The antimicrobial effectiveness of cacao shell and cacao husk combination on inhibition of pathogenic bacteria in food products

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Abstract. Cocoa shell and cocoa husk are the waste from the processing of cocoa beans whose utilization has not been done optimally. The cocoa shell and cocoa husk contain phytochemical compounds which are potential to inhibit the growth of pathogenic bacteria in food products. The aim of this study is to find the ratio of cocoa shell extracts and cocoa husk extracts that most effectively inhibited the growth of Escherichia coli, Staphylococcus aureus and Salmonella sp. The method used experimental research followed by Randomized Block Design Factorial Pattern with 9 treatments and 3 replication. The treatment consists of two factors. The first factor was 3 types of extract (1:1, 2:3 and 3:2) and the second factor was 3 bacteria types (Escherichia coli, Staphylococcus aureus and Salmonella sp). The result showed that the extract mixture of cocoa shell and cocoa husk didn’t provide interaction effects, but provided an independent effect to inhibit the various types of bacteria. The comparison of 3:2 w/w of cocoa shell and cocoa husk extract contain phenol 0.063% and flavonoid 0.0191% gave the most effective inhibitory effect on Escherichia coli with a diameter of inhibition zone 5.84 mm (resistant), Staphylococcus aureus 4.04 mm (resistant) and Salmonella sp. 21.00 mm (sensitive) and was able to reduce the total bacteria of Escherichia coli 7.67 log CFU/ml (6.19%) , Staphylococcus aureus 7.06 log CFU/ml (13.65%) and Salmonella sp. 6.49 log CFU/ml (20.62%). Phytochemical tests showed that cocoa shell extract and cocoa husk extract containing phenol, flavonoid, triterpenoid, tannin, and alkaloid.

Keywords: antimicrobial, cocoa shell, cocoa husk, pathogenic bacteria, phytochemical compounds,

1. Introduction
Cocoa (Theobroma cacao L.) is a commodity that has a bright prospect because the price is relatively high and easy to market [1]. Indonesia ranks third as the largest cocoa producing country after Ivory Coast and Ghana. The Directorate General of Plantations (2016) states that the development of cocoa production in Indonesia tends to continue to experience fluctuating and unpredictable production rates
The provisional number of cocoa production in 2016 is around 656,817 tons and it is estimated that production will increase in 2017 to 688,345 tons. The part of cocoa that is used for the benefit of the chocolate processing industry is the seed portion. Production and processing of cocoa beans produce cocoa husk waste, pulp fluid and cocoa bean shells. The existence of cocoa shell and cocoa husk is often not utilized optimally and allowed to pile up into agricultural waste so that it affects the environment. The percentage of cocoa shells produced from the overall weight of cocoa husks is around 2% [3] and the percentage of cocoa husk shells from total fresh cocoa is around 75.52% [4]. If the percentage of the cocoa production waste is calculated with the estimated amount of cocoa production in 2017, the total amount of cocoa bean shell waste produced will reach 13,766.9 tons and the amount of cocoa husk waste can reach 519,838.14 tons.

Sartini et al (2007) extracted bioactive components of the skin of dried and fresh cocoa husk which showed inhibition of the growth of pathogenic bacteria Escherichia coli, Staphylococcus aureus, Salmonella thyposa and Streptococcus mutans [5]. Antimicrobial activity of cocoa shell extract and cocoa husk extract is still in the intermediate inhibitory zone and has not been effective in inhibiting the growth of pathogenic bacteria in food products.

Pathogenic bacteria can cause poisoning in humans, especially through food that comes into contact with humans during handling, processing, storage, and serving. WHO states that there are 14 types of pathogenic bacteria that often contaminate food to cause poisoning. However, in Indonesia the most common causes of poisoning are caused by Escherichia coli, Staphylococcus aureus and Salmonella bacteria. Staphylococcus aureus generally contaminates foodstuffs that have high protein content while Escherichia coli and Salmonella generally contaminates raw food and imperfectly cooked food or undercooked food [6].

Based on the description above, to increase the effectiveness of antimicrobial against inhibition of pathogenic bacteria in food products, it is necessary to have an antimicrobial modification with a combination of cocoa bean skin extract and cocoa husk skin. Therefore it is necessary to conduct research on the antimicrobial effectiveness test of a mixture of cocoa shell extract and cocoa husk which is expected to be effective in inhibiting the growth of pathogenic bacteria in food products to sensitive zones. In this study a comparison of the concentration of cocoa bean extract and cocoa husk extracts were effective compared to inhibit the growth of pathogenic bacteria in food products.

2. Materials and methods
The research method used in antimicrobial testing with the diffusion method is an experimental method using a factorial randomized block design (RBD) repeated 3 times with a total of 2 treatment factors each with 3 levels. The first factor is the type of treatment (A), which is the ratio of the concentration of the mixture of cocoa shell and cocoa husk which consists of 3 levels, namely:

\[ a1 = 1:1 \text{ (v/v)} \]
\[ a2 = 2:3 \text{ (v/v)} \]
\[ a3 = 3:2 \text{ (v/v)} \]

The second factor is the type of bacteria used (B) for antimicrobial tests consisting of 3 levels, namely:

\[ b1 = \text{Escherichia coli} \]
\[ b2 = \text{Staphylococcus aureus} \]
\[ b3 = \text{Salmonella sp.} \]

To find out which treatment shows a real difference, a Duncan test is performed. The research process includes extraction of cocoa shell extract and cocoa husk, antimicrobial test of cocoa shell extract, cocoa husk extract and, mixture of cacao shell and cacao husk (modification of Cappucino and Sherman, 2001) [7], quantitative antimicrobial testing of cocoa (modification of Parish and Davidson, 1993) [8], testing of ethanol levels in extract [9], phytochemical component testing of
cocoa shell, cocoa husk and, mixture of cacao shell and cacao husk, includes alkaloid test [10], steroid and triterpenoid tests [11], flavonoid test, saponin test, phenolic/tanin test [11], and total phenolic and total flavonoids quantitatively (Socha et al, 2009).

3. Results and discussions

3.1. Phytochemical components of cocoa shell extract, cocoa husk extract and its mixtures
Phytochemical component testing was carried out on both extracts singly or in combination to find out whether there were differences in the extract combinations used or not. The results of testing phytochemical components qualitatively can be seen in table 1.

Table 1. The qualitative analysis phytochemical components of cocoa shell extract, cocoa husk and mixtures of cacao shell and cacao husk extract.

| Phytochemical component | Cacao shell extract | Cacao husk extract | Mixture 1:1 (v/v) | Mixture 2:3 (v/v) | Mixture 3:2 (v/v) |
|-------------------------|---------------------|-------------------|------------------|------------------|------------------|
| Phenolic                | +                   | +                 | +                | +                | +                |
| Flavonoid               | +                   | +                 | +                | +                | +                |
| Steroid                 | -                   | -                 | -                | -                | -                |
| Triterpenoid            | +                   | +                 | +                | +                | +                |
| Saponin                 | -                   | -                 | -                | -                | -                |
| Tanin                   | +                   | +                 | +                | +                | +                |
| Alkaloid                | +                   | +                 | +                | +                | +                |

Note: "+" contains the tested compound "-" does not contain the tested compound

Based on table 1, cocoa shells extract, cocoa husk extract and all three mixtures were positive contain several phytochemical compounds, namely phenolic compounds, flavonoids, triterpenoids, tannins, and alkaloids as well as negative for steroid and saponin compounds. The three mixtures tested did not have differences in the content of the phytochemical components, so it could not be seen which extract mixes had higher potential as antimicrobial.

The phenol test showed that the extract of the cocoa shell, the extract of the cocoa husk and the three mixed treatments were positive containing phenolic compounds. This is indicated by the color change to black when the extracted sample is added with 5% FeCl₃ reagent. According to Harborne (1996), FeCl₃ reagents will react with polyphenol compounds in extracts to form complex compounds and produce a blackish-blue color [12]. One of the phenolic compounds is tannin. The results of tannin testing using 1% FeCl₃ reagent showed a blackish color change. According to Sumalin et al (2015) a positive compound has tannin if the material reacted with color to green, red, purple, blue or deep black [13]. Tannins have potential as an antimicrobial because of their binding properties to proteins. Tannins can inhibit the growth of Staphylococcus aureus, Bacillus subtilis, and Bacillus stearothermophilus through the mechanism of changing the cytoplasmic membrane [14]. Tannin compounds can bind to proteins and then stop the activity of enzymes so that cell metabolism stops and cells become dead (Fuller, 1986).
The flavonoids in cocoa shell extract, cocoa husk extract, and mixed treatment were carried out using concentrated HCl and Mg. The addition of concentrated HCl serves to hydrolyze flavonoids into their aglycones by hydrolyzing O-glycosyl. According to Baud (2014) [15], a material that has a flavonoid content will be reduced by using Mg and HCl resulting in a change in color to red, yellow or orange. The results of tests conducted on the five extract samples showed a change in color to yellow and orange. This shows that the five extracts tested positive contain flavonoid compounds. The function of flavonoids in plants is as a regulator of growth, regulating photosynthesis and has antibacterial activity [16]. Flavonoids contained in cocoa shell extract and cocoa husk extract are classified as phenolic compounds that have glycoside bonds and function as antibacterial agents. These phenolic compounds will interact with bacterial cell membrane proteins through the process of adsorption by binding to the hydrophilic part of the cell membrane to make the cell membrane part into lysis and damage to bacterial cells [17].

The alkaloid on cocoa shell extract and cocoa husk extract and its mixture using Dragendorff reagent showed the results that the five extract samples tested positive contained alkaloid compounds. This is indicated by the appearance of a color change when the extract is added to the Dragendorff reagent. Alkaloid compounds contained in cocoa include caffeine and theobromine [3]. Alkaloid compounds are organic compounds that have nitrogen atoms and are alkaline (alkali) and can cause bacterial cell protein coagulation so that it causes inhibition of bacterial growth. Coagulation of bacterial cell proteins will disrupt the constituent components of peptidoglycan in bacterial cells which causes the cell wall layer to not be formed intact, thus causing bacterial cell death [18].

The content of steroid and triterpenoid compounds in the sample is marked by the occurrence of a green discoloration for steroids and a deep red color for triterpenoid compounds. The results of testing on cocoa shell extract, cocoa husk extract and mixture showed that the five extracts tested positive contained triterpenoid compounds but were negative steroid compounds. No steroid compounds were detected in the five extracts tested due to the ethanol solvent used. Ethanol is a polar solvent that can extract polar compounds from materials. According to Naufalin (2005), some steroids are non-polar to semi-polar, so in the process of isolation, it is necessary to use solvents that are also non-polar to semipolar [19]. Triterpenoids are one of the terpenoid groups whose carbon skeletons originate from six isoprene units, are crystalline, colorless, and are not volatile [12]. Triterpenoid compounds effectively inhibit the growth of the bacteria *Bacillus subtilis*, *Staphylococcus aureus* and *Escherichia coli* [19]. The terpenoid compound is a lipophilic phenol compound. The mechanism of action of terpenoids against bacterial inhibition is not different from the mechanism of action of other phenol compounds such as flavonoids, polyphenols, and alkaloids by damaging cell membranes. According to Parisa (2004), terpenoid compounds are active against the inhibition of the growth of bacteria, fungi, viruses, and protozoa [20]. Saponin testing on an ingredient is carried out using hot water dissolved in the sample, cooled and then shaken vigorously until foam forms. To see the stability of the froth produced by the sample and then drip with HCl, if the froth is stable and does not disappear, the sample is positive containing saponins. The results of saponin testing on cocoa shell extract, cocoa husk extract, and comparison of the mixture showed that the five samples tested did not cause foam, so the five samples tested negative contained saponins. The results of saponin component testing in this study are not in accordance with Kayaputri *et al* (2014) which states that extracts of cocoa bean extracts extracted using 70% ethanol solvent contain very strong saponins [21]. The results of saponin testing on cocoa husk extracts are also not in accordance with research by Rachmawaty *et al* (2017) which states that cocoa husk extracts produced from extraction using 70% ethanol still have saponin components in them [22]. Saponins are also one of the phytochemical components that can function as antimicrobial. Some saponins function in plant defense against microbial or fungal attack and fight viruses and have hemolytic properties and some are cytotoxic [23]. The presence of saponins in an extract is influenced by the type of solvent used. According to Arora and Bhardwaj (1997), saponin is a non-polar compound so it cannot dissolve in polar solvents.

The quantitative determination of phytochemical compounds is carried out on phenolic and flavonoid compounds because both of these compounds are known as phytochemical compounds that provide the
highest inhibitory effect on bacterial activity. The total phenolic testing conducted in this study was carried out by the Folin-Ciocalteu method. Phenolic compounds can react with Folin-Ciocalteu reagents. This method is based on the reducing power of phenolic hydroxy groups. The determination of total flavonoids was carried out using the aluminum trichloride (AlCl₃) colorimetric method. The results of quantitative analysis of phytochemical component can be seen in table 2.

Table 2. The quantitative analysis of phytochemical components of cocoa shell extract, cocoa husk extract and its mixtures.

| Extract of                  | Concentration (%) |          |          |
|-----------------------------|-------------------|----------|----------|
|                             | Polyphenol        | Flavonoid|
| Cacao shell                 | 0.0015            | 0.0113   |
| Cacao shell: cacao husk (3:2 v/v) | 0.0063            | 0.0191   |
| Cacao shell: cacao husk (1:1 v/v) | 0.0082            | 0.0282   |
| Cacao shell: cacao husk (2:3 v/v) | 0.0103            | 0.0380   |
| Cacao husk                  | 0.0123            | 0.0490   |

Based on table 2, the total phenol content quantitatively showed that cocoa husk extract has the highest phenol content that is equal to 0.0123% and cocoa shell extract has the lowest phenol content which is equal to 0.0015%. While the phenol content of the three mixture treatments performed showed that the mixture ratio of 1:1 (w/w) had a total phenol of 0.082%, 2:3 (w/w) of 0.103 and 3:2 (w/w) of 0.0063%. Total flavonoids produced from this study also showed that cocoa husk had the highest flavonoid content compared to other extracts of 0.0490%, cocoa shell extracts had total flavonoids of 0.0113%, while total flavonoids in the extract mixture ratio 1:1, 2:3 and 3:2 (w/w) respectively are 0.0282%, 0.0191%, and 0.0380%.

Based on the results of the total phenolic and flavonoid test, it can be seen that the total content of phenols and flavonoids in the cocoa husk is higher than the cocoa shell. Total phenol and flavonoid in each extract mixture are influenced by the amount of cocoa husk extract composition mixed, the more cocoa husk extract mixed, the higher the total phenol and total flavonoid in the extract mixture treatment. The total amount of phenols and flavonoids in the three mix ratios showed a decrease in the concentration of the total amount of phenols and total flavonoid of cocoa husks. According to Liu et al (2014) differences in the total amount of phenols and flavonoids produced in the extract mixture can be influenced by the composition of each extract, it is likely that there has been an interaction of compounds contained in the extract [24].

3.2. **Antimicrobial activity of mixture of cocoa shell extract and cocoa husk extract using diffusion method**

Antimicrobial activity of a mixture extract of cocoa shell and cocoa husk is shown by the formation of inhibitory zones in the form of a clear zone around the well that has been made. Based on the results of statistical analysis, the diameter of the clear zone area showed that the mixed treatment of cocoa shell and cocoa husk did not show any interaction with the test bacteria. Therefore the independent effect test for each factor is continued. Based on the independent effect test, each comparison of the extract mixture and the type of test bacteria has a significantly different effect on the diameter of the clear zone area produced. The average diameter of the resistance zone is presented in table 3
Tabel 3. Average diameter of clear zone area of cocoa shell and cocoa husk extract mixes against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella*.

| Treatment | The average of inhibitory area (mm) |
|-----------|------------------------------------|
| a1 = 1:1  | 9.24 ± 6.30\(^a\)                  |
| a2 = 2:3  | 9.73 ± 8.11\(^b\)                  |
| a3 = 3:2  | 10.29 ± 8.19\(^c\)                 |
| b1 = *Escherichia coli*               | 5.26 ± 0.71\(^b\)                  |
| b2 = *Staphylococcus aureus*          | 4.32 ± 1.06\(^a\)                  |
| b3 = *Salmonella* sp.                 | 19.69 ± 2.20\(^c\)                 |

Note: The average treatment marked with the same lowercase letter in the same column is not significantly different at the 5% level according to the Duncan Test.

Based on table 3, it can be seen that the most effective extract mixture ratio is the 3:2 (v/v) and the most effective inhibitory bacterial type is *Salmonella* sp. The ratio of mixtures extracts 3:2 (w/w) had the most effective inhibitory effect on the three pathogenic bacteria tested, then the mixture of 2:3 (w/w) and 1:1 (w/w). The ratio of mixture extract 3:2 (w/w) extract mixture is dominated by cocoa shell extract. This showed that the higher the composition of the cocoa shell extract added, the greater the inhibitory effect on the three pathogenic bacteria tested. The inhibitory zone formed can be seen in figure 1.

**Figure 1.** Inhibitory Zones Produced by the Ratio of Cocoa Shell and Cocoa Husk Extract of 3:2 (v/v) to Bacteria (a) *E. coli*, (b) *S. aureus* and (c) *Salmonella* sp.

The phytochemical compounds that most influence the inhibition of bacterial activity are flavonoids and polyphenols. However, in this study, the higher amount of flavonoid and polyphenol composition did not affect the inhibitory effect of test bacteria. This might need to be considered by other factors such as liking some of the organic ingredients contained in the cocoa extract. The presence of organic material in the extract during mixing which causes the phytochemical mixture in the extract does not inhibit microorganisms and contact between antimicrobial compounds with bacterial cells so that the resulting inhibition is reduced [25].

3.3. Antimicrobial activity mixture of cocoa shell extract and cocoa husk extract using contact method

The antimicrobial activity of cocoa shell and cocoa husk extracts using contact methods was carried out to determine the quantitative mixture ratio of extracts against test bacteria by counting the number of test bacteria at 24 hours incubation time (T24) after extracting in contact with test bacteria and stated in CFU/ml log. A comparison of the extract mixture tested showed a significantly different effect on the total test bacteria at T24. The total T24 test bacteria in all three extract treatments can be seen in table 4.
According to Pelczar and Chan (1988) damaging lipids in the plasma membrane of microorganisms, causing cell contents to come out. Pratiwi (2008) states that antimicrobial activity is also caused by phenolic compounds, namely by differences in polarity between the constituent lipids of DNA and the flavonoid alcohol so that bacteria will lysis and cells will die. The damage reaction of DNA lipid structure is caused by flavonoids will react and come into so that cell walls will be damaged and flavonoids enter the bacterial cell nucleus that lipids and amino acids in bacterial cell walls will react with alcohol groups in flavonoid compounds in bacterial cell walls.

Based on table 5, it can be seen that the combination of extract giving a significantly different effect on the total Escherichia coli bacteria at T24. While the percentage reduction in the amount of E. coli bacteria to each extract is 13.16% for a ratio of 1:1 (v/v), 4.84% for a ratio of 2:3 (v/v), and 6.19% for a ratio of 3:2 (v/v).

The combination of extract administration did not have a significantly different effect on total Staphylococcus aureus bacteria at T24. While the percentage reduction in the amount of S. aureus bacteria to each extract is 13.16% for a ratio of 1:1 (v/v), 9.61% for a ratio of 2:3 (v/v), and 13.65% for a ratio of 3:2 (v/v).

The combination of extract giving a significantly different effect on total Salmonella sp. on T24. While the percentage reduction in the number of Salmonella sp. of each extract, namely 8.76% for the ratio 1:1 (v/v), 13.53% for the ratio 2:3 (v/v), and 20.62% for the ratio 3:2 (v/v).

The mixture extract had a higher inhibitory effect on gram-negative bacteria compared to gram-positive bacteria. The resulting different inhibitory response is likely due to several factors. First, the structure of the cell walls of Gram-negative bacteria is more complex but has a thinner layer of peptidoglycan than Gram-positive bacteria. Jawetz et al (2001) stated that gram-positive bacteria have peptidoglycan on thicker cell walls thus forming a rigid structure [26]. The cell wall of gram-negative bacteria consists of 10% peptidoglycan, lipopolysaccharides and lipid content of 11–12%, while gram-positive bacteria consist of 60–100% peptidoglycan and 1–4% lipids [25] (Pelczar & Chan, 1988). The thickness of the peptidoglycan layer in gram-positive bacteria is thought to be more capable of blocking the penetration of antimicrobial compounds than gram-negative bacteria.

Second, gram-negative bacteria have porin proteins in the outer membrane of their cell walls. The porin protein functions as an outlet for the entry of active compounds so that the active compounds in the extract mixture being tested will more easily penetrate into cells and damage cell enzymes, causing cell damage [25].

Third, gram-negative bacteria have a high lipid content in the outer membrane of the cell wall. The presence of lipid content in cell walls can increase cell wall permeability [27]. The higher lipid content is likely to cause greater cell wall permeability and the higher the ability of antimicrobial compounds to penetrate into the cell, causing cell damage.

The pathogenic bacteria associated with lipid content is also influenced by the active compound in the extract being tested. Naiborhu (2002) added that alkaloid, flavonoid and phenol compounds inhibit bacterial growth by denaturing proteins and damaging bacterial cell membranes by dissolving fat found in bacterial cell walls [28]. Monalisa et al (2011) states that flavonoid compounds are lipophilic so that they can damage the lipid layer on the bacterial cell membrane [29]. Rustama and Lingga (2005) stated that lipids and amino acids in bacterial cell walls will react with alcohol groups in flavonoid compounds so that cell walls will be damaged and flavonoids enter the bacterial cell nucleus [30]. In the cell nucleus, flavonoids will react and come into contact with DNA and cause damage to the structure of DNA lipids so that bacteria will lysis and cells will die. The damage reaction of DNA lipid structure is caused by differences in polarity between the constituent lipids of DNA and the flavonoid alcohol groups.

Pratiwi (2008) states that antimicrobial activity is also caused by phenolic compounds, namely by damaging lipids in the plasma membrane of microorganisms, causing cell contents to come out [31]. According to Pelczar and Chan (1988) lipids in pathogenic bacteria will be extracted from the cell wall.
by phenolic compounds so that the pores expand [25]. This caused cell seepage and membrane function to be increased by uncontrolled absorption which damages the cell wall components.

4. Conclusions
The ratio of cocoa shell and cocoa husk extract does not have an interaction effect on the inhibition of various types of bacteria. Comparison of the cocoa shell and cocoa husk extract gives a significantly different independent effect on the diameter of the inhibited zone. The type of test bacteria also had a significantly different effect on the diameter of the inhibitory zone.

The mixture ratio of cocoa shell and cocoa husk extract does not have an interaction effect on the inhibition of various types of bacteria. Comparison of the cocoa shell and cocoa husk extract gives a significantly different independent effect on the diameter of the inhibited zone. The type of test bacteria also had a significantly different effect on the diameter of the inhibitory zone.

The mixture ratio of cocoa shell and cocoa husk 3:2 (w/w) containing 0.063% total phenol and total flavonoid 0.0191% provides the most effective inhibitory effect on Escherichia coli 5.26 mm (resistant zone), Staphylococcus aureus 4.32 mm (resistant zone) and Salmonella sp. at 19.68 mm (sensitive zone).

Whereas the contact method ratio of 3:2 (w/w) can reduce the total Escherichia coli bacterial test 7.67 log CFU/ml (6.19%), Staphylococcus aureus 7.06 log CFU/ml (13.65%) and Salmonella sp. 6.49 log CFU/ml (20.62%). Phytochemical components contained in cocoa peel extract and cocoa peel extract are phenolic compounds, flavonoids, triterpenoids, tannins, and alkaloids.

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