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Antibacterial Activity of Flavonoids from Ethyl Acetate Extract of Milk Banana Peel (*Musa x paradisiaca* L.)

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ABSTRACT

Infectious diseases caused by Staphylococcus aureus and *Escherichia coli* can be treated by milk banana peels (*Musa* × *paradisiaca* L.). This study aims to reveal the antibacterial activity of milk banana peel ethyl acetate extract against *Staphylococcus aureus* and *Escherichia coli*, and to identify their active compounds. Extraction was conducted by maceration at room temperature, followed by partition. Separation was carried out by column chromatography with mobile phase of chloroform: ethyl acetate (7.5:2.5) and stationary phase of silica gel 60. Antibacterial assay was performed by well diffusion method and identification of active compounds was analyzed by UV-Vis spectrophotometry and LC-MS/MS. Extraction of the peel powder produced methanol extract, partition of the methanol extract resulted *n*-hexane, ethyl acetate, *n*-butanol, and water fractions. Antibacterial activity assay shows that ethyl acetate extract was the most active to inhibit the growth of both bacteria. Separation with column chromatography resulted 5 fractions. Identification of the most active fraction with UV-Vis showed that the isolate gave maximum absorption at λ 339.00 nm (band I) and λ 262.00 nm (band II) which were thought to be flavonol (3-OH substituted). Analysis with LC-MS/MS shows that the most active fraction contained 3-methacrylate flavonols and 3-(furan- 2yl) acrylate flavonols.

1. Introduction

Bacteria are prokaryotic microorganisms that do not have a nuclear membrane but still have genetic information in the form of DNA. Various kinds of bacteria often cause problems in the health, such as *Staphylococcus aureus* and *Escherichia coli*. Various clinical syndromes associated with diseases caused by *S. aureus* include skin and soft tissue infections, pneumonia, bone and joint infections, and central nervous system infections (Liu *et al.* 2011). While Diseases caused by *E. coli* include urinary tract infections, diarrhea, sepsis, and meningitis (Jawetz *et al.* 2008). Antibiotics are usually used to treat the infection, but the use of these has side effects, one of which is causing resistance. The resistance occurs due to inappropriate use of antibiotics and obstacles in developing new antibiotics (Ventola 2015). Prevention efforts are needed by controlling the use of antibiotics, developing research on resistance mechanisms, and developing new antibiotics both synthetic and natural (Liu *et al.* 2011).

The use of new antibiotics from natural ingredients continues to be developed. Samanea saman leaf *n*-butanol and ethanol extracts show antibacterial activity against *S. aureus* and *E. coli* (Rita *et al.* 2016; Rita *et al.* 2018). Samanea saman *n*-butanol extract also shows antifungal activity towards *Candida albicans* (Silaen 2020). Essential oils of *Acorus calamus* L. rizhome strongly inhibited the growth of *E. coli* and *S. aureus* (Rita *et al.* 2017a), and *Candida albicans* (Rita *et al.* 2017b). Antimicrobial activity against *E. coli, S. aureus,* and *C. albicans* was also shown by *Acorus calamus* L. rizhome ethanol extract (Susanah *et al.* 2018) and that of *n*-hexane, ethyl acetate, and *n*-butanol extracts (Rita *et al.* 2019). Ananta *et al.* (2018) reported that several kinds of local banana (*Musa* sp.) peels from Bali have antibacterial activity against *E. coli* and *S. aureus.* Wahyuni *et al.* (2019) revealed that yellow kepok banana peel (*Musa Paradisiaca* L.) *n*-butanol extract strongly inhibited the growth of *E. coli* and *S. aureus*

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with Minimum Inhibitory Concentrationa (MIC) was 0.5% for *S. aureus* and 0.2% for *E. coli*.

Banana is a very special fruit in Bali, because it is usually used as an offering in religious ceremonies. Banana fruit is usually consumed directly or used as raw material for food, but the peel is rarely used, even though the peel can be used as an ingredient in antibacterial drugs. Rita *et al.* (2020) reported antibacterial activity of selected local banana peels cultivated in Bali. Antibacterial assay on the peels against *Staphylococcus aureus* varied between 11.00 and 14.77 mm and that of *Escherichia coli* from 9.00 to13.37 mm at concentration of 20% (b/v).

The activity of banana peel is due to the presence of secondary metabolites which synergize with one another. Ehiowemwenguan *et al.* (2014) stated that there were flavonoids, tannins, alkaloids, volatile oils, saponins, and glycosides in ethanol extract of *Musa sapientum* peels. Kibria *et al.* (2019) reported that secondary metabolites detected from banana peel (*Musa paradisiaca*) ethanol extract were flavonoids, tannins, saponins, alkaloids, and glycosides. Singh *et al.* (2016) reported that banana pulp and peel contained various phenolic compounds, such as gallic acid, catechin, epicatechin, tannins, and anthocyanins. According to Rita *et al.* (2020), banana peels from Bali contained phenolic compounds and flavonoids. Total phenolic contents varied between 177 and 276 mg GAE/100g, while total flavonoid contents varied from 1756 to 2259 mg QE/100g. Susanah *et al.* (2018) revealed that there was a positive correlation between antimicrobial activity and their flavonoid and phenolic contents.

We have conducted antibacterial assay to milk banana peels (*Musa x paradisiaca* L.) methanol extract against *E. coli* and *S. aureus*. The extract strongly inhibited *E. coli* and *S. aureus* with an inhibition of 17.50 and 10.25 mm at concentration of 25%. Therefore, it is necessary to separate the compounds in the methanol extract to determine the structure of the compound, especially the flavonoid contained in the active fraction.

2. Materials and Methods

2.1. Plant Material

Milk banana peels were collected from Gianyar Bali. The fresh peels were cuti into small pieces and dried at room temperature for 3 weeks. The dried peels were powdered and used for extraction.

2.2. Bacterial Agents

Pure cultures of *Staphylococcus aureus* and *Escherichia coli* were purchased from the Laboratory of Clinical Microbiology of Region Public Hospital Sanglah Denpasar. The isolates were purified and maintained at 4°C until use.

2.3. Extraction

One kilogram of milk banana peel powder was macerated with 10 l of methanol at room temperature (25°C) for 2 x 24 h. The macerated extract was filtered through filter paper (Whatman No. 4) and evaporated under vacuum. The extracts were then stored at 4°C until further analysis. The methanol extract was then dissolved into methanol 70%, then the methanol was evaporated to obtain a water extract. The water extract was then partitioned with *n*-hexane, ethyl acetate, and *n*-butanol respectively. The extracts were then evaluated their antibacterial activities.

2.4. Antibacterial Activity Assay

Antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was evaluated by well agar diffusion method (Rita *et al.* 2018 with modification). Antibacterial activity assay was carried out on the *n*-hexane, ethyl acetate, *n*-butanol, and water extracts with a concentration of 25%, of which 10% tween as a negative control and 0.03% amoxicillin as a positive control. The extract (20 µl) was put into each well which already contains bacteria (200 µl), then incubated at 37°C for 24 hours. Inhibition zone (in diameter) was observed after incubation period. Then, the most active extract was performed with various concentrations. The concentration of extract applied were 0, 1, 2, 3, 4, 5, 10, 15, 25, 50, 75, and 100% (w/v). The assay was repeated in triplicate. Statistical analysis was performed using ANOVA test Duncan's Multiple Range Test. Minimum Inhibitory Concentration (MIC) was determined based on the lowest concentration of extract which still provides an inhibition zone.

2.5. Separation of the Components in the Active Extract

Separation was carried out by column chromatography. To determine the best mobile phase, a Thin Layer Chromatography (TLC) separation was performed previously with various eluents. The TLC plate that has been spotted the sample was inserted

into the vessel that was saturated with mobile phase vapor in a vertical direction and allowed to elute until it reaches the predetermined limit mark. Then the TLC plate was removed and dried. The separation results were observed under a UV lamp at a wavelength of 254 nm and 366 nm. The eluent which was capable of producing the most stain separation and well separated was the best mobile phase, it was used as a mobile phase in column chromatography.

Silica gel 60 was used as the stationary phase in column chromatography. One gram of the most active extract was dissolved with the solvent and fed into the column through the column wall. The mobile phase flow was speeded to 1 ml/minute. The eluate obtained was collected every 3 ml. Eluates that have the same separation or stain pattern will be combined, then evaporated and tested for antibacterial activity.

2.6. Identification Flavonoids of the Most Active Fraction

The most active fraction was then phytochemically tested flavonoids and analyzed using Ultraviolet-Visible (UV-Vis) and Liquid chromatography-tandem mass spectrometry (LC-MS/MS) spectrometry.

The flavonoid test was carried out by addition 0.1 gram of magnesium powder and 5-6 drops of concentrated HCl into 1 ml of the fraction (Wilstatter test), forming an orange to red color (Flavone), red to dark red (Flavanol), dark red to magenta (Flavanon) (Tiwari *et al.* 2011). If concentrated HCl is added (Bate-Smith test) then heated for 15 minutes on a water bath. If it gives a red color, it will positively contain flavonoids (Lestari *et al.* 2015). A few drops of diluted sodium hydroxide (NaOH) solution was added to the fraction, yellow color was formed, and then additional with hardly any sulfuric acid made the colorless of fraction, expresses the existence of flavonoids (Sariwati *et al.* 2019).

UV-Vis spectrum measurements were carried out at a wavelength of 200-600 nm. The most active fraction was dissolved with methanol to make a concentration of 0.01 mg/ml and its absorption was observed (Markham 1982).

The most active fraction was added to the reverse phase C18 pre-column (1.7 µm 2.1 internal diameter x 20 mm, BEH was separated by UPLC acetonitrile / 0.05% formic acid (v/v) (A) and water + format acid (B). Elution was performed for 30 minutes at a rate of 0.2 ml/minute). The elluent was then analyzed in an acquity UPLC * H Class System (water USA) quadrupole time of flight mass spectrometry (water USA) equipped with positive mode Electrospray ionization (ESI) source and operated using the xevo G2-s Qtof software. All mass spectra extracted using masslynx v 4.1 software.

3. Results

The results of the partition of the water extract (from 80 g of 70% methanol extract where methanol was evaporated) obtained 15.84 g of *n*-hexane extract, 8.30 g of ethyl acetate extract, 7.58 g of *n*-butanol extract, and of 5.70 g of water extract. Each extract was tested for its antibacterial activity against *S. aureus* and *E. coli*. Antibacterial activity assay was carried out at concentration of 25%, of which 10% tween as a negative control and 0.03% amoxicillin as a positive control. The results of the assay can be seen in Table 1.

Based on Table 1, it is known that the ethyl acetate extract of milk banana peel has the highest inhibitory zone in inhibiting the growth of *S. aureus* and *E. coli*, followed by *n*-butanol, water, and *n*-hexane extracts. water and *n*-hexane extract did not inhibit the growth of *E. coli*. therefore, the ethyl acetate extract was continued for antibacterial assay with various concentration and separation of the compounds by column chromatography.

The ethyl acetate extract antibacterial activity assay was carried out with various concentrations of 100, 75, 50, 25, 15, 10, 5, 4, 3, 2, and 1%. The result of this assay is presented in Table 2. Meanwhile, Graph of the inhibitory zone of ethyl acetate extract against the growth of both bacteria is presented in Figure 1. These results indicate that the increase the extract concentration, the rise inhibitory zone of the both bacteria. Based on Table 2, it can be seen that the MIC of ethyl acetate extract in inhibiting *S. aureus* was 1% with a diameter of inhibition of 5.00 mm, while the MIC in inhibiting *E. coli* was 3% with an inhibitory

Table 1. Results of antibacterial activity assay of *n*-Hexane, ethyl acetate, *n*-Butanol, and water extracts of milk banana peels against *S. aureus* and *E. coli* at concentration of 25%

Extracts	Average inhibitory zone (mm)	
	S. aureus	*E. coli*
n-Hexane	6.0	0.0
Ethyl acetate	23.25	20.13
n-Butanol	13.25	9.75
Water	12.75	0
Tween 10%	0	0
Amoxicillin 0.03%	31.50	25.25

Table 2. Results of determination of bacterial growth inhibition of ethyl acetate extract against *S. aureus* and *E. coli*

Treatment (%)	The average inhibition zone of extracts against bacteria (mm)	
	S. aureus	*E. coli*
0	0.00±0.00[a*]	0.00±0.00[a]
1	5.00±0.13[b]	0.00±0.00[a]
2	5.50±0.23[c]	0.00±0.00[a]
3	6.25±0.33[d]	6.55±0.12[b]
4	6.38±0.14[e]	8.20±0.36[c]
5	10.38±0.19[f]	10.25±0.11[d]
10	13.13±0.17[g]	13.25±0.27[e]
15	18.38±0.23[h]	16.00±0.19[f]
25	23.25±0.47[i]	20.13±0.26[g]
50	24.25±0.31[j]	20.75±0.10[h]
75	25.25±0.40[k]	21.38±0.13[i]
100	27.38±0.37[l]	22.38±0.11[j]

*Different notations indicate significantly different values ($p<0.05$)

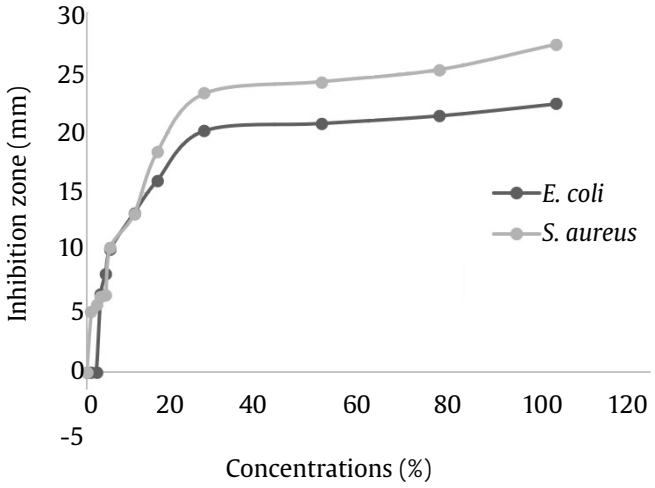


Figure 1. A curve of the inhibitory zone of ethyl acetate extract against the growth of *S. aureus* and *E. coli*

zone of 6.55 mm. Based on the Duncan's Multiple Range Test, it appears that the treatment provided a significant difference between concentrations. From Figure 1 it can be seen that *S. aureus* was more sensitive than *E. coli*.

The selection of the best eluent for the separation of compounds by column chromatography was carried out using TLC with various mobile phases, including *n*-hexane: ethyl acetate (6.5: 3.5), *n*-hexane: ethyl acetate (7.5: 2.5), and chloroform: ethyl acetate (7.5:2.5) and used a stationary phase of silica gel GF254. The best eluent is an eluent that can separate compounds with a distance between the stains from each other quite far and produce the highest number of stains. The results of TLC are presented in Table 3. TLC results showed that chloroform: ethyl acetate (7.5:2.5) was the best eluent (mobile phase).

A total of 2.00 grams of ethyl acetate extract of milk banana was separated by column chromatography using 60 g of silica gel as a stationary phase and a mobile phase of chloroform: ethyl acetate (7.5:2.5). The result of separation obtained 200 bottles of eluate. Each eluate was tested by TLC to determine the separation pattern from column chromatography in which eluents having the same separation pattern were combined. Based on the stain pattern of each eluate, 6 fractions were produced which are presented in Table 4.

The six fractions resulted from column chromatography were assayed for their antibacterial activity against *S. aureus* and *E. coli*. The results of assay for antibacterial activity can be seen in Table 5 and Figure 2. The graph of comparison antibacterial

Table 3. Selection of the best eluent

Eluent	Number of Stains	Rf value
n-Hexane: ethyl acetate (6.5:3.5)	8	0.02; 0.07; 0.13; 0.20; 0.26; 0.39; 0.48; 0.64
n-Hexane: ethyl acetate (7.5:2.5)	8	0.04; 0.09; 0.14; 0.24; 0.39; 0.47; 0.55; 0.95
Chloroform: ethyl acetate (7.5:2.5)	9	0.06; 0.14; 0.21; 0.35; 0.49; 0.65; 0.74; 0.87; 0.92

Table 4. TLC results from column chromatography

Fractions	Colour	The number of Stains	Rf value	Weight (g)
1	yellow	2	0.15; 0.95	0.0360
2	Orange	1	0.81	0.0057
3	Red	4	0.28; 0.53; 0.74	0.1113
4	Red	5	0.06; 0.18; 0.66	0.1038
5	Orange redness	2	0.24; 0.88	0.0632
6	Orange	2	0.27; 0.41	0.0624

Table 5. Results of antibacterial activity assay of column fraction on the growth of *S. aureus* and *E. coli*

Fractions	The average of inhibition (mm)	
	S. aureus	*E. coli*
F1	9.00	10.30
F2	9.75	8.25
F3	20.00	11.75
F4	30.38	20.25
F5	37.25	27.38
F6	25.88	21.63

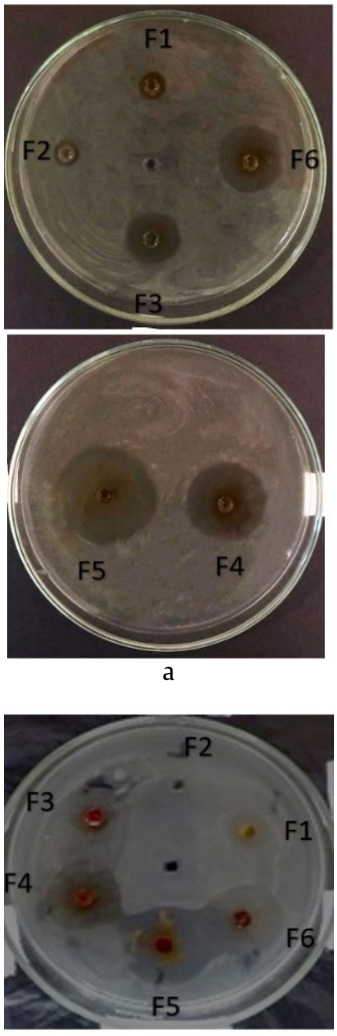


Figure 2. Antibacterial assay results for the fraction of the column chromatography against (a) *S. aureus* and (b) *E. coli*

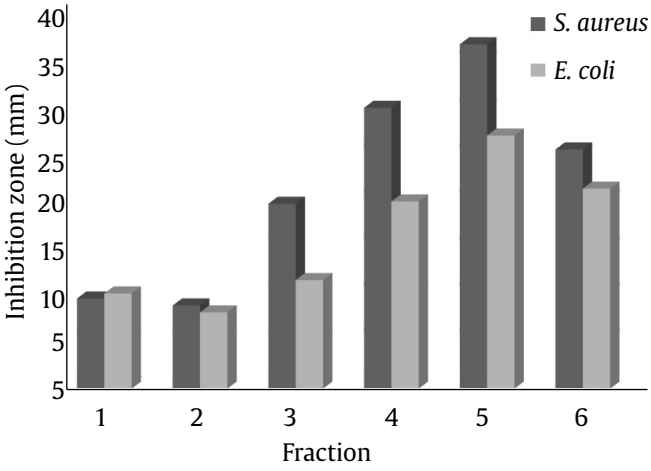


Figure 3. The graph of comparison antibacterial activity of the fractions between *S. aureus* and *E. coli*

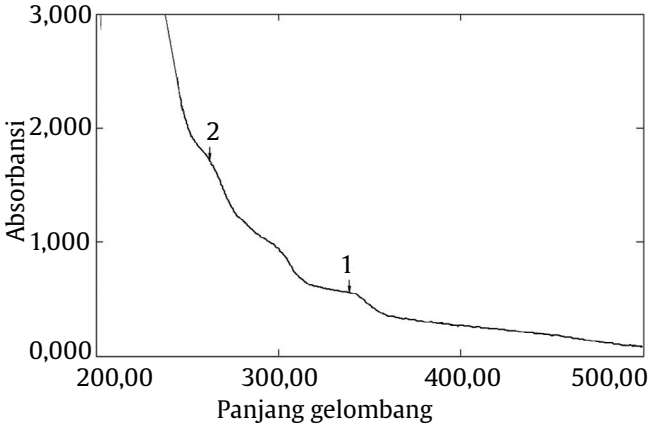


Figure 4. UV-Vis spectrum of F5

activity of the fractions between *S. aureus* and *E. coli* is presented in Figure 3. Based on the results of the antibacterial assay, it can be seen that fraction 5 (F5) was the most active fraction to inhibit both *S. aureus* and *E. coli*, followed by F6, F4, F5, F1, and F2 respectively. However, the inhibition against *S. aureus* was higher than that of *E. coli*.

Based on the phytochemical test, it was estimated that the F5 contained the flavonol group which were indicated by formation of a red color with Wilstatter reagent, an orange color with Bate-Smith reagent, and a yellow color with NaOH solution. The F5 was then identified using a UV-Vis and LC-MS/MS spectrophotometer.

The results of the analysis with a UV-Vis spectrophotometer for F5 can be seen in Figure 4. Based on the Figure, there were two absorption bands which were characteristic of flavonoids. The chromatogram pattern of F5 analysis using LC-MS/MS showed that there were peaks in the retention times of 8.462 and 9.125 which were thought to be flavonoids (substituted 3-OH). Mass spectrum of F5 with LC-MS/MS ESI positive ion at a retention time of 8.462 and 9.125 are presented in Figure 5 and 6. The results of LC-MS/MS analysis of F5 fraction and the suspected compounds can be seen in Table 6. The MS/MS spectrums are shown in Figures 5. Flavonoids that were in the active fraction were thought to be a flavonol group with the name of 3-methacrylate flavonols and 3-(furan-2il) acrylate flavonols (Figure 7).

4. Discussion

The solvents used in the partition process are based on the degree of polarity, starting with the

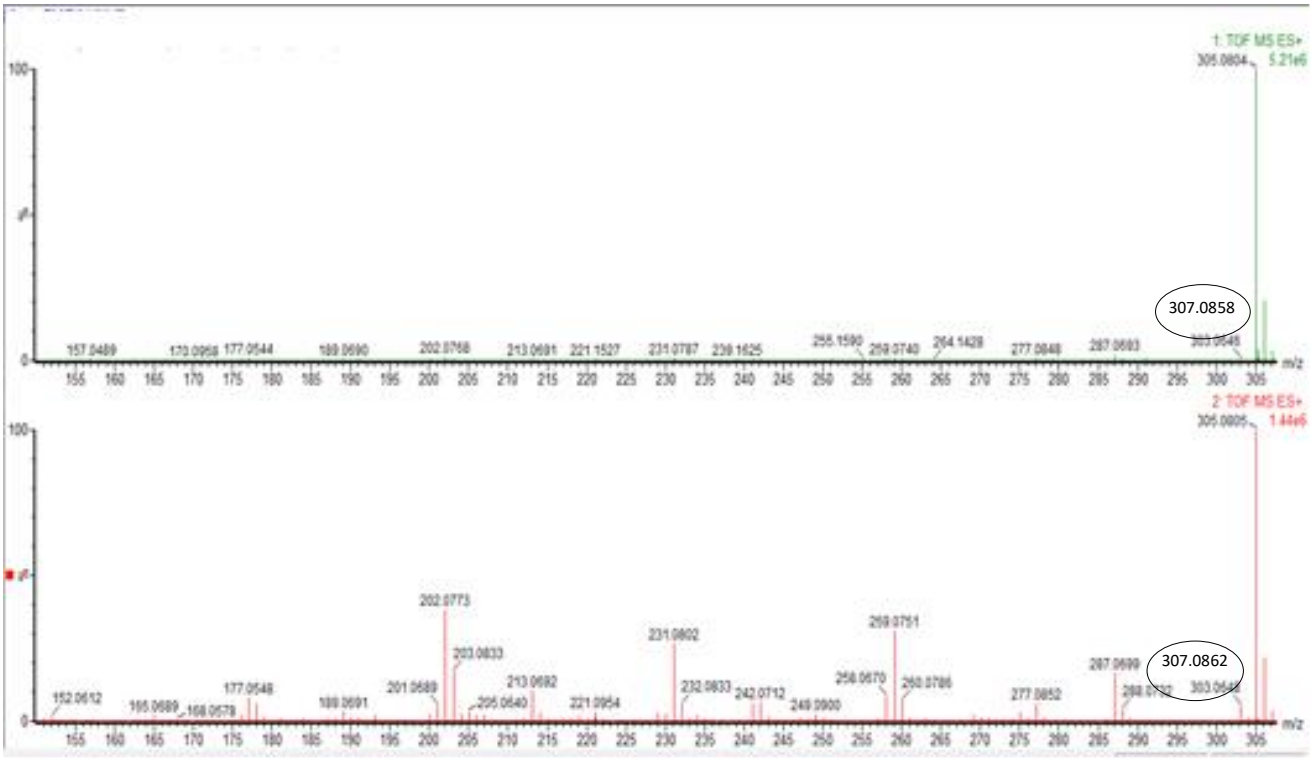


Figure 5. Mass spectrum of F5 with LC-MS/MS ESI positive ion at a retention time of 8.462

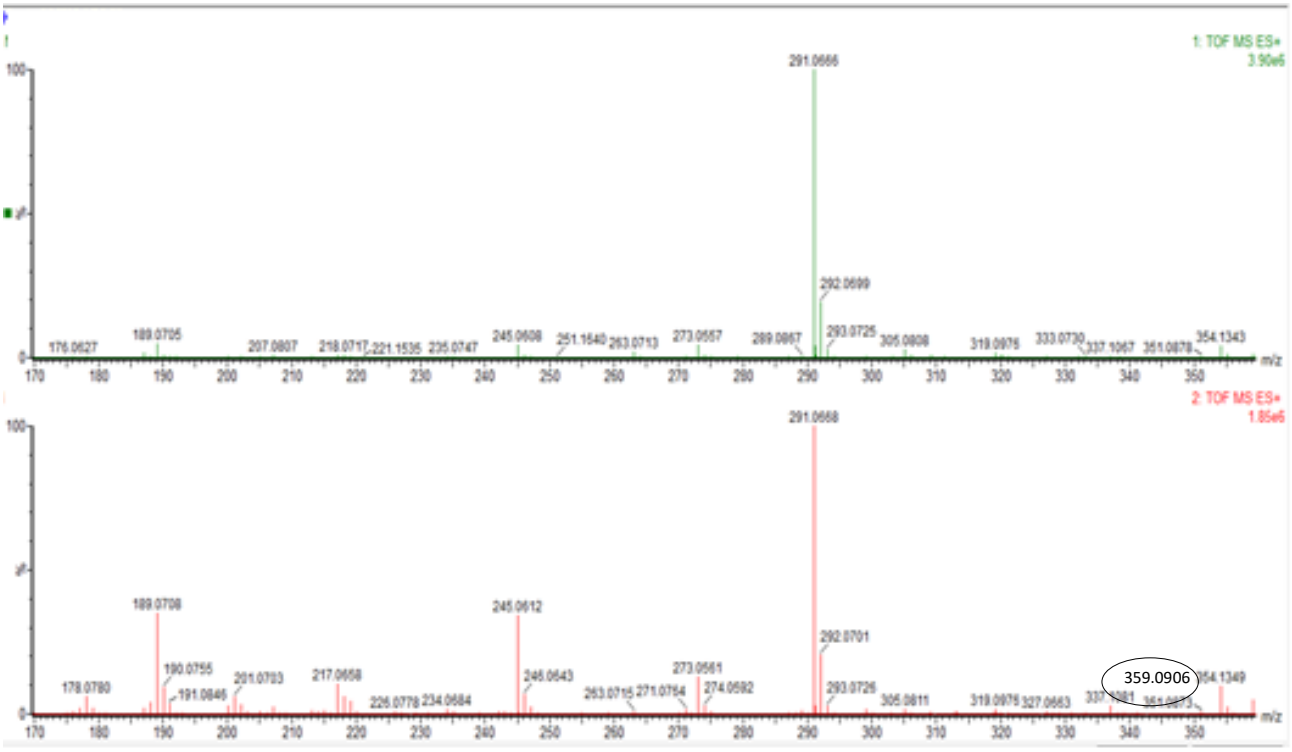
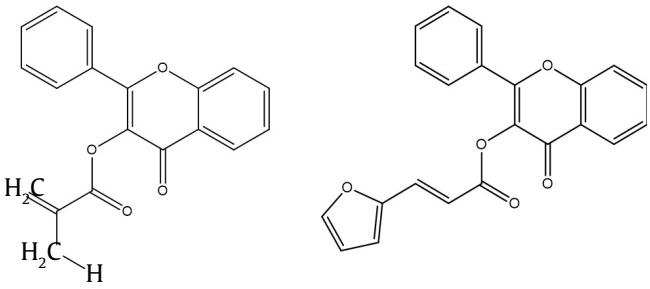


Figure 6. Mass spectrum of F5 with LC-MS/MS ESI positive ion at a retention time of 9.125

Table 6. Results of LC-MS/MS analysis of fraction 5 and suspected compounds

Retention time (Rt)	m/z (M+H)		Suspected compounds
	Precursor ion	Product ion	
8.462	307.0862	305.0805 (peak ion); 287.0669; 259.0751; 231.0802; 177.0548	3-methacrylate flavonols
9.125	359.0906	305.0811; 291.0668 (peak ion); 263.0715; 235.0748	3-(furan-2il) acrylate flavonols



(1) (2)

Figure 7. Estimated structure of compounds from LC-MS/S analysis (1) 3-methacrylate flavonol (tR 8,462) and (2) 3-(furan-2il) acrylate flavonol (tR 9,125)

non-polar solvent (*n*-hexane), followed by semi-polar (ethyl acetate) and polar (*n*-butanol). One of the semi-polar groups of compounds is flavonoids which are not bound by sugar. This was in accordance with the research of Rita *et al.* (2019) who conducted an antibacterial test on the partition extract from the rhizome of *Acorus calamus*.

The results of the antibacterial activity assay showed that the ethyl acetate extract of milk banana peels was the most active in inhibiting the growth of *S. aureus* and *E. coli*. This shows that the antibacterial compounds in the peels were semi-polar compounds. Tamokou *et al.* (2012) reported that ethyl acetate extract, fractions and compounds from stem bark of *Albizia adianthifolia* (Mimosoideae) showed antimicrobial activity against some bacteria and yeasts. Saeidi *et al.* (2014) revealed that ethyl acetate and aqueous extracts of *Mentha longifolia* L. have antibacterial activity against important human pathogens. Keikhaie *et al.* (2018) also reported that MIC of ethyl acetate Extract of *Securigera securidaca, Withania sominefra, Rosmarinus officinalis,* and *Aloe vera* Plants was lower than that of Methanol extract against Important Human Pathogense. This means that the ethyl acetate extract was more active than the methanol extract.

When compared to the inhibition zone of methanol extract, ethyl acetate extract produced a higher inhibitory zone. This is probably because the compounds contained in methanol extract were antagonistic. The methanol extract was the result of the maceration process where there were still a lot of compounds from polar to nonpolar. So that the ethyl acetate extract that has gone through the separation process produced a higher inhibition. Antibacterial assay which was carried out with various concentrations showed a positive correlation of the inhibitory zone of the extracts against the bacteria with the applied extract concentration. in addition, based on Duncan's test it was found that the inhibition was significantly different between the concentrations applied. This is in line with the studi Susanah *et al.* (2018) which states that the concentration of the extract applied affected the inhibition of bacterial growth, and changes in concentration have a significant effect on the inhibition. The MIC of ethyl acetate extract in inhibiting *S. aureus* was 1% with a diameter of inhibition of 5.00 mm, while the MIC in inhibiting *E. coli* was 3% with an inhibitory zone of 6.55 mm. MIC was determined based on the lowest concentration of extract which still provides an inhibition zone. Therefore, the lower the MIC, the greater the activity in inhibiting bacteria (Keikhaie *et al.* 2018; Mogana *et al.* 2020; Panphut *et al.* 2020). Balouiri *et al.* (2016) revealed that the method to determine antibacterial can be various, one of them is by diffusion method. This method can be used either to determine the inhibition zone, MIC or otherwise.

Antibacterial activity assay of fractions from separation with chromatography column shows that the fifth fraction (F5) produced the highest inhibition zone among the other fractions with an inhibition diameter of 37.25 mm which was included in the very strong category. The diameter of the inhibition zone produced by F5 fraction was greater than the diameter of the inhibition zone of ethyl acetate extract. This was because the compounds contained in ethyl acetate extract were antagonistic each other, so that they were more active as antibacterial in an individual or single form. In general, *S. aureus* (gram positive bacteria) was more sensitive than *E. coli* (gram negative bacteria). This is due to differences in

the cell wall complexities of the both bacteria (Pelczar 2002). *E. coli* contains peptidoglycan between the inner and outer membranes, and the outer membrane was derived from lipopolysaccharides, proteins, and lipids. Polysaccharides have a role in prevent the entry of hydrophobic compounds into cell membranes, whereas lipids played a role in preventing entry hydrophilic compound (Prescott *et al.* 2002; Pelczar *et al.* 2002).

Based on the phytochemical test, it is known that the F5 fraction contained flavonoid compounds of the flavonol group (Tiwari *et al.* 2011). The mass spectrum in Figure 5 with a retention time of 8,462, detected the peak of the precursor ion with $(M+H)^+$ 307.0862 ($C_{19}H_{15}O_4$) and the product ion at m/z 305.0805 ($C_{19}H_{13}O_4$) (peak ion), 287.0669 ($C_{19}H_{11}O_3$), 259.0751 ($C_{18}H_{11}O_2$), 231.0802 ($C_{17}H_{11}O$), 177.0548 ($C_{13}H_5O$) identified as 3-methacrylate flavonols. The mass spectrum in Figure 6 with a retention time of 9,125, detected the peak of the precursor ion with $(M+H)^+$ 359.0906 ($C_{22}H_{14}O_5$) and the product ion at m/z 305.0811 ($C_{19}H_{13}O_4$), 291.0668 ($C_{18}H_{11}O_4$) (peak ion), 263.0715 ($C_{16}H_7O_4$), 235.0748 ($C_{15}H_7O_3$) identified 3-(furan-2il) acrylate flavonols.

Based on identification using LC-MS/MS, it is known that the active compound at F5 which plays an active role as an antibacterial against *S. aureus* and *E. coli* was thought to come from the flavonol compound, namely 3-methacrylate flavonols and 3-(furan-2il) acrylate flavonols. This was also supported by the results of phytochemical tests and analysis with UV-Vis which show that the compounds contained in F5 were flavonoids in the flavonol group. Asih *et al.* (2018) also succeeded in identifying flavonoid glycoside compounds in the flavonol (3-OH substituted) group from the *n*-butanol extract of tamarillo, namely routine, quercetin 3-O rhamnoside, and kaemferol 3-O routineoside. According to Rita *et al.* (2016) activity of flavonoids and phenols as antibacterials is caused by the formation of complexes with bacterial proteins through hydrogen bonds, covalent bonds and hydrophobic bonds, so as to deactivate enzymes from bacteria.

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