ACTIVITY OF ANTIOXIDE AND ANTI MICROBA FROM ETHANOL EXTRACT OF CACAO FRUIT SKIN ON VARIOUS RATIO OF SOLID MATERIAL

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Abstract
Cacao fruit skin is a waste of plantation cultivation that contains polyphenols so that it has the potential to be utilized as a source of antioxidants and natural antimicrobials. This study aims to determine the effect of ethanol solvent extraction on antioxidant and antimicrobial activity in vitro. The research method begins with the cacao fruit skin siege which is then extracted by maceration using 5 types of treatment i.e the ratio of the material with the solvents of each R1 (1: 2); R2 (1: 3); R3 (1: 4); R4 (1: 5) and R5 (1: 6). The antioxidant test was done by DPPH method, while the antimicrobial test was done by the well diffusion method. The results showed that the highest yield of antioxidant activity was obtained with the use of the ratio of 1: 4 (R3) solvent with IC50 75.98 µg / mL, whereas the lowest antioxidant activity was obtained in the ratio of 1: 2 (R1) IC50 94,74 µg / mL. Similar results were also observed in antimicrobial activity, a higher increase in diameter inhibition was obtained at a ratio of 1: 4 (R3) for all bacterial species of E. coli bacteria of 4.3 mm - 12.15 mm, Salmonella sp of 1.23 mm - 7.45 mm and for staphylococcus aureus of 6.48 mm-15.80 mm. Thus, it was concluded that cacao fruit skin extract with ethanol solvent at an R3 ratio (1: 4) had better antioxidant and antimicrobial activity than other treatments and could be interpreted as a potential natural preservative.

Keywords: Cocoa fruit extract, antioxidant, antimicrobial, solvent ratio

1. Introduction

Cacao fruit skin (cocoa pod husk) is a waste from the cultivation of plantations that are suspected to have bioactive components such as flavonoids, polyphenols and alkaloids (Kim et al., 2004). The proportion of fruit skin waste is relatively large, about 74.30%, while the beans with pulp are only about 25.70%, with the ratio of pulp or seed is 1.52 (Wahyudi, et al., 2008). In addition, the skin of cacao fruit contains 88% dry ingredients, crude protein 8%, and crude fiber 4%. But it can be said that to produce 1 ton of dried cocoa beans it is available as much as 10 tons of cacao fruit skin waste (Anwar, at al., 2014). The existence of cocoa fruit waste that has not been utilized properly, left alone by farmers, then this agricultural waste has the potential to be a problem related to the hygiene and health of the garden. Inadequate garden conditions accelerate the physical damage of cocoa stems, becoming the site of insects and disease sources such as farmers' garden affected by PBK (Hasrini & Susilowati, 2007). In advanced conditions, cocoa productivity decreases with the tendency of damaged crops, and some farmers in Parimo and Donggala districts have converted to palm oil (Anonymous, RPKP, 2016).

The acceleration of economic development in the Sulawesi Corridor with the placement of Special Economic Zones (SEZ) in Palu City, the research on the development of cocoa downstream including the handling of by-products is important. As a first step in manufacturing of natural preservatives, it is necessary to understand more proportionately the types of solvents as well as the ratio of their use of the ingredients (cacao fruit skin flour) which exhibits the best antioxidant and antimicrobial activity. This condition is intended to produce the best solvent usage formula considering that the ethanol solvent price is high (expensive). It is recognized that this research is still the second stage of 3 years of research to produce natural preservatives, thus the antioxidant and antimicrobial activity becomes the overall determinant of the study.

Some studies show the presence of antioxidant compounds in cocoa beans, but still, less than studied the compound on the skin of cocoa fruit even though someone has done it (Sartini, 2010). Publication in this area of study is still very limited. Therefore, this study aims to determine the effect of ethanol solvent extraction on antioxidant and antimicrobial activity in vitro, which has benefit for the development of value-added products. It becomes raw material in the industrial world and requires innovation.

2. Material and Methods

2.1. Materials and Tools

The main raw material is cultivated leather of Lindak varieties from farmers built in Makmur Village, Palolo Subdistrict, Sigi Regency. DPPH reagents and chemicals 2,2diphenyl-2-picrylhydrazyl, ethanol pa (Merck), and Escherichia coli test microbes, Salmonella sp and Staphylococcus aureus (Microbiology laboratory collections of Brawijaya University of Malang. The main equipment is a rotary evaporator, analytical scales, shake shaker, and SHIMADZU Spectrophotometer Models UV 160 and FTIR.

2.2.Extraction

The process is started by drying the skin of cocoa fruit at 60^{o}C, 100 mesh-sized, packed in 100 grams plastic. Each fruit, bark flour sample was macerated three times, using 96% ethanol solvent in various solvent or solvent ratios: R 1 (1:2); R2 (1:3); R3 (1:4); R4 (1:5) and R5 (1:6).

2.3. Antioxide Activity Test

This test using the DPPH assay method at 517 nm wavelength (Krings and Berger, 2001). The initial concentration of DPPH solution was 0.1 mM and the absorbance readings were performed after 30 min. If the absorbance drops very drastically (the solution turns yellow) before 30 minutes, it is necessary to dilute the sample of the solution thoroughly. The antioxidant activity is expressed as% = {(Control-Asampel) or (Control)} X 100%.

2.4. Antibacterial Activity Test

This test use agar diffusion method (Ayad et al., 2000), perforation by the culture of test bacteria was planted 1 ose on 10 ml of liquid medium. Then, it is incubated at 37^{o}C for 24h, the culture was taken 100 µL and mixed in 20 mL of medium to 45^{o}C, silenced at room temperature until medium compacted, 8 mm diameter hole was made. The hole included 100 µL filtrate extract of the ingredients (flour) from various material or solvent ratios according to the concentrations (103, 104, 105, and 106µg / ml) and incubated at 37 ° C for 24 hours. The bright zone formed around the wellbore was measured using a sliding range. The type of bacteria used in this study includes Escherichia coli, Salmonella sp and Staphylococcus aureus.

2.5.Methodology

The study used a Completely Randomized Design (RAL), five (5) treatments and three (3)

replications. The treatments included five (5) types of material / solvent ratio ie ratio 1: 2 (R1); 1: 3 (R2; 1; 4; R3); 1: 5 (R4) and 1: 6 (R5), and repeated 3 times to obtain 15 experimental units. The observed variables started with phytochemical screening and continued with the test antioxidants and antimicrobials.

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3. Results and Discussion

Based on the measurement of the DPPH assay method, the cacao fruit skin had antioxidant activity of all treatment types with the solvent or solvent ratio. As a result, an antimicrobial assay with well diffusion method showed an increase in inhibition diameter for all types of bacteria (E. coli, Salmonella sp and Staphylococcus aureus) bacteria.

3.1. Yield and Phytochemical Test

The test was calculated by comparing the extracts obtained from a number of samples at the beginning of the extraction on dry bases. The results of calculation of the yield and phytochemical test of cacao fruit skin extract can be seen in Table 1. Phytochemical screening was performed to determine the bioactive components contained in cocoa peel extracts with ethanol solvent (the solvent was selected in the first year of study). The results of phytochemical screening show that each treatment contains phytochemical compounds such as alkaloids, flavonoids, polyphenols and tannins in the same intensity while saponin compounds are not identified.

Table 1. Yield and Screening results of phytochemical components at material or solvent ratios

Class of compounds	**Material or solvent ratios**				
	R1	R2	R3	R4	R5
Alkaloid	+	+	+	+	+
Flavonoid	+	+	++	+	+
Polifenol	+	+	++	+	+
Tanin	+	+	++	+	+
Saponin	-	-	-	-	-

Rendemen (%)	**5,27**	**5,92**	**7,44**	**7,56**	**7,69**

Chemical laboratory analysis results at FMIPA, Tadulako University, 2018.
Explanation:
- : negative reaction
+ : positive reaction
++: strong positive reaction
R 1 : 1 : 2 (100 g cacao fruit skin flour/ 200 ml solvent)
R 2 : 1 : 3 (100 g cacao fruit skin flour / 300 ml ssolvent)
R 3 : 1 : 4 (100 g cacao fruit skin flour / 400 ml solvent)
R 4 : 1 : 5 (1 part cacao fruit skin flour / 500 ml solvent)
R 5 : 1 : 6 (1 part cacao fruit skin flour / 6 parts solvent)

This may be related to the type of solvent used, in which the ethanol solvent can generally extract classes of polar compounds, such as, polyphenols and flavonoids but not for non-polar compounds. According to Tiwari et al. (2011), ethanol will be able to penetrate well into the cell membrane in the plant part as well as the active components of plants that have antimicrobial activity more frequently extracted using ethanol solvent. Phytochemical screening treatment was initiated by preparation of material sample at a moisture content of 6.78%, and the result of extraction range from 5.27% to 7.69% for all treatment of solvent or ingredient ratios. Low water levels indicate that this material as a source of antioxidants and antimicrobials can survive during storage, while high yields have the potential for the development of antioxidant and antimicrobial industries. In the treatment of the ratio of material or solvent obtained the highest yield at R5 (1: 6), which indicates that the higher the ratio of material or solvent the higher the amount of recovery yield. It is strongly suspected that the greater the ratio of the solvent/solute to the more extracted solute, affecting the solute concentration gradient between the solvent extracted material and the solute molecule mobility in the solvent (Treybal, 1980). Nevertheless, the results of statistical analysis showed that the yield obtained as well as phytochemical screening results showed that between treatments R3, R4 and R5 were not significantly different ($P<0.05$). When seen from the tendency of the number of yield obtained, the increase of the extraction has a significant impact on its ratio from Ratio R3 to Ratio R4. However, R5 and R6 have a smaller ratio than the level of extraction rate of R1 to R2 ratio as well as from the ratio of R2 to R3. This shows that at each level of the material or solvent ratio, the rate of extraction of the cocoa bean skin component from Ratio R1,

R2 to R3 is greater than the extraction rate from R4 to R5 which is shown by a relatively stable yield recovery in the ratio range between R4 to R5. The magnitude of the rate of extraction of bioactive components indicated by the high yield value obtained which is suspected as a result of a large concentration difference between the material extracted with the solvent at the beginning of the extraction process. The value of the extraction rate will decrease as the number of components extracted from the material. Then it will be a minimum value if the equilibrium concentration between the material and the solvent is reached. Furthermore, the analysis of phytochemical components shows that for all treatment the ratio of material or solvent obtained the same phytochemical compound. It is assumed that the various applied ratios have the same ability to extract the chemical compounds present in the cocoa fruit shell powder, resulting from the process of solvent removal to obtain concentrated extracts of vacuum rotary evaporator using temperature 45^{o}C and dried by using nitrogen gas so that the possible components active primarily those acting as antioxidants and antimicrobials have not undergone much change for all treatments.

3.2. Antioxidant Activity Testing (IC50).

The antioxidant activity test of the DPPH method at this stage is done through variation of concentration of the antioxidant extract sample with ethanol solvent. Subsequently, the sample was mixed with DPPH, divortex then incubated at 37 °C for 30 minutes. A further measuring of absorbance was carried out using a spectrophotometer, as results are listed in Table 2.

Table 2. Antioxidants Activity (IC 50) on material and solvent ratios

Material or solvent ratios	**IC 50 (µg/mL)**			**Total**	**Means**
	1	2	3		
R1 (1:3)	95,27	95,21	93,74	284,22	94,74
R2 (1:2)	91,50	89,20	91,85	272,55	90,85
R3 (1:4)	75,91	76,38	75,65	227,94	75,98
R4 (1:5)	77,89	80,39	78,80	238,05	79,35
R5 (1:6)	83,98	84,11	84,60	252,69	84,23

Chemical laboratory, analysis results at FMIPA, Tadulako University, 2018.

The result of variance analysis showed that the treatment of material or solvent ratio gave a very real difference (P> 0.01) to antioxidant activity. The difference in antioxidant activity of this extract is affected by several factors, such as the difference in the ability to transfer hydrogen atoms to free radicals, the chemical structure of antioxidant compounds (Widyawati et al., 2010). Furthermore, Andayani et al. (2008) state that compounds having antioxidant activity generally have substituted hydroxyl groups in ortho and para positions against the -OH and -OR groups. The data in Table 2 shows that the ratio of the material or solvent, in general, gives the result of strong antioxidant activity because the IC50 values obtained are generally at the number 75.98 - 94.74, some literature reported that if IC50 values generated in the range of 50-100 are included, strong antioxidant activity is involved. This condition may be related to the characteristics of antioxidant compounds in the skin of cacao fruits that are more soluble in ethanol so the possibility of bioactive components can be extracted perfectly. The more dissolved active components are, the more compound antioxidant content, according to Katja and Suryanto (2009) statements that solvents such as methanol and ethanol are very widely used solvents and effective for the extraction of phenolic components from natural materials. Phenolic compounds are reported to react with reactive oxygen compounds. It occurs when one or two hydroxyl groups in aromatic rings that may act as hydrochloric donors. Based on the ratio of material or solvent, then the best treatment is R3 that is the ratio of material or solvent 1:4.

3.3. Testing Antimicrobial Activity

Testing of antimicrobial activity at various ratios was intended to see the potential ratio of ingredients or solvent of cacao fruit skin extract to pathogenic bacteria, for instance, E. coli, Slamonella sp and S. aureus. The measured indicator is the ability of the extract to provide clear zones in the well area, the greater the area indicates the inhibiting ability of the extract is also higher, as can be seen in Table 3.

The data in Table 3 show that the cocoa fruit peel extract at the various material or solvent ratios showed an increase in inhibitory power as the ratio of the solvent to all test bacteria increased. This is also shown in the extract concentration treatment where the higher the concentration of the extract the greater the inhibitory power. The level of inhibitory power is evident from the treatment of R1 (1: 2) to (R2) (1: 3) to R5 (1: 6) but the greatest inhibitory gain

is obtained from the treatment of R2 (1: 2) to R3 (1: 4) at a concentration of 106 µg / ml ie 12.15 mm for E. coli, 7.45 mm for Salmonella sp and 15.80 mm for Staphylococcus aureus, respectively.

Furthermore, the extract of the material has the potential to inhibit the growth of all types of pathogenic bacteria tested, in which the greatest inhibitory effect against Staphylococcus aureus bacteria. The difference in inhibition is thought to be caused by several factors such as the diffusion ability of the extracted material, the concentration of the extract, the interaction between the medium component, and the environmental conditions. This assumption is in line with the statement Siswandono and Soekardjo (2000) that the concentration of a substance that serves as an antibacterial is one factor that determines the size of the ability of antibacterial substances in inhibiting the growth of bacteria tested.

Table 3. The diameter of inhibition (mm) on various material or solvent ratios to some pathogenic bacteria

Treat-ment	**Extract Concentration(µg/ml)**	**Clear Zone Diameter (mm)**		
		Ecoli sp	*Salmonella* sp	*S. aureus*
	1000000	8.50	3.16	12.33
R1	100000	3.00	1.76	6.67
	10000	1.65	1.26	5.44
	1000	0	0.00	2.25
	1000000	9.65	4.45	13.15
R2	100000	5.15	3.25	7.00
	10000	4.80	1.89	5.57
	1000	1.15	0.79	4.00
	1000000	12.15	7.45	15.80
R3	100000	6.65	5.25	9.77
	10000	4.5	3.89	8.45
	1000	4.3	1.23	6.48
	1000000	12.95	8.89	16.11
R4	100000	5.50	6.95	10.23
	10000	4.50	4.12	8.97
	1000	2.00	1.83	6.66

	1000000	13.15	9.37	16.45
R5	100000	7.35	7.49	10.77
	10000	5.35	5.67	9.45
	1000	2.15	2.13	6.17

Microbiology laboratory analysis results at Brawijaya University, 2018.

It is further stated that the inhibitory diameter of the extract is also influenced by the type of microorganisms tested due to differences in cell wall structure among test bacteria affecting the working of cacao fruit skin extract as an antimicrobial compound. The presence of antibacterial activity of cacao fruit skin extract is supported by photochemical screening results indicating the presence of bioactive compounds in cacao fruit skin extracts, such as alkaloids, flavonoids, polyphenols, and tannins. Flavonoid compounds reported by some researchers will disrupt the integrity of bacterial cell membranes, tannins work competitively with glycosyl transferase enzymes in reducing saccharides as the basic ingredients of glycosylation. The enzyme of glycosyltransferase is an enzyme that plays a role in the process of adding sugar groups to proteins or lipids. If this enzyme is inhibited then the formation of bacterial polysaccharides is also inhibited. Other effects as antibacterials of tannins include reactions with cell membranes, enzyme inactivation, and destruction or inactivation of genetic material functions (Juliantina et al., 2009; Agustin DW. 2010).

The test bacteria used in this study include Gram-positive bacteria represented by Staphylococcus aureus and Gram-negative bacteria represented by E. coli and Salmonella sp bacteria. The results of this test show that the inhibition diameter of cocoa leaf extract is higher than Staphylococcus aureus bacteria compared to E. coli and Salmonella sp. This difference is suspected because of differences in cell wall structure between the two types of bacteria that affect the activity of cacao fruit skin extract as an antibacterial compound. Gram-positive bacteria cell wall structure is simpler, which is layered with low lipid content (1-4%) to facilitate the bioactive compound into the cell (Hawley, 2003). Staphylococcus aureus as a gram-positive bacterium has 3 layers of cytoplasmic membrane, thick peptidoglycan layer (Jawetz et al, 2007). Gram-negative bacterial cell wall structure is more complex, layered three, namely the outer layer

of lipoprotein, the middle layer of lipopolysaccharide which acts as a barrier to the entry of antibacterial bioactive material and the inner layer of peptidoglycan with high lipid content (11-12%) (Hawley, 2003). Escherichia coli and Salmonella sp as gram negative have more complex layers and layered layers of cytoplasmic layers, peptidoglycan single layers and external membranes composed of lipoproteins and lipopolysaccharides. Gram-negative cell wall cells contain three components which are namely: the outermost membrane lipoproteins that contain protein molecules called porin and lipopolysaccharides. Porin in the outermost membrane of gram–negative bacterial cell wall is hydrophilic. Porin contained in the outer membrane causes the compounds contained in the extracts of the skin of the cocoa fruit which is difficult to enter into bacterial cells. The difference in the structure and components of this cell wall has an impact on Escherichia coli and Salmonella sp as a smaller gram-negative group inhibition diameter compared with gram-positive in various treatments. The result is similar to analysis of antioxidant activity so that the ratio of the best extract and solvent is 1: 4 ratio (R3).

4.Conclusion

Cocoa skin ethanol extract at the various material or solvent ratios contain bioactive components of theobromin group compounds, flavonoids, phenolic compounds and tannins. The treatment with the highest antioxidant activity resulted in 1: 4 (R3) solvent or solvent ratio of IC50 75.98 µg / mL and the lowest in material or solvent ratio of 1: 2 (R1) with IC50 value of 94,74 µg /mL. While for antimicrobial activity also obtained an increase in higher diameter inhibition at the ratio of 1: 4 (R3) with a diameter of inhibition between 4.3 mm and 12.15 mm. This means that the extract of cocoa fruit ethanol is very likely to produce natural preservatives.

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