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Analysis of antioxidant and antibacterial activity of cocoa pod husk extract (*Theobroma cacao* L.)

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ABSTRACT

Background: *Theobroma cacao bean manufacturing worldwide includes undesired byproducts such as cocoa pod husks. Cocoa pods contain a variety of beneficial chemicals, including polyphenols. Polyphenols have a vital function in the treatment of many illnesses and infections of the oral cavity. This is due to their vital qualities in the oral cavity, including anti-inflammatory, antibacterial, and antioxidant actions.* ***Purpose:*** *The goal of this study was to look at the antioxidant content and antibacterial activity of cocoa pod husk extract (Theobroma cacao L.) against Phorphyromonas gingivalis (P. gingivalis) and Streptococcus mutans (S. mutans).* ***Methods:*** *Cocoa pod husk extract was produced using an ultrasonic homogenizer and 70% ethanol. Thin layer chromatography and the Folin–Ciocalteu test were used to determine the phytochemical content and total phenolic content of the extract. The 2,2-diphenyl-1-picryl-hydrazyl-hydrate technique was used to measure antioxidant activity. Minimum inhibitory concentration (MIC) tests were used to measure the antibacterial activity of ethanolic extract at concentrations of 1, 4, 8, 16, 32, and 64 mg/ml using a deep-well broth microdilution technique.* ***Results:*** *The presence of alkaloids, flavonoids, tannins, saponins, and triterpenoids was discovered in the cocoa pod husk extract.* ***Conclusion:*** *The antioxidant activity of the extract was significant (IC_{50} = 62 ppm), and the MIC of P. gingivalis and S. mutans was 16 mg/ml and 8 mg/ml, respectively.*

Keywords: *antibacterial activity; antioxidant; cocoa pod husk; medicine; phytochemical*
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INTRODUCTION

The oral cavity reflects the overall health of the body since it is the point of entry for food for optimal development and wellness. However, the mouth cavity serves as an ideal microenvironment for microorganisms. Warm temperatures, humidity, and a nutrient-rich environment can all stimulate the development of microbes. Bacteria can become pathogens when the usual flora balance is disrupted. The link between the oral microbiome and human health evolves in tandem with the development of numerous oral and systemic disorders, such as caries and periodontal disease.[1] Plaque bacteria, which cause periodontal disease and tooth cavities, are the primary cause of oral infections.[2] Antibacterial medicines are used to treat periodontitis.[3]

In addition, the treatment of oral disorders is linked to the inflammatory process and wound healing. People typically take a variety of pharmaceuticals for such treatment; however, many encounter negative effects, such as gastrointestinal problems, hypertension, and bleeding.[4] As a result, we require a natural substance with little adverse effects yet significant efficiency.[5] Herbal medications have few negative effects and offer benefits in the form of pharmacological qualities. Herbal medications are recommended by the World Health Organization as natural elements to preserve health.[6] The cocoa plant is one of the plants in Indonesia that has the potential to be an antioxidant, anti-inflammatory, and natural antimicrobial.[7]

Cocoa (*Theobroma cacao* L.) comes in many types and has several health advantages. It is a plantation

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commodity with high output, and Indonesia was the world's third largest cocoa producer and exporter in 2019.[8] However, manufacturing has a negative impact on the environment, including growing cocoa pod waste, which can cause environmental concerns if not adequately handled. Cocoa pods may be extracted and utilized in a variety of culinary and health products since they are high in antioxidants, such as polyphenols, flavonoids, tannins, and phytosterols.[9]

Polyphenols are known to be natural antioxidants. They play a vital role in numerous illnesses, infections, and oral cancers in the oral cavity. This is due to their vital qualities in the oral cavity, including anti-inflammatory, antibacterial, and antioxidant actions. Polyphenols can quickly change the dentin surface characteristics, mostly through interactions with collagen and enamel, to provide superior adhesive capabilities and antibacterial activity against numerous microbes in the oral cavity.[10] Flavonoids and phenolic acids are significant antioxidant groups since they directly regulate bacterial development and reduce pathogenic activity. Antibacterial antioxidants work through three fundamental mechanisms: outer membrane permeability, cytoplasmic leakage, and nucleic acid production inhibition. The antibacterial effect of polyphenols may be due to their ability to chelate iron, which is required for the survival of practically all bacteria. Polyphenols demolish walls, enhance cytoplasmic membrane permeability, and release lipopolysaccharides.[11]

Porphyromonas gingivalis (*P. gingivalis*) is a periodontal pathogenic Gram-negative bacterium that causes gingivitis and periodontitis. Meanwhile, *Streptococcus mutans* (*S. mutans*) is a cariogenic bacterium that contributes significantly to the pathogenesis of tooth caries.[12] These two bacteria are crucial in the formation of plaque and biofilms. An increase in the quantity of bacteria will disrupt the natural flora of the oral cavity's equilibrium.[13] Exploring the biological activities of cocoa pod husk extract has the potential for drug discovery, which may be utilized for the future innovative development of pharmaceutical, medical, health, and domestic goods to boost their economic worth. Furthermore, the purpose of this study was to determine the antioxidant content and antibacterial activity of cocoa pod husk extract against *P. gingivalis* and *S. mutans.*

MATERIALS AND METHODS

The experimental process for in-vitro study was conducted in the Center of Development of Advanced Science and Technology at the University of Jember using the following materials: cocoa pod husk, aquadest, vitamin C, 70% ethanol solvent, $FeCl_3$, HCl, magnesium powder, silica gel GF254 (Merck), dimethyl sulfoxide, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, Folin–Ciocalteu reagent, *P. gingivalis* ATCC 33277, *S. mutans* ATCC 25175, Brain Heart Infusion Broth (BHI-B), a rotary evaporator, a micropipette (microlite), an analytical balance (Ohauss), thin layer chromatography (TLC) (Sigma-Aldrich), and ultraviolet (UV)-visible spectrophotometry (Genesys). Total phenolic content (TPC), antioxidant activity (IC_{50}), and lowest inhibitory concentration of *P. gingivalis* and *S. mutans* were the variables to be measured.

The Coffee and Cocoa Research Institute in Jember, East Java, provided samples of forastero type cocoa pods. They were washed, chopped into small pieces, and air-dried. Five kg of cocoa pods yielded roughly 500 g of cocoa pod powder after being cooked, mixed, and weighed. The extraction was performed at the University of Jember's Pharmacy Laboratory using the ultrasonic bath technique. For 3 3 min, 50 g of cocoa pod husk powder and 300 cc of 70% ethanol solvent were ultrasonicated.

The liquid was stirred every 3 min before being ultrasonicated again. The entire filtrate produced was then transferred to a petri plate and placed in an oven to be evaporated at 40°C until a consistent weight extract was achieved. This resulted in an extract with a concentration of 100 mg/ml being created.[14] Here, TLC and a silica plate with ethanol as a solvent were used to evaluate the extract. Silica gel GF254/plate stationary phase was made with a length of 8 cm and a breadth of 2 cm, washed with methanol, and then activated in an oven at 100°C for 10 min.

A 10-mg sample of the extract was dissolved in 1 ml of ethanol and spread over the stationary phase. The chromatogram was examined after 30 min, and the active chemicals as antioxidants displayed yellow stains on a purple background, orange or red stains, and green or blue stains. Purple stains were observed in the TLC findings for the free terpenoid/steroid group compounds, while the flavonoid compounds exhibited yellow stains. A purple stain was observed in the saponin test. Finally, the alkaloid compounds stained orange, while the polyphenol/tannin group chemicals stained black.[15]

The modified Folin–Ciocalteu reagent was used to evaluate the TPC of the cocoa pod husk extract.[16] In brief, 1.0 mg of crude extract was diluted in 1 ml of ethanol (1 mg/ml) and vortexed to produce a homogenous stock solution. This stock solution was diluted many times. The solution was then treated with 0.75 ml of Folin–Ciocalteu reagent. After 3 min, 20% (w/v) sodium carbonate was added to the solution and left to stand for 1 h at room temperature before the absorbance was measured at 765 nm with a UV-visible spectrophotometer. The TPC was estimated using the standard gallic acid solution calibration curve and represented as mg gallic acid equivalent (GAE) per gram of sample (mgGAE/g). All experiments were carried out in triplicate. To determine antioxidant activity, 1 ml of ethanol extract solutions from the cocoa pod husks (10, 20, 40, 60, and 80 mg/ml) were combined with 1 ml of the DPPH solution (40 mg/ml). The mixtures were incubated in a dark room for 30 min before the absorbance was measured using the UV-visible spectrophotometer at 520 nm. The measurements were repeated three times using vitamin C as a control. The ethanol extract from the cocoa pod husks was treated in the same way. The following formula was used

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to obtain the percent value of radical scavenging indicated by the IC_{50} value:[17]

$$\% \text{ radical scavenging} = \frac{(\text{A Blank} - \text{A Extract})}{\text{A Blank}} \text{ x } 100\%,$$

where A Blank denotes absorbance of ethanol DPPH solution and A Extract denotes sample absorbance

The calculation of the percentage of radical scavengers was inputted into the regression equation with the *x*-axis of extract concentration (µg/ml) and the percentage value of antioxidant inhibition. The IC_{50} value is calculated when the percentage value of radical scavenging is 50 using the equation $y = ax + b$.[18]

The antibacterial activity of *P. gingivalis* and *S. mutans* was determined using Gram staining on the isolates, *P. gingivalis* ATCC 33277 and *S. mutans* ATCC 25175. Both isolates were collected from the Microbiology Research Center Laboratory at Universitas Airlangga's Faculty of Dental Medicine. The liquid dilution technique, as modified by Okeke et al,[19] was utilized in this work, with concentrations of 1, 4, 8, 16, 32, and 64 mg/ml of cocoa pod husk extract.

A 3.7-g sample and 100 ml of sterile distilled water were placed in an Erlenmeyer tube, mixed with a spatula, and heated. The tube was then wrapped in cotton and sterilized in an autoclave at 121°C for 15 min.

A 2-ml solution of sterile BHI-B was mixed with 2 ml of *P. gingivalis* and *S. mutans* suspension in a test tube. As with *S. mutans*, one dosage of P. *gingivalis* was placed in a desiccator and incubated at 37°C for 24 h. Turbidity in the medium characterized the development of *P. gingivalis* and *S. mutans*. Six tubes containing 1, 4, 8, 16, 32, and 64 mg/ml of the cocoa pod husk extract and 1 ml of BHI-B were used for successive dilution of each bacterium. Each tube received a *P. gingivalis* suspension with a turbidity level of 1.5 108 CFU/ml McFarland before being incubated at 37°C for 24 h. When there was no bacterial growth, as indicated by the absence of turbidity in the test tube, the lowest concentration was observed. For the *P. gingivalis* and *S. mutans* groups, the minimum inhibitory concentration (MIC) test was performed three times.

RESULTS

Here, TLC was used to perform a qualitative phytochemical analysis of the cocoa pod husk extract to identify stains in the sample (Table 1). The stationary phase (adsorbent) and mobile phase (eluent) determined the separation of chemical components. Furthermore, due to the adsorbent's propensity to absorb chemical components based on their polarity, the chemical components in the sample migrated up following the mobile phase. The findings indicated that secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, triterpenoids, and steroids, exist. Plants have many secondary metabolites that are employed in industry and medicine. The TLC findings indicted that cocoa pod husk extract is an antioxidant and includes anti-inflammatory and antibacterial properties.

The Folin–Ciocalteu assay was used to assess the total phenol concentration of the extract. Based on electron transport, this test technique delivers a reduction capability reported as phenolic content. The TPC and yields of plant extracts are affected by the solvent used for extraction. The results of measuring the absorbance of standard gallic acid at concentrations of 20, 40, 50, and 60 mg/ml were gallic acid standard curves, as shown in Figure 1. Gallic acid's standard curve has a linear regression equation of $y = 0.011x + 0.005$ with a regression coefficient of $R^2 = 0.9912$. The TPC can be calculated using these formulae, as indicated in Table 2.

These findings suggest that cocoa pod husk extract includes phenolic chemicals. This is consistent with the TLC results, which indicated alkaloids, flavonoids, phenolic compounds, tannins, triterpenoids/steroids, and saponins. The DPPH technique was used to perform a quantitative evaluation on the antioxidant activity of the cocoa pod husk extract. The antioxidant activity test employed vitamin C as a positive control, which was due to the ability of the chemicals in vitamin C to diminish or fend off free radicals, which is extremely beneficial and frequently employed by researchers. The IC_{50} value of the cocoa pod husk extract sample is shown in Table 3.

The antioxidant test revealed that there is an antioxidant concentration (ppm) that may reduce free radicals by 50% (IC_{50} value). The IC_{50} value of cocoa pod husk extract is 62 ppm, which is a powerful antioxidant when compared with vitamin C, also an extremely strong antioxidant (5.31 ppm). The stronger the antioxidant activity, the lower the

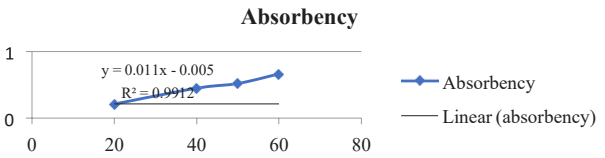

Figure 1. The standard curve for gallic acid.

Table 1. Results of phytochemical analysis of cocoa pod husk extract

Chemistry compound	Extract result	Characteristics
Alkaloid	+	Orange
Flavonoid	+	Yellow
Tannin	+	Black
Saponin	+	Purple red
Triterpenoid	+	Purple
Steroid	+	Purple

Table 2. Total phenol content of cocoa pod husk extract

Repetition	Sample weight	Absorbance	Total rate (mgGAE/g)	Average rate (mgGAE/g) x±SD
1	0.084	0.412	4.910	
2	0.095	0.562	5.915	5.64 ± 1.39
3	0.110	0.670	6.050	

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IC_{50} value. Compounds with IC_{50} values of <50 ppm are classed as extremely strong antioxidants, those with 50–100 ppm as strong antioxidants, and those with 100–150 ppm as weak antioxidants.[20]

The MICs of *P. gingivalis* and *S. mutans* were used to assess the antibacterial activity. The MIC test findings on *P. gingivalis* after a 24-h incubation revealed that there was no turbidity starting at a concentration of 8 mg/ml. However, there was some turbidity at values of 4, 2, 1, and 0.5 mg/ml, suggesting bacterial proliferation. Meanwhile, the MIC for *S. mutans* was determined to be 16 mg/ml (Table 4). The MIC values for *P. gingivalis* (8 mg/ml) and *S. mutans* (16 mg/ml) suggested that cocoa pod husk extract has bacteriostatic action. Figure 2 depicts the results of a 24-h incubation at 37°C. When there was no bacterial growth, as indicated by the absence of turbidity in the test tube, the lowest concentration was observed.

DISCUSSION

Overall, TLC with UV-visible spectrophotometry is a useful approach for identifying unsaturated bonds in plant components, which can be used to differentiate between conjugated and non-conjugated systems.[21] The presence of alkaloids, flavonoids, tannins, saponins, triterpenoids,

Table 3. The antioxidant activity of the ethanol extract from cocoa pod husk

Sample	Concentration (ppm)	% radical scavenging	IC50 (ppm)	Category of antioxidant
Ethanol extract from cocoa pod husk	10	23.08	62.02	Strong antioxidant
	20	47.71		
	40	61.56		
	60	58.34		
	80	67.39		
Vitamin C	10	34.90	5.31	Very strong antioxidant
	20	52.56		
	40	29.17		
	60	61.29		
	80	69.72		

Table 4. Minimum inhibitory concentration on *P. gingivalis* and *S. mutans*

Sample extract of cocoa pod husk (mg/ml)	*P. gingivalis*	*S. mutans*
4	-	-
8	V	-
16	V	V
32	V	V
64	V	V

Note: V = no bacterial growth

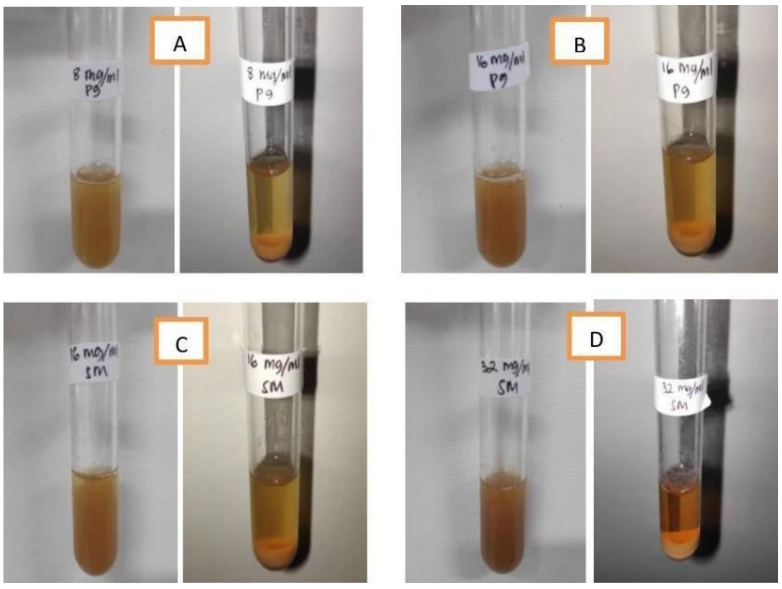

Figure 2. MIC before and after incubation: (A) *P. gingivalis* 8 mg/ml, (B) *P. gingivalis* 16 mg/ml, (C) *S. mutans* 16 mg/ml, (D) *S. mutans* 32 mg/ml.

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and steroids was discovered in the phytochemical analysis of an ethanol extract from cocoa pod husks. Alkaloids are chemical molecules with nitrogen atoms that are basic in nature and can induce bacterial cell protein coagulation. They interfere with the peptidoglycan components of bacterial cells, causing the cell wall layer to fail and the bacteria to perish.[22]

Flavonoids are the most abundant polyphenolic substance in cocoa pod husks. These active chemicals may have antioxidant and antibacterial properties. Flavonoids act as antimicrobials through mechanisms such as nucleic acid synthesis inhibition, cytoplasmic membrane function inhibition, energy metabolism inhibition, attachment and biofilm formation inhibition, porin inhibition in cell membranes, changes in membrane permeability, and pathogenicity attenuation.[23]

Tannins are also one of the active antibacterial substances found in cocoa pods, and they operate by targeting bacterial cell wall polypeptides that induce bacterial lysis and faulty cell wall construction. Tannins have been demonstrated to suppress Gram-positive and Gram-negative bacterial growth.[24] Furthermore, tannins can prevent germs from adhering to the cell surface, resulting in cell death. Tannins can also prevent the absorption of carbohydrates and amino acids, depriving bacteria of an energy source.[25] Triterpenoids are terpenoid group substances that function by interacting with porins (transmembrane proteins) on the bacterial cell wall's outer membrane and generating strong polymer bonds that destroy the porin, causing bacterial cells to lack nutrition.[26]

Saponins are antibacterial chemicals found in cocoa pod husks that operate by hydrolyzing bacterial cell walls. Bacterial cell metabolism will then be interrupted, as would the process of ATP synthesis for cell growth. If this process continues, cells will die. Saponins also have antioxidant capabilities, which decrease superoxide to create intermediate hydroperoxide cells and prevent free radical damage to biomolecular structures.[27] Saponins attack bacterial proteins, causing cell membrane integrity to be compromised, cell membrane dysfunction, and bacterial cell death.[28] The TLC examination of the phytochemical analyses of cocoa pod husks revealed antioxidant components, including flavonoids, that can serve as antioxidants by catching free radicals. The extract's TPC reflects its antioxidant effectiveness.[29,30]

The plant's phenolic chemicals have redox characteristics that function as antioxidants. Here, the TPC of the cocoa pod husk extract was 5.64 mgGAE/g. A high enough phenolic concentration in an extract is responsible for its bioactivity, which includes antioxidant and antibacterial properties.[31] Martinez et al.[32] discovered that a high TPC was substantially linked with antioxidant capacity. This is consistent with the findings of the current investigation. The capacity of the ethanol extract of cocoa pod husks to trap DPPH free radicals indicates that the test sample possessed antioxidant activity, as demonstrated by a decrease in DPPH absorbance. Antioxidants in the extract will neutralize free radicals by donating electrons to DPPH, resulting in a color shift to yellow or a reduction in the intensity of the brown hue of the extract. The IC_{50} parameter is used to determine antioxidant activity, which is the sample concentration necessary to capture 50% of DPPH radicals. The greater the antioxidant activity, the lower the IC_{50} value.[17]

Cocoa pod husk extract has a high antioxidant capacity, with an IC_{50} of 62.02 ppm. The antioxidant activity of cocoa pod husk extract is greater than that of ethanol extracts from young cocoa pods (76,094 ppm), ripe cocoa pods (91,884 ppm),[33] and extracts of cocoa bean husks with an IC_{50} value of 181.2 ppm.[34] The extract of cocoa pod husks has been proven to prevent the growth of both Gram-negative and Gram-positive bacteria. *P. gingivalis* is a Gram-negative bacterium with a thin cell wall structure of around 10–15 nm that consists of three layers: an outer membrane, an inner membrane, and a thin peptidoglycan layer on the inside with a high lipid content (11%–21%). The outer membrane is made up of two layers: lipopolysaccharide and lipoprotein. *S. mutans* is a Gram-positive bacterium with a simpler cell wall structure. The cell wall is around 25–30 nm thick and single-layered, with peptidoglycan as the main component and a modest lipid content (1%–4%). This type of bacterium is more vulnerable to the activity of antibacterial components, such as phenolic compounds and penicillin. Due to the cell wall's basic structure, antibacterial chemicals may easily enter the cell and locate a target to work on.[35]

However, in this study, the lowest inhibitory concentration of the cocoa pod husk extract against *P. gingivalis* (8 mg/ml) was lower than that of *S. mutans* (16 mg/ml). According to Hirao et al.,[36] cocoa has substantial antibacterial activity against periodontal pathogenic bacteria, such as *P. gingivalis, Fusobacterium nucleatum*, and *Prevotella intermedia*, and polyphenols are to blame. According to Smullen et al.,[37] unfermented cocoa demonstrates higher antibacterial activity than fermented cocoa, with MICs of 4 mg/ml and 8 mg/ml, respectively. Gram-positive and Gram-negative cell walls differ greatly in composition since Gram-positive bacteria have a strong peptidoglycan layer containing lipoteichoic acid but no outer membrane. The outer membrane of Gram-negative bacteria is made up of phospholipids, proteins, lipopolysaccharide, and a thin coating of peptidoglycan. Meanwhile, the cell walls of Gram-negative and Gram-positive bacteria are extremely crucial in osmotic protection. Any cell wall damage reduces the cell's tolerance to osmotic pressure and ionic strength. Natural antioxidants operate as antibacterials by inhibiting energy metabolism, causing membrane damage, and resulting in nucleic acid production.[38]

This study revealed that cocoa pod husk extract has a high antioxidant capacity and inhibited bacteria, with MICs of 16 mg/ml for *P. gingivalis* and 8 mg/ml for *S. mutans*. These findings suggest that cocoa pod husk extract could be used as an alternative therapy for periodontal disease and tooth caries. However, to strengthen the scientific evidence, these findings must be supported by in-vivo and in-vitro methodologies.

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