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Isolation of secondary metabolite compounds of *Coffee Benalu* leaves (*Loranthus parasiticus* (L.) Merr.) and its antibacterial activity test

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ABSTRACT

Coffee parasite leaves (*Loranthus parasiticus* (L.) Merr) is one of the plants that can be used as a medicinal material commonly obtained in various subtropical regions or tropical regions. This study aims to isolation of secondary metabolite compounds from methanol extract of coffee leaves and antibacterial activity by of disc diffusion and microdilution methods against *S. aureus* bacteria, *S. mutans*, and *S. viridians*. The result of antibacterial activity against *S. aureus* bacteria, *S. mutans*, and *S. viridians* respectively is 7.5 mm; 7.9 mm; and 8 mm which indicates that the ability to inhibit the growth of methanol extract bacteria 1% coffee leaves belongs to the medium category. The results of the microdilution method in determining the value of KHM against bacteria *S. Aureus*, *S. Mutans*, and S. *Viridians* are equal to the KHM value of 5000 µg/mL. Meanwhile, the MBC value for *S. aureus* was >5000 g/Ml, for *S. mutans* was >5000 g/mL and for *S. viridians* it was 5000 g/mL, indicating that the methanol extract of the coffee parasite leaves was only an inhibitor. Isolation of secondary metabolite compounds is carried out by fractionation using Liquid Vacuum Chromatography and Gravitational Column Chromatography which are further characterized using the GC-MS instrument. The results of the isolation of secondary metabolites from the methanol extract of the leaves of the coffee parasite (*Loranthus parasiticus* (L.) Merr.) showed that the leaves of the coffee parasite contained any 9 compounds.

1. Introduction

Indonesia is a country that belongs to the tropics and there are various plants of a biological origin that can be used as traditional medicinal ingredients and in the form of secondary metabolite compounds consisting of alkaloids, flavonoids, steroids, terpenoids, and phenylpropanoids (Paramudita et al. 2017). Secondary metabolite compounds are one of the chemical compounds that will never run out (Rohama & Zainuddin, 2021; Simorangkir et al. 2022). One of the plants that can be used as a medicinal ingredient is the leaves of the coffee parasite. Flavonoids are polyphenol substances that are often found in the epidermis of leaves and fruits with important functions, namely antioxidants, antimutagenics, antineoplastics, and vasodilator activity (Yulian & Safrijl, 2018).

Tannins have antioxidant activeness, can clog tumor breeding, and can also clog enzymes, namely reserve transcriptase and DNA topoisomerase. While saponins can play a role in

antipathogenic (Diningsih & Aswan, 2019). According to Yulian & Safrijal (2018) the technique for knowing the substances that can be found in the leaves of coffee is using the technique of phytochemical screening approach (phytopharmacology screening approaches). As for the results of phytochemical tests that have been carried out, the coffee parasite leaves test material has secondary metabolite compounds of alkaloid groups, terpenoids, and flavonoids, while the ethanol extract of coffee parasite leaves has alkaloid compounds and flavonoids. Based on this, it is important to conduct further research on the methanol extract of coffee leaves to produce new antibacterial alternative sources and find out the secondary metabolite compounds contained in coffee parasite leaves extract (*Loranthus parasiticus* (L.) Merr.).

2. Method

2.1. Material and Sample

The main ingredients used are the leaves of the coffee parasite (*Loranthus Parasiticus* (L.) Merr.). The process of extracting active compounds from the leaves of the coffee plant parasite using methanol as a solvent. For the isolation of secondary metabolites, N-hexane, ethyl acetate, aquadest, silica gel 60 GF254, and for the antibacterial test, Muller Hinton Agar (MHA), Muller Hinton Broth (MHB) media, Dimethyl sulfoxide (DMSO), Chloramphenicol, NaCl 0,9%, bacterial cultures of *S. aureus, S. mutans, and S. viridians*.

2.2. Antibacterial Activity

Solid agar media is prepared from MHA as much as 3.8 grams in 100 mL of aquades and liquid agar media is made from MHB as much as 3.6 grams in 100 mL of aquaades (Leliqia et al. 2021). Then the substrate is heated. Furthermore, the media and equipment to be used are sterilized using an autoclave with a temperature of 121ºC within 15 minutes (Utomo et al. 2018; Zega et al. 2021). Then *S. aureus* bacteria, *S. mutans*, and *S. viridians* rejuvenated by inoculating test bacteria in nutrient agar (NA) media incubated at 37ºC for 24 hours and then suspended in a test tube containing 10 mL of sterile NaCl solution of 0.9% until bacterial suspension turbidity is obtained equal to McFarland's standard 0.5 (Foni et al. 2019). Antibacterial activity tests carried out at a consentration of 1% and carried out by two methods, namely the paper disc diffusion method using paper discs that have been dripped with coffee parasite leaf extract, DMSO 2% as the negative control, and chloramphenicol as positive control and then using microdilution methods with Muller Hinton Agar (MHA) and Muller Hinton Broth (MHB) media's to determine Minimum Kill Concentration (MHB) and Minimum Inhibitory Concentration (MIC) (Suhardiman et al. 2019).

2.3. Isolation of Secondary Metabolites

Separation of compounds in *coffee parasite* leaf methanol extract (*Loranthus parasiticus* (L.) Merr.) is carried out using 2 column methods, namely Liquid Vacuum Chromatography (KVC) and Gravity Column Chromatography (KKG). Separation begins with the initial TLC followed by the KVC method using eluent. n-hexane, n-hexane: Ethyl acetate, ethyl acetate, ethyl-acetate: methanol, methanol with variations in eluent ratio based on polarity level (Alen et al. 2017). Further separation of compounds is carried out using Gravitational Column Chromatography (KKG) with an eluent ratio of n-hexane: methanol (1: 1) (Rahmi et al. 2016). Identification of compounds contained in parasiticus coffee leaves (Loranthus parasiticus (L.) Merr.) is carried out using the GC-MS instrument. A total of 0.014 grams of 15 fraction isolate isolates were dissolved using n-hexane and ethyl acetate and put in a vial bottle.

3. Results and Discussion

3.1. Antibacterial Activity Test Result Disc Diffusion Method

Antibacterial activity test are performed with disk diffusion method (Kirby Bauer) using disc paper to determine the inhibitory ability characterized by the formation of clear zones around the disk paper. The bacteria used are Staphylococcus aureus, Streptococcus mutans, and Streptococcus viridians. The results of antibacterial tests of the extract were compared to negative control and positive control. The negative control used is DMSO, which is a solvent used to dissolve samples. DMSO is used as a negative control aimed at the comparison that the solvent used as a thinner does not affect the antibacterial test results of the compound to be tested. The positive control used is a standard antibiotic commonly used in medicine, namely chloramphenicol (Natheer et al. 2012).

The results of the antibacterial activity of coffee parasite leaves extract against *S. Aureus*, *S. Mutans*, and *S. Viridians* bacteria showed the antibacterial activity of methanol extract of coffee benalu leaves characterized by the formation of clear zones around the disc with an average inhibitory zone can be seen in Table 1.

Table 1. Result of Inhibition Zone Diameter Measurement In Antibacterial Test With Paper Disc Diffusion Method.

No	Test sample concentration % (mg/mL)	Inhibition zone diameter (mm)								
		S. aureus			*S. mutans*			*S. viridans*		
		d_1	d_2	$\bar{d}$	d_1	d_2	$\bar{d}$	d_1	d_2	$\bar{d}$
1.	K^+	32.6	35.6	34.1	20.2	20.4	20.3	29.2	27.7	28.45
2.	K^-	0	0	0	0	0	0	0	0	0
3.	1	7.5	8.0	7.75	6.7	9.1	7.9	8.1	7.9	8

*Note: d1 = Bacterial inhibition zone diameter 1, d2 = Bacterial inhibition zone diameter 2, = Average value of bacterial inhibition zone

In the classification of the category of bacterial inhibitory power by Davis & Stout (1971) stated that if the diameter of the bacterial inhibition zone is equal to or smaller than 5 mm then it is weak, if the inhibition zone is at a diameter of 5-10 mm it is categorized as moderate, then if the inhibition zone is at 5-10 mm diameter is categorized as moderate. ranged from 10-20 mm, it was categorized as strong, whereas if the diameter of the bacterial inhibition zone was more than 20 mm, it was categorized as very strong.

Based on the Table 1, the results of the antibacterial activity of leaf extract of the plant *Loranthus parasiticus* (L.) Merr. shows that the leaf extract of the plant Loranthus Parasiticus (L.) Merr. has antibacterial activity against *S. aureus*, *S. mutans*, and *S. viridans* with the moderate category at a concentration of 1% with an average inhibitory zone of 7.75 mm against *S. aureus* bacteria, 7.9 mm against *S. mutans* bacteria and 8 mm against *S. viridans* bacteria. The diameter of the inhibitory zone formed is influenced by the high low number of active compounds or substances contained in the fraction. The high low concentration used depends on the number of active ingredients contained in the research material (Purwanto, 2015; Silaban et al. 2022; Simorangkir et al. 2022).

3.2. The Result of Antibacterial Activity Test with Microdilution Method

To find out the minimum levels of antibacterial compounds that can inhibit bacterial growth is carried out by the microdilution method by mixing substances in media which is then suspended with bacteria and then inserted into each microplate and then incubated. In the microdilution method if each microplate is still visible turbidity it indicates that there is no antibacterial activity on the microplate. Concentrations in which the extract cannot inhibit the growth of *S. aureus*, *S. mutans*, and *S. viridans* bacteria are used as reference levels to determine MIC and MBC values. MIC is the

smallest level that can inhibit the growth of bacteria. MBC is the smallest level that can kill bacteria. MBC is a continuation of MIC by growing bacteria on gelatin plates. The results of measuring the MBC value using the microdilution method for *S. aureus*, *S. mutans*, and *S. viridans* bacteria that have been grown on agar media are shown in Table 2.

Table 2. Results of Measuring the value of MIC and MBC using the Microdilution Method for *S. aureus, S. mutans* and *S. viridans* bacteria

Test Bacteria	Test concentration	Test value	
		MIC (µg/ml)	MBC (µg/ml)
S. aureus	Extract 5000 ppm	5000	>5000
	Chloramphenicol 500 ppm	31.25	125
S. mutans	Extract 5000 ppm	5000	>5000
	Chloramphenicol 500 ppm	62.5	250
S. viridans	Extract 5000 ppm	5000	5000
	Chloramphenicol 500 ppm	62.5	250

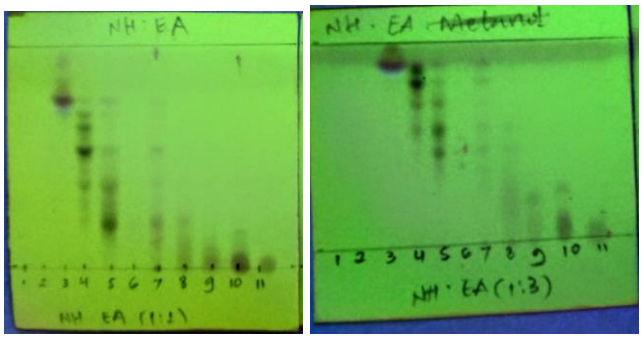
The results of the antibacterial activity test using the method of a microdilution of the leaf extract of the *Loranthus parasiticus* (L.) Merr plant against three test bacteria showed the same activity with a MIC value of 5000 µg / mL and mbc values for the bacteria *S. aureus*, *S. mutans*, and *S. viridans*, respectively > 5000. µg/mL, >5000 µg/mL, and 5000 µg/mL. Based on the table, leaf extracts of the plant *Loranthus parasiticus* (L.) Merr. Showed the highest activity against *S. viridans* bacteria with a MIC value of 5000 µg/ml and MBC 5000 µg/mL, but did not have a higher activity when compared to chloramphenicol antibiotics showing higher activity against *S. aureus* (MIC 31.25 µg/mL and MBC 125 µg/mL). According to Kuete et al. (2010), an extract is categorized as strong when its MIC value is reduced from 100 µg /mL, moderate when it ranges from 100 < MIC < 625 µg/mL, and low when the MIC value > 625 µg/mL. And the smaller the value of MBC MIC produced, the higher the antibacterial activity. From the results of measuring MIC values in methanol extract leaves of the Plant *Loranthus parasiticus* (L.) Merr. All three bacteria have a MIC value of 5000 µg / mL so methanol extract leaves of the plant *Loranthus parasiticus* (L.) Merr. it's just an inhibitor.

3.3. Isolation of Secondary Metabolic Compounds

Before separating secondary metabolite compounds contained in the leaves of coffee parasite first carried out preparative TLC. Preparative Thin Layer Chromatography is performed to identify and determine the eluent capable of providing good separation that will be used for column chromatography and to provide good coloring of substances. Furthermore, the separation of active compounds using Liquid Vacuum Chromatography using eluent n-hexane, n-hexane : ethyl acetate, ethyl acetate, ethyl acetate : methanol, and methanol by increasing the pattern in a row by adjusting the ratio of 21 fractions of the sample eluent produced by 21 fractions. After that, the solvent is removed from each fraction using a rotary evaporator and then n-hexane : ethyl acetate fraction or Fraction 1-11 are monitored by TLC using eluent n-hexane : ethyl acetate (1:1) and (1:1) to see the same chromatogram stain and determine which eluent is best to use in the gravitational thin layer chromatography that can be seen in (Figure 1a) for n-hexane : ethyl acetate (1:1) and (Figure 1b) for n-hexane : ethyl acetate (1:3). Based on the same chromatogram pattern fractions 4, 5, 6, 7, and 8 are then combined for further separation using column chromatography with eluent n-hexane : ethyl acetate (1:1).

The combined fraction of 4-8 is further fractionated using gravity column chromatography with eluent n-hexane : ethyl acetate (1:1) resulting in 52 fractions which are then monitored using TLC with eluent n-hexane : ethyl acetate (1:1) (Figure 2), then eliminated solvent using rotary evaporator. After being monitored with TLC using eluent n-hexane: ethyl acetate (1:1), it can be seen that each resulting

fraction still has more than one spot of stain indicating that the resulting isolate is not yet pure. After removing the solvent using a rotary evaporator, fraction 15 (F–15) obtained a result of 0.023 grams which is the most weight. So the fraction was chosen to do GC-MS to find out the content of compounds contained in coffee leaves (*Loranthus parasiticus* (L.) Merr.).



a b

Figure1. TLC Result of Fraction N-hexene:Ethyl Acetate Separation With KVC By Comparison (a) N-hexane:Ethyl Acetate (1:1) and (b) N-Hexane : Ethyl Acetate (1:3).

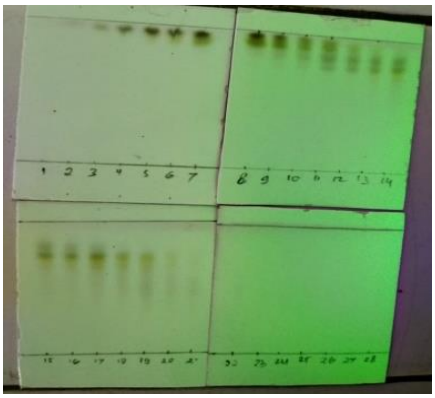


Figure 2. TLC Result N-hexane Fraction: Ethyl Acetate From Separation With Column Chromatography With Eluent N-hexane : Ethyl Acetate (1:1).

3.4. Identification of Secondary Metabolite Compound Structures

To determine the type of compound contained in the leaves of the coffee parasite (*Loranthus parasiticus* (L.) Merr.) then switched fraction 15 (F15) for analysis using the GC-MS instrument to identify the type of compound based on the molecular weight of the compound. Based on the results of identification, the resulting chromatogram shows that immature isolates are characterized by the appearance of several peaks. Each peak formed shows the presence of compounds contained in the fraction of 15 extracts of methanol parasitic coffee leaves shown in Figure 3.

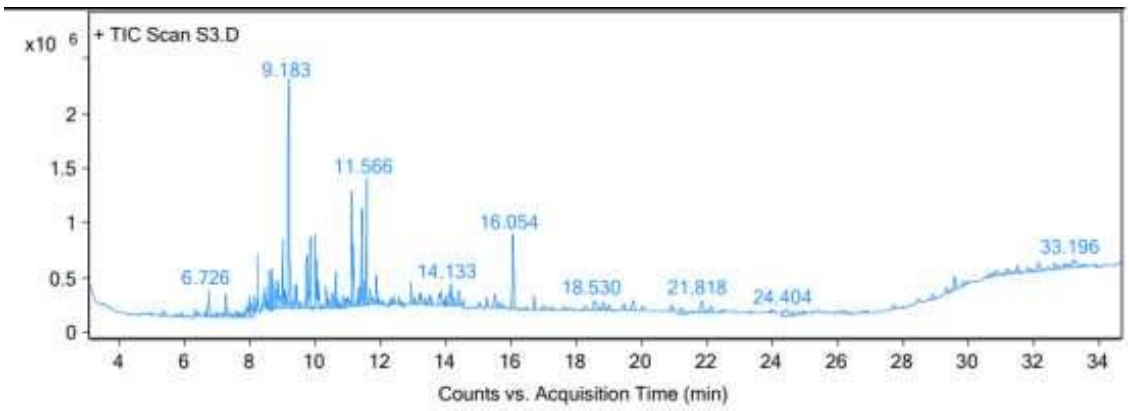


Figure 3. Separation Spectrum In GC-MS

The results of the analysis using the GC-MS instrument showed that there were 9 compounds contained in fraction 15 of the coffee leaf parasite methanol extract, in which the GC-MS chromatogram displayed the main peaks and included the highest of the analysis spectrum with the most abundance. The peaks that appeared dominant at retention times of 9.183 and 11.566 were the compounds with the greatest abundance contained in the fraction of 15 methanol extracts of coffee leaves parasite. From the results of the analysis, it can be seen that the Retention Time (RT), % Area, Compound Name, molecular formula, and similarity of the 10 compounds are in Table 3.

Table 3. Components of Chemical Compounds in the Chromatogram

Peak	Retention Time (RT)	%Area	Name	DB Formula	*Similirity*
1	8.222	16.27	Diethyltoluamide	$C_{12}H_{17}NO$	98
2	8.998	29.16	2-Cyclohexen-1-one, 3,5,5-trimethyl4-(3-oxobutyl)-	$C_{13}H_{20}O_2$	89.15
3	9.183	100	1,11-Tridecadiene	$C_{13}H_{24}$	67.34
4	9.737	27.35	6-Hydroxy-4,4,7a-trimethyl-5,6,7,7atetrahydrobenzofuran-2(4H)-one	$C_{11}H_{16}O_3$	75.2
5	9.829	31.01	2-Cyclohexen-1-one, 4-hydroxy-3,5,6- trimethyl-4-(3-oxo-1-butenyl)-	$C_{13}H_{18}O_3$	93.99
6	11.104	53.83	5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1- oxaspiro[2.5]octan-4-one	$C_{14}H_{20}O_3$	72.87
7	11.418	27.26	5,6,6-Trimethyl-5-(3-oxobut-1-enyl)-1- oxaspiro[2.5]octan-4-on	$C_{14}H_{20}O_3$	72.76
8	11.566	42.24	Ethane, 1,2-diphenyl-1,2-bis(azetidinyl-1)-	$C_{20}H_{24}N$	88.22
9	16.054	41.11	Phthalic acid, di(2-propylpentyl) ester	$C_{24}H_{38}O_4$	98.16

Based on the similarity value in table 3, it can be seen that 3 compounds have similarity values above 90. In this study, one compound with a high similarity value of 98% was considered a diethyltoluamide compound with a base peak at 119.1 and the molecular formula C12H17NO. The peak of fragmentation of diethyltoluamide compounds can be seen in Figure 4.

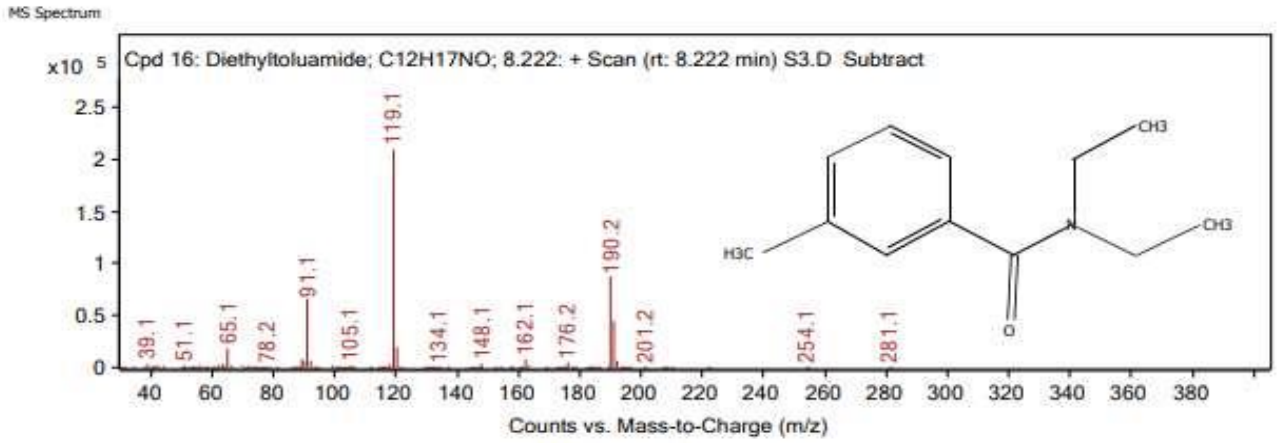


Figure 4. The Peak of Compound Fragmentation on GC-MS Analysis

Fragmentation of the compound in the semi-polar fraction F15 showed the presence of a peak base peak at m/z 119.1. At the m/z peak 190.1 comes from C12H17NO+ which is caused by the release of -C2H5 from the molecular ion followed by the release of -C2H5 to form -C10H12NO which appears at m/z 162. This ion then releases C2H5 forming a base peak at m/z 134. A rearrangement of C12H17NO+ occurs by the release of NC4H10 to form C8H7O- which is seen at m/z 119. Then C12H17NO+ also occurs from the release of CONC4H10 to form -C7H7 which is seen at m/z 91. Based on this analysis, the fragmentation pattern of the compound Dithyltoluamide can be seen in Figure 5.

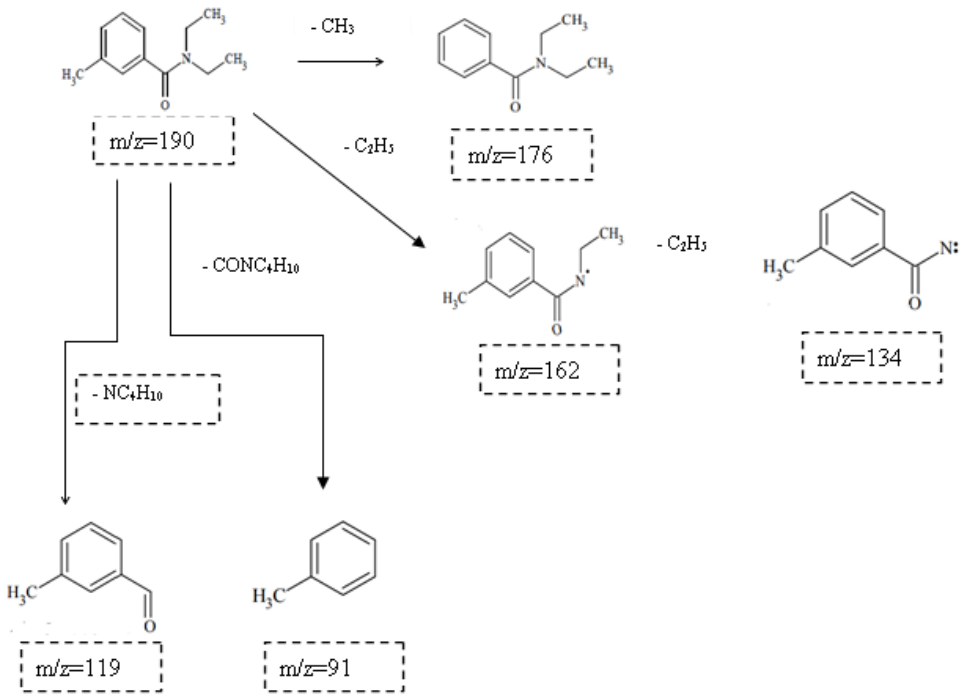


Figure 5. Diethyltoluamide Compound Fragmentation

Diethyltoluamide is an aromatic amide compound. Diethyltoluamide is prepared from m-tolyl chloride and diethylamine in benzene or ether. Diethyltoluamide, commonly abbreviated as DDET, is an active substance that is usually used in repellants that do not have an odor but can It causes a burning sensation when DEET comes in contact with the eyes. DEET is also toxic and very corrosive, these chemicals can be absorbed by the skin so it can cause irritation and can even trigger skin cancer if used continuously (Nurfadillah & Moektiwardoyo, 2020). Diethyltoluamide is a compound that has been isolated from methanol extract of the coffee parasitic leaf (*Loranthus parasiticus* (L) Merr.) which has a similarity index of 98%. The results of the fraction (F15) do not show the presence of secondary metabolites of alkaloids, flavonoids, tannins, saponins, and terpenoids. The absence of these groups may be due to alkaloids, flavonoids, tannins, saponins, and terpenoids are not easily identified by GC-MS related to the non-volatile nature of the compound.

In this plant, it is not possible to predict which compounds will act actively in inhibiting the growth of these bacteria. Therefore, to determine the process of inhibiting bacterial growth, further molecular research needs to be carried out. The research was to analyze cell damage using a Scanning Electron Microscope (SEM) (Utomo et al. 2018).

4. Conclusion

The results of the antibacterial activity test for the disc diffusion method with an extract concentration of 1% against the bacteria *S. aureus*, *S. mutans*, and *S. viridians*. were 7.75 mm, respectively; 7.9 mm; 8 mm which indicates that the ability to inhibit bacterial growth of 1% extract is

in the moderate category. Meanwhile, the MIC values for *S. aureus*, *S. mutans*, and *S. viridans* were the same, namely 5000 g/mL. The MBC value for S. aureus, S. mutans, and S. viridans bacteria, respectively >5000 g/mL, >5000 g/mL, and 5000 g/mL, indicating that the methanol extract of the coffee parasite leaf was only an inhibitor. The results of isolation of secondary metabolite compounds from the methanol extract of the coffee parasite stem which was analyzed using GC-MS concluded that fraction 15 contained 9 components with the similarity of 98% which were Diethyltoluamide.

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