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To cite this article: Rachmawaty *et al* 2018 *J. Phys.: Conf. Ser.* **1028** 012013

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Active Compounds Extraction of Cocoa Pod Husk (*Thebroma Cacao* L.) and Potential as Fungicides

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Abstract. The research on extraction bioactive compound of cocoa pod husk waste and antifungal activity assay by in vitro has been done. The aim of research was to get a phytochemical components extracted from the rind of cocoa, phenolic content and test the bioactivity of extract cocoa pod husk against on pathogenic fungus *Fusarium oxysporum*. Pod husks used are dried. Samples were extracted by maceration with acetone-water (7: 3) and 70% ethanol. Each extracts were then analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) to determine the active compounds found in the extract cocoa pod husk. The results obtained by GC-MS analysis showed three major components of the compound in ethanol *ie*: Methyl-9,12-Dienoate Octadeca; 9-octadecenoic acid (Z) -, Methyl Ester; Hexadecanoic acid, 15-methyl, Methyl Ester. Whereas in acetone solvent compounds showed 4 major components, namely: Isopropyl myristate; 1,2-Benzenedicarboxylic Acid, Dioctyl Ester; 9-octadecenoic acid (Z) -, Methyl Ester; Octadecanoic Acid, Methyl Ester. Acetone extract phenolic levels of 94.92 mg GAE / g extract and ethanol extract of 49.92 GAE / g extract. Antifungal testing by the agar diffusion method showed of extract cocoa pod husk with acetone able to inhibit the growth of the fungus *Fusarium oxysporum* (fungus pathogen on tomatoes), meaning that the extract has the potential to be developed as natural fungicides to suppress the growth of pathogenic fungi.

Keywords: Cocoa Pod Husk, Phenolic, Fungicides, *Fusarium oxysporum*

1. Introduction

Cacao (*Theobroma cacao* L.) is one of the country's export commodities Indonesia with high selling value. The Ministry of Agriculture reports that in 2010, Indonesian ranks third as the country's largest cocoa producer in the world with total exports amounting to 900 thousand tons [1]. At harvest time, farmers usually harvest the cocoa beans to be processed into chocolate and cocoa fruit leather waste quite a lot. The existence of these wastes are often not utilized well and sometimes go unpunished become agricultural waste. Waste pod husks produced in large quantities would be a problem if not handled properly because the production of solid wastes reaches more than 60% of the total fruit production [2].



Cocoa fruit skin has a chemical composition that is quite complex. One of the chemicals it contains is phenol. Phenol is a chemical compound to be antimicrobial. Antimicrobial compound is a biological or chemical compounds that can inhibit the growth and activity of microbes. Based on this, the cocoa fruit skin has the potential to be used as an alternative controller to inhibit the growth of microbial pathogens that cause plant diseases. The chemical composition of the cocoa fruit skin is Water 12.98% Total 32.52% N, 9.65% protein, 0.15% fat, crude fiber and ash 33.9% 10.8% [4].

Utilization of scientific research pod husks as vegetable fungicide has not been done. So it requires deeper study on the ability of the corresponding pod husks in suppressing the growth of fungi *F. oxysporum*.

F.oxysporum fungus is one type of fungus that is very important to know in carrying out cultivation. This type of fungus, into the host so many plant species, ranging from plants, which means positioned to hedge in the garden farmers. *F.oxysporum* have a high variety of species, about 100 species and caused extensive damage in a short time with the intensity of the attack reached 35% [5]. *F.oxysporum* fungus is one type of soil borne pathogens deadly, because it has a pathogenic strain that can be dormant for 30 (thirty) years before continuing virulence and infect plants. *F.oxysporum* fungus is the cause wilt and stem rot disease on different types of crops, horticulture and gardening. The host of this pathogen are vegetables, onions, potatoes, tomatoes, cabbage, radish, chinese cabbage, mustard-finding meeting, watermelon, melon, papaya, bark, chrysanthemum, orchid, green beans, peppers, cucumber, guava and ginger. Another plant pathogen known to be the host of this oil palm, coconut, pepper, vanilla, and cotton [6].

Study pod husks as natural fungicide has not been widely studied by previous researchers. While research to find alternatives plant disease control that are effective, safe and has great potential is a challenge for agriculture today. Pod husks one of natural fungicide that could be developed in the field of agricultures because it contains phenolic as the antimicrobial. So that the necessary studies on cocoa pod husk as natural fungicide to control fusarium wilt caused by *F. oxysporum*.

Based on the description above, it is necessary to investigate phytochemical components extracted from pod husks and know the phenolic content of each solvent used and its potential as a natural fungicide.

2. Materials and Methods

2.1. Material

The main materials used in the experiment is a skin cacao (*Theobroma cacao* L.) dry, ethanol, acetone and aquabidest. Raw material for qualitative analysis of phytochemicals is distilled water, hydrochloric acid, magnesium, sulfuric acid, acetic acid anhydride, gelatin 2% and reagent Dragendof. Materials used to test the antifungal activity of phenolic and testing is gallic acid, Folin-Ciocalteu reagent, paper disc, Potato Dextrose Agar (PDA) and isolates of *Fusarium oxysporum*.

2.2. Methods

The method used is an experimental method that analyzed descriptively by comparing the two types of extraction treatments are:

- extraction using acetone-water 7: 3 (v/v)
- extraction using ethanol 70%

2.3. Extraction of Cocoa Pod Husk

Pod husks dry milled using a grinder to form a powder. Bark powder sifted cocoa beans using sieve size of 80 mesh. The powder is then weighed each of 20 g and then put in a flask. The solvent is added about 200 mL respectively, so that the ratio between the sample and the solvent is 1:10, then do maceration for 24 hours at room temperature in an enclosed and protected from direct light. The filtrate was separated from the residue, then concentrated using a rotary evaporator at a temperature of 45°C, to obtain a thick rind extract of cocoa.

2.4. Analysis Using Gas Chromatography - Mass Spectrometry (GC-MS).

The sample used in 1 mL, then injected into the GC-MS. Characteristics of the GC-MS is used for the brand Shimadzu with type QP2010S, the temperature of the injector 280°C, injector split mode, the sampling time of 1 minute, the column temperature 40 - 2700c with setting the initial temperature of 40°C was held for 5 minutes, and 10 minutes to reach a temperature of 2700c (230C / min) were detained for 60 minutes, so the total time 88 minutes program, detector temperature 280°C, a temperature of 250°C interval, He carrier gas, the main pressure 500-900, flow control pressure mode, the pressure of 10.9 kPa, total flow 58, 8 ml / m, column flow of 0.55 ml / m, linear acceleration 26.0 cm / sec, purge flow 3.0 ml / m, split ratio of 99.8, RTX-5 ms column type, column length 30.00 m, thickness μm 0:25 , diameter of 0.25 mm.

2.5. Determination of Phenolic Content

0.1 g sample extract is then dissolved 10 mL of distilled water. 0.2 mL of the extract sample was added 15.8 mL of distilled water and 1 mL of reagent Folin-Ciocalteau, mixed. Let stand 10 minutes and add 3 mL of 20% Na_2CO_3 solution, let stand for 2 hours at a temperature of 30°C. Measure by UV absorption at a wavelength of 765 nm.

2.6. Test bioactivity fungicides.

2.6.1. Preparation of fungal cultures for testing [6]. Stock fungus *Fusarium oxysporum* used for testing were grown in agar slant. Before use, the fungus *F. oxysporum* rejuvenated in advance to obtain optimum growth conditions. Aseptically inoculated mushroom mycelium on PDA medium (Potato Dextrose Agar) in a petri dish. Inoculum are incubated for 72 hours at a temperature of 30°C. Furthermore, the inoculum can be used for testing.

2.6.2. Formulation extract cocoa pod husks. The crude extract pod husks fungicide formulated into a vegetable by adding water mixed with Tween-80. the percentage concentration of the extract consisting of 0% (control), of 0.02%; 0.2%; 2% (g / mL). As a positive control (+) is used antifungal Nystatin. The formulation may further be used to test the antifungal activity against *F. oxysporum* in vitro.

2.6.3. Antifungal activity extract cocoa pod husk. Testing is done by testing the antifungal activity of the crude extract of the cocoa pod husk against *F. oxysporum*. Petri dish that already contains 10 ml of PDA and 200 μl suspension of *F. oxysporum* allowed to solidify. After a solid, well diffusion made each 2 pieces on each Petri dish using a 5 mm diameter cork borer. Each well is filled with 20 μl diffusion rough rind extract cocoa extract concentration is different. Inhibition was determined by measuring the diameter of inhibition zone extract on the growth of fungal colonies, as measured on the second day after inoculation. As a positive control used antifungal (Nystatin).

3. Results and Discussion

3.1. Phytochemical components

Qualitative test phytochemical components have been carried out and reported in the extracts tested. Qualitative determination can be seen from the change in color or the formation of scum and sludge if the sample was treated with certain chemicals. Results of qualitative testing phytochemical components cocoa fruit skin extracts indicate that cocoa fruit peel extract on every acetone and ethanol contains phytochemical components such as alkaloids, flavonoids, tannins and saponins, triterpenoids whereas only identified in the extract using ethanol 70% [7]. The presence of compounds such as alkaloids, flavonoids, tannins, saponins and triterpenoid showed that the cocoa pod husks extract has potential as an antimicrobial.

3.2. Analysis using GC-MS.

Continuing phytochemical test, then analyzed using GC-MS. Type of phytochemical found in the cocoa pod husk extract can be seen in Table 1.

Table 1. Data Analysis Using GC-MS

No.	Smples	Compound
1	ethanol	- Octadeca Methyl-9,12-Dienoate - 9-octadecenoic acid (Z) -, Methyl Ester - Hexadecanoic acid, 15-methyl-Methyl Ester
2	acetone	- Isopropyl myristate - Benzenedicarboxylic Acid - 9-octadecenoic acid (Z) -, Methyl Ester - Octadecanoic Acid, Methyl Ester

The results of GC-MS on the samples showed that the fraction was detected seven main peak of the second solvent, the seventh highest main compound Methyl-9,12-Dienoate Octadeca, 9-octadecenoic acid (Z) -, Methyl Ester, hexadecanoic Acid, 15-Methyl - Methyl Ester, Isopropyl myristate; Benzenedicarboxylic Acid; 9-octadecenoic acid (Z) -, Methyl Ester and Octadecanoic Acid, Methyl Ester. Table 2 component compounds and potential utilization of extract cocoa pod husk.

Table 2. Components of the compound and its utilization

No.	component compound	potential activity	Reference
1	Octadeca Methyl-9,12-Dienoate	antimicrobials	[8]
		antioxidants	[9]
2	9-octadecenoic acid (Z) -, Methyl Ester	antioxidants	[10]
		Anticancer	[11]
		antimicrobials	[12]
3	Hexadecanoic acid, 15-methyl-Methyl Ester	antioxidants	[13]
		antimicrobials	[14]
		antifungal	[15]
4	Isopropyl myristate	antioxidants	[16]
5	Benzenedicarboxylic Acid	antimicrobials	[17]
		antioxidants	[18]
6	9-octadecenoic acid (Z) -, Methyl Ester	antimicrobials	[19]
7	Octadecanoic Acid, Methyl Ester	antimicrobials	[19]

3.3. Analysis of total phenolic content

Determination of the phenolic content of the extract cocoa pod husk conducted to determine the potential as an antimicrobial. Phenolic analysis using a standard solution of gallic acid. The use of gallic acid as a standard solution for the gallic acid is a derivative of hydroxybenzoic acids are classified as simple phenolic acids [8]. Total phenolic content of each extract expressed as gallic acid equivalents or Gallic Acid Equivalent (GAE). GAE is a common benchmark for measuring the amount of phenolic compounds contained in a material [20].

From the data calculation, acetone has a total phenolic extract the highest of 94.92 GAE / g extract compared to extract ethanol 49.92 GAE / g. The solubility of phenolic compounds depends on the solvent used. Cocoa fruit peel extract with the highest phenolic levels found in the extract in acetone: water (7: 3), while the lowest levels in the extract in ethanol. Phenolic bound to a sugar molecule at position 3-O-glycoside will tend to dissolve in polar solvents. In addition, the hydrogen bonds between the atoms O of acetone with the H atom of a hydroxyl group of phenolic compounds glycosylated also affect solubility. The presence of the methyl group is the driver of the electrons causes the electrons of O atoms to be rich. Thus the hydrogen interaction with phenolic compounds is easier. Although the same thing occurs in 50% acetone, but with the amount of water, the likely interaction of hydrogen

tends to occur between acetone and water. In the methanol and ethanol, H atoms in the solvent would reduce the possibility of hydrogen interaction with the sample for a stronger interaction with O atoms in the molecule itself. This will reduce the chances of the hydrogen bond with the H atom of the -OH group of phenolic compounds samples. [21] This will reduce the chances of the hydrogen bond with the H atom of the -OH group of phenolic compounds samples. [21] This will reduce the chances of the hydrogen bond with the H atom of the -OH group of phenolic compounds samples. [21]

Hydrogen bonding will occur between oxygen atoms have a free electron pair of hydrogen atoms of phenolic compounds with solvents and vice versa. Phenolic still bound to a sugar molecule on the skin are polar cocoa pods will tend to be more soluble in the solvent that has been diluted with distilled water. Because the bound glycoside has good solubility in water, compared to the still in a pure state, the type of solvent that has been diluted with distilled water to be able to extract the phenolic compounds with more number. [22]

3.4. Antifungal activity cocoa pod husk extract

The diameter of the resistor is a measure of the strength of the substrate antifungal barriers. Width diameter clear zone is formed is determined by the concentration of the compound which is the basis of testing quantitative and indicate the compound to diffuse freely throughout the medium. The test results antifungal activity cocoa pod husk extract can be seen in Table 3. The test results showed that the extract of acetone: water (7: 3) have a diameter greater inhibition than the ethanol extract.

Table 3. The results of the antifungal activity test

Extract	Inhibition zone diameter (cm) at <i>F.oxysporum</i>
Acetone: water (7: 3)	1.1
ethanol	-

3.5. Growing Minimum Inhibitory Concentration Acetone: water extract

Test minimum growth inhibitory concentration (KHTM) is an effort to find the lowest concentration that was able to inhibit the growth of microbial organisms. The test results KHTM acetone water (7: 3) extract are shown in Table 4. It is seen that the higher the concentration of extract of the more extensive zone of inhibition against the *F. oxysporium*

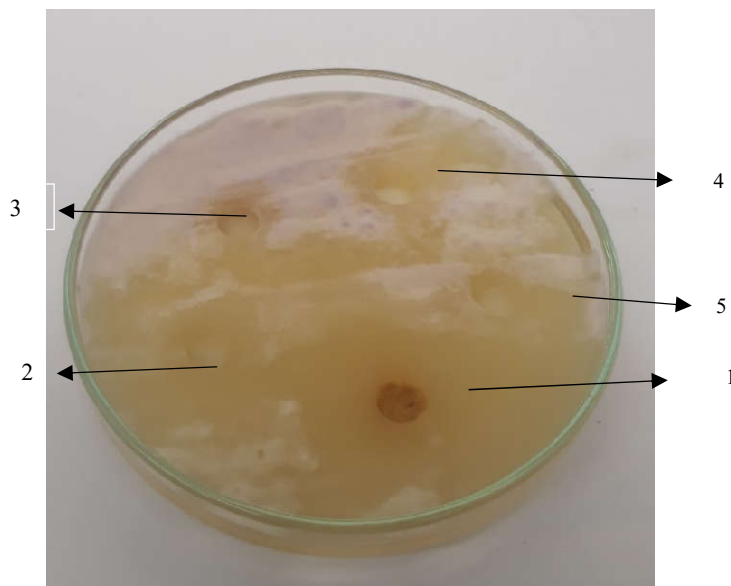
Table 4. The test results KHTM cocoa fruit peel extract

Concentration Acetone: water pod husks extract (%)	Inhibition zone diameter (cm) at <i>F.oxysporum</i>
0:02	0:09
0.2	0:43
2	0.68
K (+)	0.3
K (-)	0

The data in Table 4 and Figure 1 shows the MIC value was lowest in the extract of acetone: water (7: 3) with the extract concentration of 0.02%, broad zones of inhibition at 0:09 cm. The higher the concentration of the extract increased inhibition zones. Inhibition zone to extract concentration 0.2% greater than the positive control inhibition zone. Positive control used is Nystatin, whereas the negative control used is distilled water. Nystatin has fungistatic and fungicidal activity as in various pathogenic and non-pathogenic fungi. Nystatin bind with sterols, especially ergosterol in fungal cell walls, thereby disrupting the permeability of the fungal cell wall that serves as a selective barrier. The high ability cocoa fruit peel extract in inhibiting the growth of fungi *F.oxysporum*, this is because the phenolic compounds contained in extracts of nature can damage cell membranes, causing changes in cell permeability which can result in inhibition of cell growth or cell death [11]. Phenol compounds can also denature proteins and constrict the cell wall of cells that can lyse fungal cell walls. In addition, the phenolic compound through the hydroxyl group binds to sulfihidril group of fungal protein so as to alter the conformation of the target cell membrane protein [18]. In addition to

flavonoids and polyphenols, saponins in pod husks allegedly also contribute to the antimicrobial activity. However, it still needs further verification. Saponins have activity as an antifungal.

From the antifungal activity test data (Table 3), showed the pod husks ethanol extract does not inhibit the growth of fungi *F. oxysporum*. It deals with the condition of which is still not a pure extract. The amount of the compounds contained in the crude extract of ethanol has resulted in their mutually exclusive properties (antagonist) between the compounds in the extract, thereby reducing the antimicrobial activity of ethanol extract pod husks. Separation or fractionation of compounds in the extract more important role in improving the inhibitory ability against microbes. Kader et al [9] reported that ethanol extracts that have been fractionated have the lowest inhibitory ability of 128-256 mcg disk-1 when tested in the bacterium *E. coli*, *P. aeruginosa*, *S. typhi*, *B. cereus*, *S. lutea* and *V. parahemolyticus*.



- Remarks: 1. The extract of acetone: water (7: 3) 2%
 2. The extract of acetone: water (7: 3) 0.2%
 3. The extract of acetone: water (7: 3) of 0.02%
 4. Positive control (+)
 5. The negative control (-)

Figure 1. The clear zone formed on extracts of acetone: water (7: 3) when tested at *F.oxysporum*

4. Conclusion

Pod husks are extracted with acetone: water (7: 3) and ethanol contains chemical compounds such as flavonoids, phenolics, tannins and saponins. Extract acetone: water (7: 3) have a total content of phenolic and antifungal activity higher than the ethanol extract. Value KHTM extract acetone: water (7: 3) to the fungus *F. oxysporum* at 0:02%, 0.2% and 2% with each inhibition zone 0:09 cm, 0:43 cm and 0.68 cm. At a concentration of 0.2% inhibitory zone is greater than the positive control inhibition zone. This shows that the extract of acetone: water (7: 3) potential as vegetable fungicide to inhibit the growth of pathogenic fungi.

Acknowledgments

The research was funded by the Applied Research Products in 2017 by the Ministry of Research, Technology and Higher Education, for which we thank him profusely to Ministry of Research, Technology and Higher Education.

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