocoa pod husk protein extract, PBC 159 clone, is more actively and effective to gram positive bacteria. This result proved that the crude extract of cocoa pod husk protein can be used as source for antibacterial compounds due to its ability to inhibit the bacteria with lowest MIC value 0.390mg/ml.

Table 4: MIC dan MBC value for cocoa pod husk protein extract

Pathogen	Concentration (mg/ml) PBC 159	
	MIC	MBC
Bacillus amyloliquefaciens	0.390	0.780
Pseudomonas aeruginosa	0.780	1.56
Serratia marcescens	12.50	25.00
Candida albican.	6.250	12.50

CONCLUSIONS

The contents of cocoa beans have high demands but nowadays a cocoa pod husk also represents a valuable food industry by-product. Cocoa pod husk are a rich source of dietary fibre and protein, as well as valuable bioactive compounds. From this finding, shows the potential of cocoa pod husk protein in Antioxidant and Antimicrobial activity. The extract contains certain compounds which can be the reason of the biological activities present in the extract. In this finding show the valuable contribution for obtaining cocoa pod husk (CPH), from an abundant, inexpensive, renewable, and sustainable source, to be apply as a high-value resource and will be provided advantages in terms of nutrition and health for the consumers.

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