

RESEARCH ARTICLE

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**COMPARISON OF SOXHLETATION EXTRACTION METHODS AND UAE
ON ANTIFUNGAL ACTIVITIES *Candida albicans* ON COCOA BEAN
EXTRACT (*Theobroma cacao* L.)**

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ABSTRACT

Chocolate beans (*Theobroma cacao* L.) is the main part of the chocolate fruit and can be consumed after it becomes a food or beverage product. Cocoa beans contain quite high levels of active antioxidant compounds, some of which are 33-42% catechins, 23-25% leukocyanidins, and 5% anthocyanins. One of the bioactive compounds that are efficacious as natural antioxidants and antimicrobials is polyphenols. This study aims to determine the comparison of soxhletation and UAE extraction methods on antifungal activity *Candida albicans* on cocoa bean extract. The research design is an experimental laboratory. Processed using the soxhletation and UAE extraction methods with methanol as a solvent, then a phytochemical screening was carried out to determine the chemical compound content in cocoa beans and an antifungal test was carried out on *Candida albicans* by hole cup diffusion method with a concentration of 50%. The results showed that the yield values of the soxhletation and UAE method were 3.81 ± 0.0022 and 4.18 ± 0.0012 . Methanol extract of cocoa beans by soxhletation method and UAE contains chemical compounds of alkaloids, flavonoids, saponins, tannins and polyphenols. The results of the antifungal test by the soxhletation and UAE methods with a concentration of 50% produced an average inhibition zone diameter of 5.44 ± 0.5652 mm and 6.60 ± 0.6372 mm. It can be concluded that the extraction method does not affect the content of chemical compounds in cocoa beans and has an effect on antifungal activity *Candida albicans* which was indicated by the diameter of the inhibition zone but did not show a significant difference.

Keywords: Chocolate Beans, Soxhletation, UAE, *Candida albicans*

INTRODUCTION

Indonesia is a tropical country with fertile land with high temperature and humidity, making it a good place for mushroom growth. Therefore, diseases caused by fungi often attack people in Indonesia. *Candida* is the type of fungus that most often causes infection. *Candida* occurs on the surface of the skin, digestive tract, oral cavity, and vagina (Puspitasari *et al*, 2019). Growth *Candida albicans* in mouth which

causes canker sores. Thrush is a common condition of oral disease (Sandy and Irawan, 2018).

Indonesia is one of the world's largest cocoa producers and exporters. In 2020, Indonesia will become the third largest cocoa exporter in the world after Ivory Coast and Ghana (Arfitasari, 2015). Cocoa is a plant with a scientific name *Theobroma cacao* Linn. This plant originally came from the rain forests of South America and is now widespread in various tropical regions (Farhanandi and Indah, 2022). Cocoa plants are known to be rich in bioactive compounds, especially polyphenols, which act as natural antioxidants and antibacterials (Laboko and Nurhasafah, 2020). Cacao is also a plantation and industrial crop which is known as an export commodity besides oil and gas so it has very good prospects apart from cloves (Aprillia and Suryadarma, 2020).

Polyphenol compounds found in cocoa beans consist of flavonoids, catechins, procyanidins, anthocyanins and complex tannins (Diyantika *et al*, 2017). The overall percentage of polyphenolic compounds in cocoa powder is higher than that in wine and tea. The polyphenols that are abundant in cocoa are flavonoids, 15-carbon compounds found in cocoa beans which consist of two benzene rings connected by a carbon chain (Aprillia and Suryadarma, 2020).

The cocoa bean can be divided into three main parts: cotyledons (87.10%), husks (12%), and cotyledons (0.9%). The number of seeds per fruit is around 20-60 and the fat content is 40-59%. The oval-shaped seeds are slightly flat, measuring 2.5 x 1.5 cm. Cocoa beans are covered with white pulp. Seeds in pods stored at 5–10 °C die within 2 days (Martono, 2014). Cocoa beans contain 50-70% fat (cocoa butter), of which 34% stearic acid (18:0), oleic acid (18:1) and 25% palmitic acid (16:0), and 2% linoleic acid (18:1). 0) (Aprillia and Suryadarma, 2020). According to Damar (2020) in Lili *et al* (2022), cocoa beans are a source of monounsaturated fatty acids and contain healthy vitamins, minerals, fiber, natural carbohydrates, and protein (Lili *et al*, 2022).

These secondary metabolites are essential for plants to defend themselves and attract insects that aid in pollination. Secondary metabolites are available for other organisms (Julianto, 2019). Extraction is the process of separating the active ingredients from the resulting mixture by soaking them in a certain solvent (Prayudo *et al*, 2015). Extraction is divided into cold extraction (maceration, percolation, UAE) and hot extraction (socracy, reflux, digestion, decoction, infusion) (Hikmawanti *et al*, 2021).

Antifungals are substances produced by microorganisms, especially fungi (fungi) that can inhibit or destroy other types of microorganisms that are lethal or inhibit the growth of bacteria, but their toxicity to humans is relatively low (Minarni *et al*, 2020). The main goal of fungal control is to prevent the spread of disease and infection, eradicate the fungus in infected hosts, and prevent decay and destruction caused by the fungus itself (Munawwaroh, 2016).

Measurement of antimicrobial activity can be done by two main methods, namely dilution or diffusion (Fitriana *et al*, 2020). The diameter of the inhibition zone can be calculated in millimeters (mm) using a vernier caliper. The diameter of the inhibition zone is then classified as having antifungal activity (Kandoli *et al*, 2016).

RESEARCH METHODS

Production of Cocoa Simplicia Powder

Chocolate beans (*Theobroma cacao* L.) that has been harvested is carried out by wet sorting, after that the cocoa beans are taken by breaking the cocoa beans using a tool and the seeds are taken. Then it is fermented and roasted at 50 °C. Then the cocoa beans are separated from the seed coat which will later be made into a paste and pressed to

remove the cocoa bean fat. After that, it is roasted again and sieved (Coffee and Cocoa Research Center, 2023).

Extract Manufacturing Soxhletation

100 grams of cocoa powder is weighed with filter paper and tied with twine. Next, prepare 500 ml of methanol. The extraction process was carried out at 50 °C until the color of the drop cycle was clear. The resulting liquid extract was then concentrated using a rotary evaporator to obtain a thick extract (Samudra *et al.*, 2022) replicated 3 times.

Ultrasound Assisted Extraction (UAE)

100 grams of cocoa powder that has been weighed and then put in an Erlenmeyer and added 500 mL methanol solvent, and extracted in an ultrasonic bath with a frequency of 37 kHz for 20 minutes. Solvent Then remove the solvent using *rotary evaporator* with a speed of 40 rpm and a temperature of 45 °C, then concentrated in a water bath at 50 °C (Tutik *et al.*, 2022) replicated 3 times.

Dilution of Cocoa Seed Extract

This study used an extract concentration of 50% based on research from Angelina (2012). Weigh the extract 2.5 g dissolved in 5 mL of 10% DMSO.

Positive Control and Negative Control

The positive control used in this study was a suspension of 100,000 IU/mL nystatin and a negative control of 10% DMSO. Micropipette 20 µL of each positive and negative control 10% DMSO and pipet into the hole cups that have been prepared (Liliany, 2018).

Phytochemical Screening

Alkaloid

As much as 0.5 gram of concentrated extract was put into a test tube, dissolved with 5 mL of 2N HCl, then divided into three tubes. Each tube is added a different reagent *Mayer*, *Dragdroff* and *Wagner*. Positive results when on the reagent *Mayer* a white precipitate forms in the reagent *Dragendroff* yellowish-orange precipitate formed, and the reagent *Wagner* brownish red precipitate formed (Ramadhan *et al.*, 2020).

Flavonoid

0.5 g concentrated extract was added to 5 ml of distilled water, heated for about 5 minutes and filtered. 0.05 mg of magnesium and 1 ml of concentrated HCl were added to 2 ml of the filtrate and then shaken until homogeneous. The result is considered positive if a yellow-orange color is formed (Ramadhan *et al.*, 2020).

Saponin

0.5 g concentrated extract was added to 5 mL of boiling distilled water and shaken vigorously for about 10 seconds. If the bubbles remain approximately 1 cm, this is considered positive (Ramadan *et al.*, 2020).

Tannin

2 mL of concentrated extract was put into a test tube and 2 mL of FeCl₃ was added. The result is considered positive if it forms a greenish black color (Herman *et al.*, 2020).

Polyphenols

3-4 drop of FeCl₃ added to 1 mL of concentrated extract. The color change from bluish black to dark indicates the presence of polyphenols (Manongko *et al.*, 2020).

Tool Sterilization

The tool is wrapped in brown paper, before use it is sterilized first using an autoclave at 121 °C and 1.5 psi pressure for 15 minutes. Tools that cannot stand heat can be sterilized with 70% alcohol (Munawwaroh, 2016).

PDA Media Preparation (*Potato Dextrose Agar*)

Weigh 7.8 grams, then 200 mL of distilled water is dissolved in Erlenmeyer. Then heat the media on a hot plate and magnetic stirrer until the solution is homogeneous. After complete dissolution, sterilize in an autoclave at 121 °C for 15 minutes (Fadli, 2017).

Preparation and Antifungal Activity Test

Mushroom Rejuvenation *Candida albicans*

Take 1 ose culture *Candida albicans* pure on PDA media, then streaked aseptically on oblique PDA media that has solidified in a test tube and incubated at 37 °C for 2x24 hours (Purnamasari, 2021).

Making Turbidity Standard 0.5 *McFarland*

The most commonly used turbidity standard in clinical microbiology laboratories is the standard *McFarland* 0,5. Pipette 0.05 mL of BaCl₂ solution 1% into a volumetric flask, add 9.95 mL of H₂SO₄ 1%, and homogenize with a vortex. Then pour the 1% BaCl₂ and 1% H₂SO₄ into a tube as much as 10 mL (Simpson *et al.*, 2014).

Suspension Manufacturing *Candida albicans*

Pure mushroom culture *Candida albicans* which has been rejuvenated is then taken using an ose needle that has been ignited previously. Then resuspended in 10 ml of 0.9% NaCl solution and homogenized using a vortex. Suspension *Candida albicans* then measured using a spectrophotometer with standard turbidity *McFarland* of 0.5 at 625 nm in order to obtain a standard inoculum suspension equivalent to turbidity *McFarland* (Sanny, 2022). For the same turbidity, the concentration of the fungus is 10⁸ CFU/mL which means the density of 100 million fungi in 1 mL of suspension (Fitria, 2020).

Determination of Antifungal Activity by Hole cup Method

Antifungal activity *Candida albicans* in this study using hole cup diffusion. PDA media was poured into sterile Petri dishes. After the PDA media solidified, 100 µL of the test mushroom inoculum was pipetted with a micropipette and then put into a Petri dish. Gradually flatten the mushroom inoculum with the L rod until it is even on the PDA media which already contains the fungal inoculum (Purnamasari, 2021). Hole cups are made using *corn born*. Add 40 µL of cocoa bean extract to the prepared hole cups and incubate at 37 °C for 24 hours. The clear area seen around the hole cup was observed and measured (Nurhayati *et al.*, 2020).

Data Analysis

The data research were processed using the SPSS statistical test (*Statistical Product and Service Solutions*) namely test *One-Way ANOVA*, with a significant difference of 5% p-value (Sig.) < 0.05 to determine the bacterial inhibition zone *Candida albicans* on the methanol extract of cocoa beans and for the content of chemical compounds were analyzed using descriptive analysis.

RESULTS and DISCUSSION

Cocoa bean extract was prepared by the Soxhletation and UAE extraction methods using methanol as a solvent. Methanol solvent was chosen in this study because

it contains few carbon atoms which is an organic solvent that is more polar than ethanol. Therefore, the concentration of total flavonoids in methanol is higher than in ethanol because the compounds that bind to ethanol are more non-polar than methanol. This is in accordance with research by Apriasari (2015) that the methanol extract using Maui banana stems contains more flavonoids compared to the ethanol extract of Maui banana stems. Therefore, the methanol extract has higher antifungal activity compared to the ethanol extract.

Tabel 1. Cocoa Bean Extraction Results

Extraction Method	Replication	Thick Extract (grams)	Yield (%)	Average $\pm$ SD
Soxhletation	1	3,75	3,75	3,81 $\pm$ 0,2219
	2	4,06	4,06	
	3	3,63	3,63	
UAE	1	4,19	4,19	4,18 $\pm$ 0,1206
	2	4,05	4,05	
	3	4,29	4,29	

From table 1 it can be seen that the Soxhletation extraction method gives the highest results, while the UAE extraction method gives the lowest results. The higher the yield value, the more valuable the resulting extract. The yield value determined in this study still meets the requirements for the yield value of cocoa bean extract according to Indonesian Herbal Pharmacy (FHI), provided that the yield of the two extracts is 10% or more.

In addition, a phytochemical screening test was performed for both extraction methods. Phytochemical screening of cocoa beans includes alkaloids, flavonoids, saponins, tannins and polyphenols. Phytochemical screening is the first step in phytochemical research and its purpose is to provide an overview of the class of substances contained in the plant being examined (Agustin, 2022).

Tabel 2. Phytochemical Screening Results of Cocoa Beans

Compound	Reagent	Soxhletation	UAE
Alkaloid	Mayer	(+)	(+) (+)
	Dragendroff	(+)	(+)
	Wagner	(+)	(+)
Flavonoid	Serbuk Mg dan HCl pekat	(+)	(+) (+)
Saponin	Aquadest	(+) (+)	(+)
Tannin	FeCl ₃	(+)	(+) (+)
Polyphenols	FeCl ₃	(+)	(+) (+)

From table 2. It can be seen that the results of examination of the chemical compound class showed that shallot skin extract contains alkaloids, flavonoids, saponins, tannins and polyphenols. The results of this phytochemical screening are in line with research conducted by Nuraskin *et al*, (2022) showed that the results of the identification test of methanol extract of cocoa beans contained alkaloids, terpenoids, saponins, flavonoids, phenolics and tannins. The alkaloid test uses 3 reagents *Mayer*, *Dragdroff* and *Wagner*. The test results are indicated by the presence of a white precipitate with the reagent *Mayer*, an orange-yellow precipitate with the reagent *Dragdroff* and brownish red precipitate with reagent *Wagner*. Testing for flavonoids is indicated by the presence of orange-yellow color. The saponin test was shown by the presence of stable foam with a length of 2 cm or less, within 10 minutes the foam did not disappear (Ramadhan *et al*,

2020). The tannin test showed the formation of a greenish black color (Herman *et al.*, 2020). This polyphenol test shows the presence of a greenish black color (Manongko *et al.*, 2020).

Antifungal activity was measured by the agar diffusion method using hole cups. This method was chosen because it can easily measure the inhibition zones formed around the hole cups and the active bacteria that live not only on the top but also on the bottom of the agar media, the procedure is simple, and the expected results can be obtained.

Tabel 3. Inhibition Zone Measurement Results

Sample	Replication	Cocoa Bean Extraction Method			
		Soxhletation (mm)	Average±SD	UAE (mm)	Average ±SD
K+	1	24,74		23,02	
	2	25,64	24,47±0,6574	24,51	23,88±0,7712
	3	26,02		24,11	
K-	1	0		0	
	2	0	0	0	0
	3	0		0	
Treatment	1	4,79	*	6,74	*
	2	5,66	5,44±0,5652	7,15	6,60±0,6372
	3	5,85		5,90	

Description: * : Significant difference with K+

The results of the antifungal test can be seen in table 3 which shows the methanol extract of cocoa beans inhibits the *Candida albicans* fungus. This is indicated by the presence of a clear zone around the formed hole. The results of the UAE antifungal test showed greater inhibition with an inhibition value of 6.60 mm ± 0.6372 compared to 5.44 mm ± 0.5652 with the soxhletation method. In other words, it shows the ability to destroy the test bacteria in the body. Cocoa seed extract was best inhibited by the UAE method. According to Ibrahim *et al.*, (2015), if the extraction temperature is too high and the extraction time is too long then the compound may be lost in solution through evaporation, and vice versa if the extraction temperature is too low, not all of the active substance is extracted from the material and forms a weak active compound. Adapted from Handayani and Sriherfyna (2016) in Yuliantari's research *et al.*, (2017) stated that bioactive ingredients such as flavonoids cannot survive at temperatures above 50 °C, which can cause structural changes and reduce the rate of extraction. If the extraction temperature is too low or the extraction time is too short, the yield will be low

The difference in the size of the inhibition zone can be seen in Figure 1. which is formed due to differences in the content of the active antifungal ingredients contained therein and the diffusion speed of the agar medium used. According to Handayani (2016) in Agustin (2022) that the extraction using the sonication method will contain more flavonoid compounds, therefore the results of the extraction using the sonication method have a large inhibitory effect. In Angelina's research (2012) that at concentrations of 12.5%, 25% and 50% showed a decrease in the number of fungal colonies *Candida albicans*. The decrease in the number of colonies was caused by the higher the concentration of cocoa bean extract, the more polyphenolic compounds in the solution. Polyphenols have microbicidal activity which is a compound that functions to reduce microbial infections such as viruses, bacteria or fungi. In addition, polyphenols also carry out other interactions by reducing microbial attachment activity required for host colonization and affecting protein transport in cell membranes (Angelina, 2012).

The formation of an inhibition zone around the hole cups is caused by the activity of compounds that act as antifungal against *Candida albicans*. The difference in the

content of these compounds has a different mechanism of action in inhibiting the fungus *Candida albicans*. Screening results showed that cocoa bean extract contains phytochemicals such as alkaloids, flavonoids, saponins, tannins and polyphenols which can inhibit the growth of fungi.

Tabel 4. Normality Test Results

Treatment Group	Statistic	df	Sig.	Result
Positive Control	0,971	6	0,896	Usual
Negative Control	-	3	-	-
Soxhletation 50%	0,879	3	0,323	Usual
UAE 50%	0,962	3	0,626	Usual

Tabel 5. Homogeneity Test Results

		Levene Statistic	df1	df2	Sig.	Result
Test Results	Based on Mean	2,044	3	11	0,166	Homogeneous
	Based on Median	1,730	3	11	0,219	Homogeneous
	Based on Median and with adjusted df	1,730	3	8,021	0,238	Homogeneous
	Based on trimmed mean	2,029	3	11	0,168	Homogeneous

Tabel 6. One-Way ANOVA Test Results

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1611,495	3	537,165	811,554	0,000
Within Groups	7,281	11	0,662		
Total	1618,775	14			

Tabel 7. Duncan's Post-Hoc Test Results

Duncan ^{a,b}				
Perlakuan	N	Subset for alpha = 0.05		
		1	2	3
Control - DMSO 10%	3	0,0000		
Socletation 50%	3		5,4333	
UAE 50%	3		6,5967	
Control + Nystatin	6			24,6733
Sig.		1,000	0,088	1,000

Based on table 4. it can be seen that the results of the normality test indicate that the data is normally distributed with a significant value *Shapiro Wilk* for each treatment group of $P > 0.05$, then the test can be continued *One-Way ANOVA*. Based on table 5. it can be seen that the results of the homogeneity test indicate that the significant value of $P > 0.05$, which means that the data is homogeneous, therefore it can be continued with the test *One-Way ANOVA*. Table 6 shows that the antifungal activity test of cocoa bean extract using the soxhletation and UAE extraction methods had a significant value of 0.000 with a real value of $P < 0.05$. So it can be seen that the extraction method affects the diameter of the growth inhibition zone of the fungus *Candida albicans*.

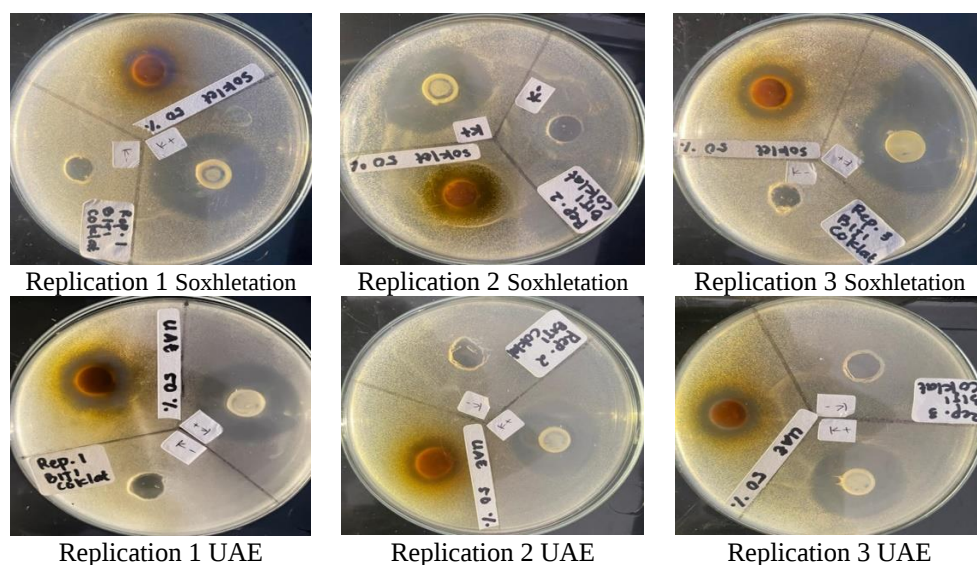


Figure 1. Antifungal Test Results of Methanol Extract of Cocoa Beans

The results of the antifungal activity test were processed using the SPSS statistical test, namely test *One-Way ANOVA* can be seen in table 7. The requirements for the ANOVA test are normality and homogeneity. This study resulted in a significant positive control value $p = 0.896$, for the soxhletation method 50% $p = 0.323$ and for the UAE method 50% $p = 0.626$. Because the significance value of each sample is $p > 0.05$, it can be concluded that the variance of the data is normally distributed. Then a homogeneity test was carried out so that a significant value was obtained with the *Levene* statistic $p = 0.238$. The significance value of the sample is $p > 0.05$ so it can be concluded that the data is spread homogeneously. The data for the normality and homogeneity tests meet the requirements, so the data can be continued with the test *One-Way ANOVA* with a significant value of $p < 0.05$.

In addition, as a follow-up test, a test was carried out *Post-Hoc* Duncan to see if there is a significant difference. Test *Post-Hoc* Duncan in this study showed that there was no significant difference between cocoa bean extraction methods on antifungal activity *Candida albicans*. This is supported by research conducted by Sanny (2022) at a concentration of 50% ethanol extract of cocoa beans using disc diffusion that the extraction method did not show any significant differences in results.

CONCLUSION

Based on the results of the antifungal test it can be concluded that the extraction method does not affect the chemical compound content but affects the antifungal activity *Candida albicans* indicated by the diameter of the inhibition zone. Where the value of the diameter of the inhibition zone between the soxhletation method and the uae method did not show any significant difference.

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