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Anti-Typhoid Activity Test Of Cacao Leaf Fraction (Theobroma Cacao L.) Against *Salmonella typhi* Bacteria In Vitro

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ABSTRACT

Cocoa leaves (*Theobroma cacao* L.) are known to contain compounds with potential antibacterial properties. This study aims to investigate the antibacterial activity of various cocoa leaf fractions against Salmonella typhi. Methods: This experimental study involved the extraction of cocoa leaves followed by fractionation using polar, semi-polar, and non-polar solvents. The antibacterial activity was tested using the agar diffusion method to measure the inhibition zones produced by each fraction against Salmonella typhi. The positive control group used chloramphenicol at a concentration of 400 ppm, while the cocoa leaf fractions were tested at various concentrations. An ethanol-free test was also conducted to ensure no ethanol contamination in the fractions tested. Results: The results showed that the water, ethyl acetate, and n-hexane fractions of cocoa leaves exhibited significant antibacterial activity against Salmonella typhi. The water fraction at a concentration of 600 ppm showed the largest inhibition zone with a diameter of 27.30 mm, which is close to the effectiveness of the positive control chloramphenicol, which had an inhibition zone of 27.70 mm. The ethanol-free test confirmed that no ethanol was detected in the three fractions. Conclusion: Based on the data obtained, cocoa leaf fractions, especially the water fraction, have the potential to be effective natural antibacterial agents against *Salmonella typhi*. This finding provides a basis for further development in the use of cocoa leaf extracts as an alternative treatment for bacterial infections.

Keywords : Agar diffusion. Cocoa leaves, antibacterial activity, *Salmonella typhi*,

INTRODUCTION

Typhoid fever is a serious infectious disease caused by the bacteria Salmonella typhi, which infects the bloodstream. It is included in the category of enteric fevers along with paratyphoid fever caused by Salmonella paratyphi. Both types of fever show similar symptoms, but paratyphoid fever generally produces milder clinical symptoms.

Transmission of typhoid fever is mainly through consumption of contaminated water or food. Clinical symptoms of the disease include prolonged high fever, abdominal pain, diarrhea, anemia, leukocytosis, gastrointestinal bleeding, intestinal perforation, septic shock, and other complications such as Superior Mesenteric Artery Syndrome (SMAS) and Splenic Vein Thrombosis, which can be fatal if not treated properly.

According to global epidemiological data, typhoid fever causes between 11 and 21 million cases with about 200 thousand deaths each year. Paratyphoid fever also adds to the burden of this disease with about 5 million cases per year worldwide.

In the context of traditional medicine, various plants have been used to treat bacterial infections.[4]One of them is cocoa leaves (Theobroma cacao L.), which are known to contain flavonoids, saponins, and tannins, which have the potential to have antibacterial activity. This study aims to test the effectiveness of cocoa leaf extract fractions in inhibiting the growth of Salmonella typhi bacteria.

METHODS

This study is a true experimental study aimed to evaluate the antibacterial activity of cocoa leaf extract fractions (Theobroma cacao L.) against Salmonella typhi bacteria. Cocoa leaves were obtained from plantations in Nangkaan Village, Bondowoso Regency, East Java. After the leaves were obtained and determined, the leaves were then washed, dried in an oven at a temperature of no more than 60°C, then ground and sieved using sieve no. 40.

Extraction was carried out by maceration using 70% ethanol for 5 days, followed by evaporation using a rotary evaporator to obtain a thick extract. The extract was then fractionated using water, ethyl acetate, and n-hexane as solvents. Each fraction was tested for its phytochemical content using TLC to identify flavonoids, tannins, saponins, alkaloids, and terpenoids and steroids.

Antibacterial activity was tested by the well diffusion method using Mueller Hinton Agar (MHA) medium and Salmonella typhi obtained from the Pharmaceutical Microbiology Laboratory of the Strada Indonesia Institute of Health Sciences. The positive control in this study was chloramphenicol. The test results were seen from the clear zone formed around the well, which indicated inhibition of bacterial growth.

The materials and tools used include various standard laboratory equipment and chemical reagents needed for extraction and antibacterial testing. Research materials used include cocoa leaves (Theobroma cacao L.), distilled water, FeCl3, NaCl, HgCl2, Violet Crystals, acetic acid, ammonia, HCL, 96% ethanol, 70% ethanol, Mg powder, ethyl acetate, N-Hexane, Salmonella typhi bacteria, Mc Farland solution, Mueller Hinton Agar (MHA) media, Mueller Hinton Broth (MHB), Salmonella Shigella Agar (SSA), DMSO 10%.

The tools used in this study include Erlenmeyer flasks, test tubes, funnels, measuring flasks, droppers, glassfirn, volume pipettes, petri dishes, stirring rods, measuring cups, spoons, well punches, autoclaves, rotary evaporators, incubators, microbiology safety cabinets, busen, ose needles, filter paper, analytical scales, rulers, sieves no. 40, silica gel plates F254. The research was conducted at the Pharmacy Study Program Laboratory of the Strada Indonesia Institute of Health Sciences for 1-2 months.

Data analysis in this study used SPSS for Windows software with the One Way Analysis of Variance (ANOVA) method to compare the average data between one treatment group. The level of significance used was 0.05. The ANOVA test was chosen because the data was numerical with one independent variable and one treatment group. Before the ANOVA test, a normality test was performed to ensure the normal distribution of the Salmonella typhi bacterial inhibition zone diameter data, as well as a homogeneity test to assess the uniformity of the data. If the ANOVA test results showed a significant difference ($P < 0.05$), a Post Hoc Duncan test was performed using the LSD (Least Significant Difference) method to identify significant differences between the test and control groups. This analysis aims to assess the effect of cocoa leaf extract fractions on the growth of Salmonella typhi bacteria by measuring the diameter of the bacterial inhibition zone after 24 hours.

RESULTS

In this study, the characteristics of the test sample involved cocoa leaf fractions (Theobroma cacao L.) obtained from Nangkaan Village, Bondowoso Regency, East Java. The object of this study used the Salmonella typhi bacterial population obtained from the Pharmaceutical Microbiology Laboratory of the STRADA Indonesia Institute of Health Sciences.

The bacterial preparation process includes rejuvenation, identification, and making a suspension that meets the Mc Farland 0.5 turbidity standard. The sampling technique used is random sampling, ensuring that each member of the population has an equal opportunity to be selected as a sample.

This study consisted of five treatment groups, including positive and negative controls, and three treatment groups with water, ethyl acetate, and n-hexane fractions from cocoa leaves. Evaluation of antibacterial activity was carried out using the well diffusion method, with inhibition zone measurements using a caliper to ensure data consistency and validity.

Determination

Plant identification is the process of identifying plant species based on morphological, anatomical, ecological, and other characteristics. It involves the use of identification keys or guides to distinguish between different types of plants (Callahan, CG, et al., 2015).

The plants used in this study were cocoa plants that had been identified at the UPT Materia Medica Batu City, Malang, East Java. Based on the determination results, the cocoa plant was identified as Theobroma cacao L. using a determination key consisting of the following series 1b-2b-3b-4b-6b-7b-9b-l 0b-11 b-12b-1 3b-14a-15a-l 09b-ll 9b-120b-128b-129b-135b-136b-139b--140b-142b-143a-144b-l 45b: Sterculiaceae -lb-3b-4b-5b-6b: Theobroma -7: T.cacao other characteristics.

Examination of Characteristics of Simple Drugs

1) Drying Shrinkage Test of Simple Drugs

The results of the dry shrinkage test on cocoa leaves, where the wet weight of the cocoa leaves was initially 5.3 kilograms, decreasing to 2.15 kilograms after the drying process.

The test results showed that the percentage of drying shrinkage obtained was 59.43%. This indicates that the drying process successfully reduced the water content in cocoa leaves by 59.43%, thereby increasing the durability and quality of cocoa leaves for the next stage. The results of the Dry Shrinkage Test can be seen in the following table:

Table 1. Cocoa Leaf Drying Shrinkage Test Results

Sample	Wet Leaf Weight	Dry Leaf Weight	% Results
Cocoa Leaves	5.3 kg	2.15 kg	59.43%

2) Water Content Test of Simple Powder

The results of the water content test of the simplicia powder showed a percentage value of 7.10%, this indicates that the drying process has reduced the initial weight of the cocoa leaf simplicia by 7.10%. The water content test of the cocoa leaf simplicia powder using the Moisture Analyzers tool can be seen in Table 2.

Table 2. Cocoa Leaf Simplicia Powder Water Content Test Results

Sample	Initial Weight	Final Weight	% Results
Cocoa Leaf Simplisia	2,001 g	1,859 g	7.10%

Examination of the Characteristics of Cocoa Leaf Extract (Theobroma cacao L.)

1. Organoleptic

ResultsOrganoleptic evaluation of cocoa leaf extract in this study noted that the extract has characteristics in the form of a dark brown color, a thick consistency, and a distinctive aroma that resembles the smell of cocoa leaves.

2. Extract Yield

The purpose of calculating the yield percentage is to compare the initial weight of cocoa leaf simplicia powder with the weight of the resulting extract, so that it can provide information on the quality of the extract. The results of the calculation of the yield of cocoa leaf extract can be found in Table 1.3.

Table 1**.** Cocoa Leaf Extract Yield Results

Sample	Weight of Simple Ingredients	Extract Weight	% Results
Cocoa Leaves	2.15 kg	146 g	6.79%

Cocoa Leaf Fraction (Theobroma cacao L.)

The cocoa leaf extract then undergoes a fractionation process to separate the compounds contained in the cocoa leaves based on their level of polarity, according to the method explained by Sari (2012).[6]

Fractionation was carried out using solvents of different polarities, namely Water as a polar solvent, Ethyl acetate as a semi-polar solvent, and n-hexane as a non-polar solvent. The results of the cocoa leaf fractionation process can be found in Table 4.

Table 2 Cocoa Leaf Extract Fraction Yield Results

No.	Sample	Extract Weight	Fraction Weight	Yield Calculation Results
1.	Water Fraction	146 grams	117.74 grams	80.64%
2.	Ethyl Acetate Fraction	146 grams	8.15 grams	5.58%
3.	N-Hexane Fraction	146 grams	0.49 grams	0.33%

Results of TLC Identification of Cocoa Leaf Fractions (Theobroma cacao L.)

The results of compound testing can be seen in Table 1.3 with the results of various fractions and mobile phases indicating the presence of alkaloid, tannin, flavonoid, saponin, and steroid/terpenoid compounds in all fractions except for steroids/terpenoids in the water fraction which were absent. The following are the results of the identification of TLC compounds of cocoa leaf fractions at 254 nm UV light:

Table 4 Results of TLC Compound Identification of Cocoa Leaf Fractions

Test Sample	**Results of Compound Identification Using the TLC Method**				
Water Fraction					
Ethyl Acetate Fraction					
N-Hexane Fraction					
Test Compound	Alkaloid	Flavonoid	Tannin	Saponins	Steroids/ Terpenoid

Identification of *Salmonella typhi* ATCC 14048 Bacteria

The purpose of bacterial identification is to identify the identity of the test bacteria used in the study. The bacteria tested in this study were Salmonella typhi obtained from the Pharmaceutical Microbiology Laboratory of University Strada Indonesia.

Bacterial identification was conducted at the Pharmaceutical Microbiology Laboratory of University Strada Indonesia and involved a series of tests, including tests on selective SSA agar media, microscopic tests (Gram staining), macroscopic tests, and biochemical tests (TSIA & LIA). The results of the identification of Salmonella typhi ATCC 14048 bacteria can be seen in Figure 1.1.

(a) (b) (c) (d) (e)

Figure 1. 1 Results of bacterial identification *Salmonella typhi*

Selective agar media test SSA, (b) Microscopic test (Gram staining), (c) Macroscopic test, (d) Biochemical test TSIA method and (e) Biochemical test LIA method.

Ethanol Free Test

The results of the ethanol-free test in various fractions showed that no ethanol was detected in all fraction samples tested, using both the odor test and the color test.

Overall, this test showed that no ethanol was detected in all three fractions. The following is the data from the ethanol-free test results for each fraction:

Table 7. Ethanol Free Test Results

No.	Testing	Sample	Reagent	Faction	Observation result	Conclusion
1.	Smell Test	2-3 drops	Add 2-3 drops acetic acid & sulfuric acid then heated (Tenda et al., 2017)	Water Fraction	No typical ethanol odor & forming black sediment	(-) Ethanol
				Ethyl Acetate Fraction	There is no characteristic odor of ethanol & forms a black precipitate	(-) Ethanol
				N-Hexane Fraction	There is no characteristic odor of ethanol & forms a black precipitate	(-) Ethanol
2.	Color Test	2-3 drops	Add 2 drops of concentrated sulfuric acid & 1 ml of potassium dichromate (Natasya et al., 2021)	Water Fraction	Brownish orange	(-) Ethanol
				Ethyl Acetate Fraction	Brownish orange	(-) Ethanol
				N-Hexane Fraction	Brownish orange	(-) Ethanol

Antibacterial Activity Test of Cocoa Leaf Fractions

The results of the antibacterial activity test of cocoa leaf fractions in Table 1. 7 show that the water fraction, ethyl acetate fraction, and n-hexane fraction each have antibacterial activity at various concentrations. The best antibacterial activity test results were found in the 600 ppm water fraction with an inhibition zone diameter of 27.30 mm. The results of this test were stated to be almost equivalent to the diameter of the inhibition zone of the 400 ppm chloramphenicol positive control, which was 27.70 mm. The following are data on the results of the antibacterial activity test on each fraction, positive control and negative control:

Table 5. Antibacterial Activity Test Results on Cocoa Leaf Fractions

No.	Sample	Concentration	Inhibition Zone Diameter (mm)	Well Diameter (mm)	Results	Average diameter of inhibition zone (mm)
					Inhibition Zone Diameter	
1	Water Fraction	200 ppm	25.44	10.00	15.44	16.13
			26.54	10.00	16.54	
			26.42	10.00	16.42	
		400 ppm	29.70	10.00	19.70	18.67

			27.84	10.00	17.84	
			28.46	10.00	18.46	
		600 ppm	38.62	10.00	28.62	27.30
			35.54	10.00	25.54	
			37.73	10.00	27.73	
2	Ethyl Acetate Fraction	200 ppm	26.50	10.00	16.50	17.44
			27.43	10.00	17.43	
			28.40	10.00	18.40	
		400 ppm	30.75	10.00	20.75	20.88
			31.22	10.00	21,22	
			30.66	10.00	20.66	
		600 ppm	34.34	10.00	24.34	23.97
			34.33	10.00	24.33	
			33.23	10.00	23.23	
3	N-Hexane Fraction	200 ppm	26.34	10.00	16.34	16.26
			25.67	10.00	15.67	
			26.78	10.00	16.78	
		400 ppm	29.89	10.00	19.89	19.74
			30.10	10.00	20.10	
			29.22	10.00	19.22	
		600 ppm	33.76	10.00	23.76	23.37
			32.89	10.00	22.89	
			33.45	10.00	23.45	
4	Positive Control	Chloramphenicol 400 ppm	39.80	10.00	29.80	27.70
			37.56	10.00	27.56	
			35.74	10.00	25.74	
5	Negative Control	0 ppm	0.00	10.00	0.00	0.00
			0.00	10.00	0.00	
			0.00	10.00	0.00	

Description: Antibacterial activity was evaluated based on the diameter of the inhibition zone, by grouping into categories of weak (< 5 mm), moderate (6 – 10 mm), strong (11 – 20 mm), and very strong (≥ 21 mm) (Susanto et al., 2012).

SPSS Data Processing Results

1) Data Normality Test

Based on the results of the normality test using *Kolmogorov-Smirnov*, it can be concluded that all data groups in Table 1.7 are normally distributed. All data groups have significant values, namely the valueSig. of 0.152 ; 0.200 ; 0.200in the ρ > 0.05 test. The following is a table of data normality test results:

Table 6. Normality Test Results *Kolmogorov-Smirnov*

Tests of Normality[a]				
		Kolmogorov-Smirnovb		
	Sample Treatment	*Statistics*	df	Sig.
Inhibition Zone Diameter	Water Fraction	.243	9	.152*
	Ethyl Acetate Fraction	.153	9	.200*
	N-Hexane Fraction	.174	9	.200*

2) Data Homogeneity Test

The results of the homogeneity test of variance using data (Table 7) Levene's statistics show that all have a significance value (Sig.) of 0.119; 0.506; 0.527; 0.132, meaning it is greater than 0.05, this shows that the variance between groups is homogeneous. The following is a table of the results of the homogeneity test using data *Levene's Statistics* :

Table 7. Homogeneity Test Results Using Data *Levene's Statistics*

Tests of Homogeneity of Variances				
	Levene Statistics	df1	df2	Sig.
Based on Mean	1,859	9	20	.119
Based on Median	.951	9	20	.506
Based on Median and with adjusted df	.951	9	9,578	.527
Based on trimmed mean	1,795	9	20	.132

3) Test *One Way* ANOVA

The results of the One Way ANOVA test for the diameter of the inhibition zone of the cocoa leaf fraction showed that there was a significant difference between the data groups. in Table 7 tested. With a significance value (Sig.) of 0.001 which is smaller than 0.05. This shows that the difference between the groups is very statistically significant.The following is a table of test results.*One Way* ANOVA:

Table 8**.** Test Results *One Way* ANOVA

ANOVA					
Inhibition Zone Diameter					
	Sum of Squares	Df	*Mean Square*	F	Sig.
Between Groups	493,668	9	54,852	54,703	.001
Within Groups	20,055	20	1.003		
Total	513,723	29			

4) Test *Post Hoc* LSD

Post Hoc test results of data on Table 1.7 conducted to determine whether a test sample group has a significant difference to other test groups from the results of the inhibition zone diameter test of cocoa leaf fractions. The Post Hoc LSD test was conducted because test results *One Way* ANOVA significance value (Sig.) of 0.001 means it is smaller than 0.05.

The conclusion of the Post Hoc LSD test that identified significant differences between many pairs of concentration groups, showed that the variation in the inhibition zone diameter of cocoa leaf fractions among these groups was statistically significant. Significant mean differences are indicated by an asterisk (*) at a significance value (Sig.) less than 0.05.Table 9. Test Results Post Hoc LSD

***Multiple ComparisonsPost Hoc Test*LSD**						
Dependent Variable: Inhibition Zone_Diameter						
					95% Confidence Interval	
(I) Concentration	**(J) Concentration (ppm)**	***Mean Difference*(IJ)**	**Std. Error**	**Sig.**	***Lower Bound***	***Upper Bound***
Water Fraction 200 ppm	Water Fraction 400 ppm	-2.53333*	.81761	.006	-4.2388	-.8278
	Water Fraction 600 ppm	-11.16333*	.81761	.001	-12.8688	-9.4578
	Ethyl Acetate Fraction 200 ppm	-1.41000*	.81761	.025	-3.0155	.3955
	Ethyl Acetate Fraction 400 ppm	-4.74333*	.81761	.001	-6.4488	-3.0378

	Ethyl Acetate Fraction 600 ppm	-7.83333*	.81761	.001	-9.5388	-6.1278
	N-Hexane Fraction 200 ppm	-.13000	.81761	.175	-1.8355	1.5755
	N-Hexane Fraction 400 ppm	-3.60333*	.81761	.001	-5.3088	-1.8978
	N-Hexane Fraction 600 ppm	-7.23333*	.81761	.001	-8.9388	-5.5278
	Positive Control	-11.56667*	.81761	.001	-13.2722	-9.8612
Water Fraction 400 ppm	Water Fraction 200 ppm	2.53333*	.81761	.006	.8278	4.2388
	Water Fraction 600 ppm	-8.63000*	.81761	.001	-10.3355	-6.9245
	Ethyl Acetate Fraction 200 ppm	1.22333	.81761	.150	-.4822	2.9288
	Ethyl Acetate Fraction 400 ppm	-2.21000*	.81761	.014	-3.9155	-.5045
	Ethyl Acetate Fraction 600 ppm	-5.30000*	.81761	.001	-7.0055	-3.5945
	N-Hexane Fraction 200 ppm	2.40333*	.81761	.008	.6978	4.1088
	N-Hexane Fraction 400 ppm	-1.07000	.81761	.205	-2.7755	.6355
	N-Hexane Fraction 600 ppm	-4.70000*	.81761	.001	-6.4055	-2.9945
	Positive Control	-9.03333*	.81761	.001	-10.7388	-7.3278
Water Fraction 600 ppm	Water Fraction 200 ppm	11.16333*	.81761	.001	9.4578	12.8688
	Water Fraction 400 ppm	8.63000*	.81761	.001	6.9245	10.3355
	Ethyl Acetate Fraction 200 ppm	9.85333*	.81761	.001	8.1478	11.5588
	Ethyl Acetate Fraction 400 ppm	6.42000*	.81761	.001	4.7145	8.1255
	Ethyl Acetate Fraction 600 ppm	3.33000*	.81761	.001	1.6245	5.0355
	N-Hexane Fraction 200 ppm	11.03333*	.81761	.001	9.3278	12.7388
	N-Hexane Fraction 400 ppm	7.56000*	.81761	.001	5.8545	9.2655
	N-Hexane Fraction 600 ppm	3.93000*	.81761	.001	2.2245	5.6355
	Positive Control	-.40333	.81761	.627	-2.1088	1.3022
Ethyl Acetate Fraction 200 ppm	Water Fraction 200 ppm	1.31000*	.81761	.025	-.3955	3.0155
	Water Fraction 400 ppm	-1.22333*	.81761	.040	-2.9288	.4822
	Water Fraction 600 ppm	-9.85333*	.81761	.001	-11.5588	-8.1478
	Ethyl Acetate Fraction 400 ppm	-3.43333*	.81761	.001	-5.1388	-1.7278
	Ethyl Acetate Fraction 600 ppm	-6.52333*	.81761	.001	-8.2288	-4.8178
	N-Hexane Fraction 200 ppm	1.18000	.81761	.164	-.5255	2.8855
	N-Hexane Fraction 400 ppm	-2.29333*	.81761	.011	-3.9988	-.5878
	N-Hexane Fraction 600 ppm	-5.92333*	.81761	.001	-7.6288	-4.2178
	Positive Control	-10.25667*	.81761	.001	-11.9622	-8.5512
Ethyl Acetate Fraction 400 ppm	Water Fraction 200 ppm	4.74333*	.81761	.001	3.0378	6.4488
	Water Fraction 400 ppm	2.21000*	.81761	.014	.5045	3.9155
	Water Fraction 600 ppm	-6.42000*	.81761	.001	-8.1255	-4.7145
	Ethyl Acetate Fraction 200 ppm	3.43333*	.81761	.001	1.7278	5.1388
	Ethyl Acetate Fraction 600 ppm	-3.09000*	.81761	.001	-4.7955	-1.3845
	N-Hexane Fraction 200 ppm	4.61333*	.81761	.001	2.9078	6.3188
	N-Hexane Fraction 400 ppm	1.14000	.81761	.179	-.5655	2.8455

	N-Hexane Fraction 600 ppm	-2.49000*	.81761	.006	-4.1955	-.7845
	Positive Control	-6.82333*	.81761	.001	-8.5288	-5.1178
Ethyl Acetate Fraction 600 ppm	Water Fraction 200 ppm	7.83333*	.81761	.001	6.1278	9.5388
	Water Fraction 400 ppm	5.30000*	.81761	.001	3.5945	7.0055
	Water Fraction 600 ppm	-3.33000*	.81761	.001	-5.0355	-1.6245
	Ethyl Acetate Fraction 200 ppm	6.52333*	.81761	.001	4.8178	8.2288
	Ethyl Acetate Fraction 400 ppm	3.09000*	.81761	.001	1.3845	4.7955
	N-Hexane Fraction 200 ppm	7.70333*	.81761	.001	5.9978	9.4088
	N-Hexane Fraction 400 ppm	4.23000*	.81761	.001	2.5245	5.9355
	N-Hexane Fraction 600 ppm	.60000	.81761	.472	-1.1055	2.3055
	Positive Control	-3.73333*	.81761	.001	-5.4388	-2.0278
N-Hexane Fraction 200 ppm	Water Fraction 200 ppm	.13000	.81761	.875	-1.5755	1.8355
	Water Fraction 400 ppm	-2.40333*	.81761	.008	-4.1088	-.6978
	Water Fraction 600 ppm	-11.03333*	.81761	.001	-12.7388	-9.3278
	Ethyl Acetate Fraction 200 ppm	-1.18000	.81761	.164	-2.8855	.5255
	Ethyl Acetate Fraction 400 ppm	-4.61333*	.81761	.001	-6.3188	-2.9078
	Ethyl Acetate Fraction 600 ppm	-7.70333*	.81761	.001	-9.4088	-5.9978
	N-Hexane Fraction 400 ppm	-3.47333*	.81761	.001	-5.1788	-1.7678
	N-Hexane Fraction 600 ppm	-7.10333*	.81761	.001	-8.8088	-5.3978
	Positive Control	-11.43667*	.81761	.001	-13.1422	-9.7312
N-Hexane Fraction 400 ppm	Water Fraction 200 ppm	3.60333*	.81761	.001	1.8978	5.3088
	Water Fraction 400 ppm	1.07000	.81761	.205	-.6355	2.7755
	Water Fraction 600 ppm	-7.56000*	.81761	.001	-9.2655	-5.8545
	Ethyl Acetate Fraction 200 ppm	2.29333*	.81761	.011	.5878	3.9988
	Ethyl Acetate Fraction 400 ppm	-1.14000	.81761	.179	-2.8455	.5655
	Ethyl Acetate Fraction 600 ppm	-4.23000*	.81761	.001	-5.9355	-2.5245
	N-Hexane Fraction 200 ppm	3.47333*	.81761	.001	1.7678	5.1788
	N-Hexane Fraction 600 ppm	-3.63000*	.81761	.001	-5.3355	-1.9245
	Positive Control	-7.96333*	.81761	.001	-9.6688	-6.2578
N-Hexane Fraction 600 ppm	Water Fraction 200 ppm	7.23333*	.81761	.001	5.5278	8.9388
	Water Fraction 400 ppm	4.70000*	.81761	.001	2.9945	6.4055
	Water Fraction 600 ppm	-3.93000*	.81761	.001	-5.6355	-2.2245
	Ethyl Acetate Fraction 200 ppm	5.92333*	.81761	.001	4.2178	7.6288
	Ethyl Acetate Fraction 400 ppm	2.49000*	.81761	.006	.7845	4.1955
	Ethyl Acetate Fraction 600 ppm	-.60000	.81761	.472	-2.3055	1.1055
	N-Hexane Fraction 200 ppm	7.10333*	.81761	.001	5.3978	8.8088
	N-Hexane Fraction 400 ppm	3.63000*	.81761	.001	1.9245	5.3355
	Positive Control	-4.33333*	.81761	.001	-6.0388	-2.6278

Positive Control	Water Fraction 200 ppm	11.56667*	.81761	.001	9.8612	13.2722
	Water Fraction 400 ppm	9.03333*	.81761	.001	7.3278	10.7388
	Water Fraction 600 ppm	.40333	.81761	.627	-1.3022	2.1088
	Ethyl Acetate Fraction 200 ppm	10.25667*	.81761	.001	8.5512	11.9622
	Ethyl Acetate Fraction 400 ppm	6.82333*	.81761	.001	5.1178	8.5288
	Ethyl Acetate Fraction 600 ppm	3.73333*	.81761	.001	2.0278	5.4388
	N-Hexane Fraction 200 ppm	11.43667*	.81761	.001	9.7312	13.1422
	N-Hexane Fraction 400 ppm	7.96333*	.81761	.001	6.2578	9.6688
	N-Hexane Fraction 600 ppm	4.33333*	.81761	.001	2.6278	6.0388

*. The mean difference is significant at the 0.05 level.

DISCUSSION

This study used cocoa leaves (Theobroma cacao L.) from the third to middle stalks because of their high secondary metabolite content. Fresh leaves as much as 8.5 kg were washed, sorted, and cut before being dried to reduce water content, reaching a dry weight of 2.15 kg. The leaf powder was ground and filtered to facilitate extraction with 70% ethanol. The drying shrinkage test showed a weight loss of 59.43%, while the water content test showed a content of 7.10%, below the maximum limit of 10% to prevent mold growth.

Maceration extraction was carried out by soaking 2.15 kg of leaf powder in 70% ethanol for seven days, producing a filtrate which was then evaporated into a thick extract weighing 146 g with a yield of 6.79%, indicating good extract quality. Liquid-liquid fractionation using n-hexane and ethyl acetate solvents separated compounds based on their polarity. The fractionation results showed the highest yield in the water fraction (80.64%) due to the content of polar compounds such as flavonoids and tannins, followed by the ethyl acetate fraction (5.58%) and the n-hexane fraction (0.33%).

After fractionation, the fractions were tested free of ethanol to ensure that there was no antibacterial effect from ethanol. The test results showed that all fractions were free of ethanol, allowing for accurate testing of the antibacterial activity of the cocoa leaf fractions. These steps ensure the quality of the extracts and fractions produced, as well as the validity of the antibacterial test results for the cocoa leaf fractions. This study focused on the identification of bioactive compounds in cocoa leaf fractions (Theobroma cacao L.) and the evaluation of their antibacterial activity against Salmonella typhi ATCC 14048. Thin Layer Chromatography (TLC) method was used to identify various bioactive compounds, including alkaloids, tannins, flavonoids, saponins, and steroids/terpenoids in the solvent fractions tested. The optimal RF value range in TLC is between 0.2 to 0.8 to ensure clear spot visualization and accurate interpretation.

Alkaloids were detected in all fractions, showing yellow spots with high RF values and showing positive reactions to reagents that produce blue or yellow colors. Alkaloids are known to have inhibitory properties against bacterial growth, contain basic nitrogen atoms, and are often pharmacologically and therapeutically active.

Tannins, which were also found in all fractions, produced green spots and positive reactions to FeCl3 reagent, indicating the presence of phenolic compounds that can form colored complexes. Tannins act as antibacterials. Flavonoids were detected in ethyl acetate and n-hexane fractions, producing bluish green spots with a positive reaction to AlCl3 reagent. Flavonoids function as bacteriostatics through mechanisms involving inhibition of nucleic acid synthesis, disruption of the cytoplasmic membrane, and inhibition of bacterial energy metabolism.

Saponins, which were present in all fractions, showed yellow spots and positive reactions to the Liebermann-Burchard reagent. Saponins reduce the surface tension of bacterial cell walls, bind lipopolysaccharides in the cell wall, and cause increased permeability which eventually results in bacterial death. While steroids/terpenoids were detected in the ethyl acetate and n-hexane fractions, adding to the complexity of the chemical composition of the cocoa leaf fractions.

Identification of Salmonella typhi bacteria was carried out through a series of tests at the University Strada Indonesia Pharmaceutical Microbiology Laboratory. This test includes Salmonella Shigella Agar (SSA) mixing media, Gram staining, and biochemical tests (TSIA and LIA). The results of Gram staining showed that the bacteria were red or pink, indicating Gram-negative bacteria. SSA media supports the growth of Salmonella spp. with the characteristics of black colonies and yellow agar zones around them. The TSIA test shows H2S production with a change in agar color to black, while the LIA test shows lysine fermentation and H2S production.

The antibacterial activity of water, ethyl acetate, and n-hexane fractions against Salmonella typhi was tested using the well diffusion method. The positive control using chloramphenicol showed an inhibition zone of 29.95 mm, indicating very strong antibacterial activity. The results of the water fraction test showed an inhibition zone of 27.30 mm at a concentration of 600 ppm, approaching the effectiveness of the positive control. The ethyl acetate and n-hexane fractions also showed antibacterial activity, although lower than the water fraction.

Statistical analysis through normality and homogeneity of variance tests showed that the data met the assumptions required for reliable statistical analysis. One Way ANOVA and Post-hoc test (LSD) confirmed significant differences between groups.

Overall, this study showed that cocoa leaf fractions have significant antibacterial activity against Salmonella typhi, especially in the aqueous fraction with a concentration of 600 ppm. Bioactive compounds such as flavonoids, saponins, tannins, and alkaloids in cocoa leaves contribute to this antibacterial effect, offering potential as a natural therapeutic agent.

CONCLUSION

Based on the results of the study, it can be concluded that all concentrations of water, ethyl acetate, and n-hexane fractions of ethanol extract of cocoa leaves (Theobroma cacao L.) showed anti-typhoid activity against Salmonella typhi ATCC 14048 bacteria. The most active fraction was the water fraction with a concentration of 600 ppm, which showed the highest antibacterial activity with an inhibition zone diameter of 27.30 mm, almost equivalent to the positive control of chloramphenicol 400 ppm which had an inhibition zone diameter of 27.70 mm. The next antibacterial activity was followed by the ethyl acetate fraction with a concentration of 600 ppm which had an inhibition zone diameter of 23.97 mm, and the n-hexane fraction with a concentration of 600 ppm of 23.37 mm. For further research, it is recommended to conduct further in vivo testing using cocoa leaf samples, as well as explore the antibacterial properties of cocoa leaves using positive controls other than chloramphenicol.

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