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Research article

Antifungal Activity of Phenolic Compounds From Samanea Saman Leaves Against Stem Rot Disease on Dragon Fruits Caused by Fusarium Solani

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Abstract.
Fusarium solani is a pathogen that causes dragon fruit stem rot. Phenolic compounds are thought to slow the progression of the disease. The aim of this research was to determine the quantity of phenolic compounds in *Samanea saman* leaf extract, identify the phenolic compounds, and evaluate their antifungal activity against *F. solani*. Extraction was done by maceration with 96% ethanol, followed by fractionation. Total phenolic content was determined by UV-Vis spectroscopy. Purification was performed by chromatographic technique. The isolate was identified by phytochemical test and spectroscopy technique. Antifungal assay was carried out by well diffusion agar. The total phenolic content of the acetone extract was 6589.12 mg GAE/100g extract. The extract strongly inhibited the growth of the pathogenic fungi with an inhibitory zone of 25.50 mm. The active isolate strongly inhibited the growth of *F. solani* with an inhibition zone of 35.50 mm. The phytochemical test and UV-Vis spectroscopy analysis showed that the isolate was hydrolyzed tannin, which absorbs ultraviolet light with a wavelength of 264.90 nm. The infrared spectroscopy analysis showed that the functional groups of tannin are OH (phenol), -CH aliphatic, -C=O, -C=C aromatic, and -CO.

Keywords: Antifungal activity, Dragon Fruit, Phenolic compounds, Samanea sama

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1. Introduction

Dragon fruit (*Hylocereus* sp.) becomes more popular in Indonesia, particularly in Bali, because of its wealth of benefits. The fruit was rich in nutrients and low in calories. Studies reported that dragon fruit may help prevent chronic diseases. It also contains fiber which is useful in the digestive process and neutralizes toxic substances [1]-[2].

Dragon fruit relatively easy to maintain, but in cultivation there are always pests and diseases that could lead to the low production and suffer losses. Disease can cause both bacteria and fungi. The fungal pathogen that infected the dragon fruit involved *Botryosphaeria dothidea*, *Colletotrichum gloesporioides*, and *Bipolaris cactivora* [3].

Dragon fruit developed on dragon fruit's orchard in Sobangan village, Mengwi Bali cannot bear fruit to the maximum, while market demand was quite a lot of, so supply dragon fruit in the supermarkets in Bali brought from outside Bali. This is also because the presence of pathogens infecting the crops, particularly fungal pathogens. Rita *et al*. [4] stated that the major pathogen was stem rot diseases. The diseases were caused by pathogenic fungi, *Fusarium solani*. *Fusarium solani* act as decomposers in soil and plant material, they are also causing some diseases of a number of important agricultural crops [5].

The use of botanical pesticides in overcoming plant diseases has been widely reported, however, only increased over the last twenty years. Rita *et al*. [6] studied antifungal activity of 30 plant extracts against pathogenic fungi causing stem rot disease on dragon fruit grown in Bali, *Fusarium solani*. The result showed that the most active extract was ethanol extract of *S. saman*. The extract inhibited the growth of *F. solani* with an inhibitory zone of 30.0 mm and MIC of 0.9 %. The extract significantly inhibited the fungal colony growth, the total biomass, and sporulation. Phytochemical analysis showed that the extract contained tannin, flavonoid, phenolic compounds, alkaloid, triterpenoid, and steroid. Rita *et al*. [7] revealed that the ethanol extracts also inhibited the growth of *Escherichia coli* with a moderate inhibition (8.33 mm) and *Staphylococcus aureus* with a strong inhibition (13.6 mm) at the concentration of 4%.

Rita *et al*. [8] reported that the activity of n-butanol extract of rain tree leaf to inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* positively correlated to its phenolic and flavonoid contents. According to Suteja *et al*. [9], the active compound from the butanol extract that inhibited the *E. coli* growth was suspected as isoflavon with a hydroxy group at C-5 and C-7. Sariningsih *et al*. [10] revealed that flavonoid isolated from ethyl acetate extract of the rain tree was also able to inhibit *Fusarium* sp., the fungal pathogen on dragon fruit. The active compounds were suspected as 3,7,8,4',5'-pentahydroxy flavonol, 3,5,4'-trihydroxy flavon, 3,5,7,8,3',4'-hexahydroxy anthocyanin.

Sari *et al*. [11] stated that the *E. coli* growth can be inhibited by phenolic compound (tannin) from acetone extract of the rain tree leaf. Salisbury and Ross [12] reported that the main advantage of tannin is to protect plant from bacterial and fungal infection. Due to high antimicrobial activity, especially antifungal, and phenolic content, this research aims to study the activity of phenolic compounds in the leaves of rain tree against the *Fusarium solani*, pathogenic fungi on dragon fruit causing stem rot disease.

2. Methodology

2.1. Sample preparation

Fusarium solani used were fungal pathogen isolated from dragon fruit stems. It was grown on PDA media.

Rain tree leaves were collected and aired. Furthermore, the dried leaves were mashed to powder and the water content was measured by heating the powder at 100 ˚C to a constant weight. The water content was calculated by the formula:

MC (%) = w1 – w2 x 100 %

w1

Where MC = Water content

w1= powder weight before heated

w2= powder weight after heated (constant weight)

3. Isolation of Phenolic Compounds

Extraction of phenolic compounds from the leaves of rain tree was started by extracting with ethanol 96% at room temperature (maceration method). The extract was evaporated to get crude extract, then the extract was dissolved into ethanol 70%, after that ethanol was evaporated, so it obtained water extract. The water extract was into n-hexane, chloroform, ethyl acetate, and acetone respectively, so that 5 extracts are obtained, there were n-hexane, chloroform, ethyl acetate, acetone, and water extracts. These extracts were then tested for phenolic compounds. The extract containing phenolic compounds was determined the total phenolic contents, then subsequently separated and purified using chromatographic techniques to obtain a pure active compound.

3.1. Phytochemical Analysis

Phytochemical analysis was carried out on acetone extract of trembesi leaves. The extract was tested for the presence of flavonoids, phenolic compounds (tannins), alkaloids, triterpenoids, steroids, and saponins [7].

The flavonoid test was carried out with the Wilstatter and Bate Smith-Metcalfe reagents. An orange-red color with Wilstatter reagent and a red color with Bate Smith-Metcalfe reagent indicated the presence of flavonoid.

The test of phenol group compounds was carried with 1% $FeCl_3$ solution, the presence of phenol will give a blue to greenish black precipitate. Tannin compound test can be

done by color test with 1% $FeCl_3$ reagent and gelatin solution. If the extract contains tannins, a blackish green or dark blue color will be formed with 1% FeCl3, and a white precipitate will form with a gelatin solution.

Examination of alkaloid compounds was carried out with Dragendroff, Meyer, and Wagner reagents. Red precipitate was formed with Dragendroff's reagent, a brown precipitate was formed with Wagner's reagent, and a white precipitate was produced with the addition of Meyer's reagent.

Examination of triterpenoid and steroid compounds was carried out with the addition of Leibermann-Burchard reagent. The formation of a green-blue color indicated the presence of steroids and red-purple for triterpenoids.

Saponin test (foam test) was carried out by: 2 mL of extract in a test tube was shaken for two minutes. The formation of stable foam indicated the presence of saponins.

3.2. The Total of Phenolic Content Determination

Determination of total of phenolic content was performed following the method from Rita *et al*. [13]. This procedure used the Folin-Ciocalteu reagent and it was analyzed using a UV-vis spectrophotometer. It used standard solution of gallic acid with various concentrations. Each concentration of gallic acid and the acetone extract was evaluated its absorbance at a wavelength of 760 nm.

3.3. Antifungal Activity Assay

The assay was carried out to the acetone extract. Ten mL of Potato Dextro Agar (PDA) and 200 L of the fungal suspension was added into petri dish and wait until solid. After that, 2 diffusion wells were made in each petri using a cork borer, each diffusion well was filled with 20 L of acetone extract of rain tree leaf (concentration of 10% b/v). The mixture was incubated for 24 hours and the inhibition was determined. if the zone of inhibition was >20 mm, so the inhibition was very strong; 10-20 mm was strong inhibition; 5-10 mm was medium inhibition; and < 5 mm was weak inhibition [14].

3.4. Separation and Purification

Separation and purification of the acetone extract was carried out by thin layer chromatography (TLC) and column chromatography. TLC was done to determine the best mobile phase to be used in column chromatography. The stationary phase used is

silica gel GF254, while the mobile phase was a mixture of various solvents based on the difference in polarity.

After obtaining the best mobile phase in TLC, then separated using column chromatography. The stationary phase used was silica gel 60 and the mobile phase used was the best mobile phase from TLC. From this column chromatography, several bottles of eluate were produced which were then carried out by TLC to determine the stain pattern produced from each bottle of eluate. The eluates that produced the same stain pattern were combined so that several fractions were obtained, then an antifungal test was carried out on each of these fractions. The most active fraction was separated again by column chromatography if it still contained more than three stains. Further separation was carried out by preparative High Performance Liquid Chromatography (HPLC).

3.5. Identification of the structure of antifungal active compounds

Identification of the most active compounds was carried out by phytochemical tests with detection reagents of phenolic compounds and spectroscopic methods such as UV-vis and IR.

4. Result and Discussion

About 5 kg of *S. saman* dried leaf powder were extracted by ethanol, it produced 404.3 grams of concentrated green ethanol extract. Furthermore ethanol extract was dissolved into ethanol 30%, the ethanol was evaporated to get water extract. Water extract was partitioned successively with 3L of n-hexane, 2L of chloroform, 2 L ethyl acetate, and 2 L acetone to obtain a concentrated extract of n-hexane, chloroform, ethyl acetate, acetone and water (Table 1).

TABLE 1: Results of n-Hexane, Chloroform, ethyl acetate, Acetone, and Water extracts.

Extract	Weigth (gram)	Colour
n-hexane	152.29	Green
Chloroform	12.46	Green
Ethyl acetate	21,86	Brown
Acetone	71.82	Brown
Water	109.56	Brown

Phenolic compounds, especially tannins are expected to be isolated in acetone extracts, because acetone is highly efficient solvent for extracting tannins according

to Ukoha *et al* [15]. Phytochemical test of tannins in acetone extracts could be done using ferry chloride ($FeCl_3$) which gives a blue black color and a gelatin solution to form a white precipitation. Beside tannins, there were also flavonoids, triterpenoids and saponins based on the qualitative test (Table 2).

TABLE 2: Phytochemical Analysis of Rain Tree Leaf Acetone Extract .

No.	**Reagents**	Change color/precipitates	**Results**
1.	$FeCl_3$	Green to blue-black	Tannin (+)
2.	Gelatin	White precipitates	
3.	Wilstatter	Green to yellowish green	Flavonoid (+)
4.	Bate Smith-Metcalfe	Green to yellowish green	
5.	Wagner	No brown precipitate formed	Alkaloid (-)
6.	Meyer	White precipitates	
7.	Liberman-Burchard	Brown to purple	Triterpenoid (+)
8.	Aquadest, heated, shaken	foam formed	Saponin (+)

The acetone extract was then determined the total phenolic content. The calibration curves of gallic acid to determine total phenolic contents can be seen in Figures 1. The equation of gallic acid obtained was y=0.0091x + 0.009 (R^2=0.9976). A summary of the calculation of the total phenolic contents was presented at Table 3. The total phenolic contents were 6589.1208 mg GAE/100g extract.

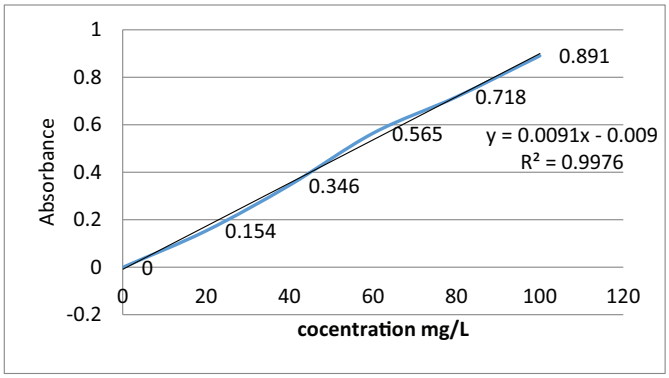

Figure 1: The calibration curves of gallic acid.

TABLE 3: A summary of the calculation of the total phenolic contents.

Mass extract	Abs	cons (x)	V tot	Dilution factor	Concentration of phenolic compounds (GAE)	
mg		mg/L	(0,0012L)	10–100	%	mg/100 g
25.90	1.112	142.2152	0.0012	10	6.5891	6589.12

There were high levels of phenolic compounds in acetone extract of *Samanea saman*, because acetone is highly efficient solvent for extracting tannins [16]. According

to Rita *et al*. [7], the phenolic content in the ethanol extract was 2544.6154 mg GAE/100g extract, it was lower than the acetone extract. Meanwhile, the total phenolic content in rain tree leaf n-butanol extract was 2398.40 mg GAE/100g extract [8].

Antifungal activity assay against *Fusarium solani* of the acetone extract was conducted by well diffusion method using PDA media. The acetone extracts of *S. saman* leaves strongly inhibited the growth of the fungal pathogen with a diameter of inhibition of 25.50 mm. Antifungal assay results are presented at Figure 2. The presence of phenolic compound in the extract caused the leakage of nutrient from within the cell because of changing the permeability of the cytoplasmic membrane. As a result, the fungus died [17].

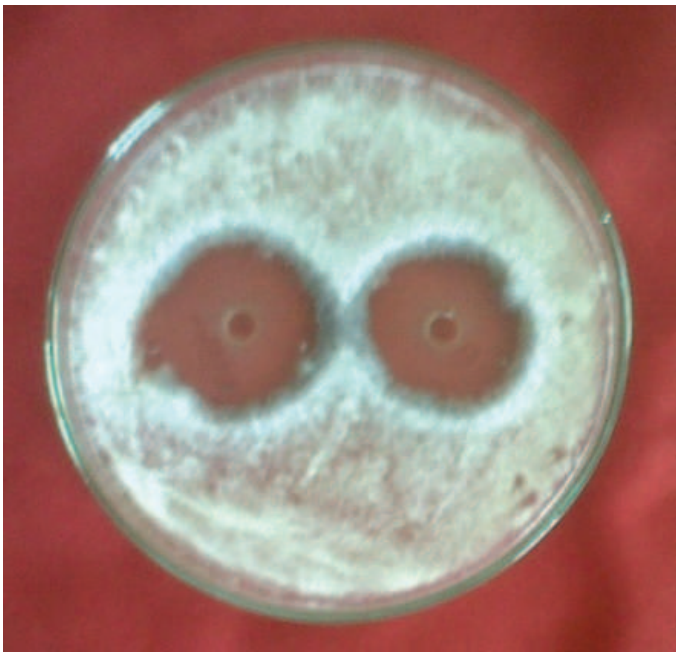

Figure 2: The antifungal assay of the acetone extracts against *Fusarium solani,* pathogenic fungi causing stem rot disease on dragon fruit.

The separation of the compounds in acetone extracts was carried out by chromatographic techniques. Selection of the best mobile phase used in the separation process by column chromatography was done by thin layer chromatography (TLC). Several mobile phases used was ethanol: petroleum ether: chloroform (5:1:3); ethanol: petroleum ether: chloroform (4:2:3), n-butanol: acetic acid: water (4:1:5); ethanol: chloroform: water (5:3:1); ethanol: chloroform (3:7), ethanol: chloroform: hexane (5:1:4), and ethanol: petroleum ether: chloroform (2:6:2). The TLC results can be seen in Table 4.

Based on Table 4, it can be seen that the best mobile phase for the separation of phenolic compounds, especially tannins in the acetone extract was n-butanol: acetic acid: water (4:1:5) (BAA). Separation of compounds was then performed by column

Table 4: TLC results of acetone extracts with different mobile phase.

No	Mobile fase	Spot Number
1	ethanol:petroleum ether:chloroform (5:1:3)	4
2	ethanol:petroleum ether:chloroform (4:2:3)	3
3	n-butanol:acetic acid:water (4:1:5)	6
4	ethanol:chloroform:water (5:3:1)	3
5	ethanol:chloroform (3:7)	1
6	ethanol:chloroform:hexane (5:1:4)	2
7	ethanol:petroleum ether:chloroform (2:6:2)	1

chromatography with mobile phase of BAA and the stationary phase of silica gel 60. Fractions were collected every 3 mL. TLC for each fraction was performed to determine the pattern of the stain. Fractions with the same stain patterns were combined, 5 fractions (FA-FE) were obtained (Table 5).

Table 5: TLC profile of fractions obtained from column chromatography.

No	Fractions	No. fraction	The number of Spots
1	FA	1-19	1
2	FB	20-33	3
3	FC	34-46	3
4	FD	47-100	2
5	FF	101-151	1

Fractions were then concentrated and each tested for antifungal activity. Antifungal test results of each are presented in Figure 3 and Table 6.

Table 6: Results of antifungal test results column chromatography fractions of acetone extract.

No	Fractions	Inhibitions (mm)
1	FA	21,75
2	FB	22,25
3	FC	22,00
4	FD	39,50
5	FE	35,50

Based on Table 5 and Figure 2, it can be seen that the most active fraction was FD with an inhibition of 39.50 mm, followed by FE with an inhibition of 35.50 mm. However, FD fraction still had 2 spots, while the FE fraction had 1spot, so analysis was then performed to FE fraction if the fraction containing tannins. Tannin in the fraction was detected by phytochemical analysis. Isolate with $FeCl_3$ solution gives a color change from yellow to blue-black, with gelatin produced a white precipitate, the test showed that the FE positive isolates contain tannins (Table 7).

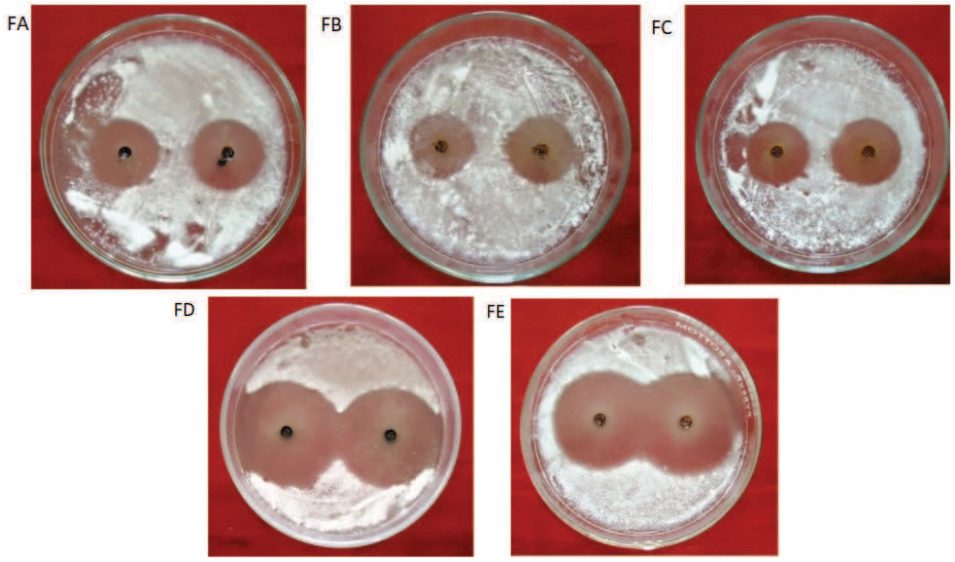

Figure 3: The results of the antifungal test of fractions from column chromatography against *Fusarium solani*.

TABLE 7: Phytochemical Test of Tannin in FE isolate.

Reagents	Color changes / precipitate	**Information**
$FeCl_3$	yellow to blue-black	Tannin (+)
Gelatine	White precipitation formed	

Khanbabaee and Ree [18] reported that tannins consisted of hydrolyzed and condensed tannins. Hydrolyzed tannins were polymeric gallic or ellagic acid esters bonded to a sugar molecule, while condensed tannins were polymers of flavonoids, especially cathecin (flavan-3-ol) units with carbon-carbon bond. Tannin compounds played a role in protection from predators, and perhaps also as a pesticide and in the growth of plant growth regulation.

Tannins are compounds that contain phenols because they are polyphenolic compounds. This test was also positive for other compounds which also contain phenol. The presence of phenol was indicated by a change in the color of the sample to blue with $FeCl_3$. The formation of this color was because tannins formed a metal-tannin complex with Fe^{3+} ions [19], as shown in Figure 4.

The FE isolate was then analyzed by preparative HPLC with 2% methanol as mobile phase and Luna 5u C18 column. From the HPLC analysis, several peaks were not completely separated, but were tried to accommodate the major peaks with a retention time of 14 minutes (Figure 5).

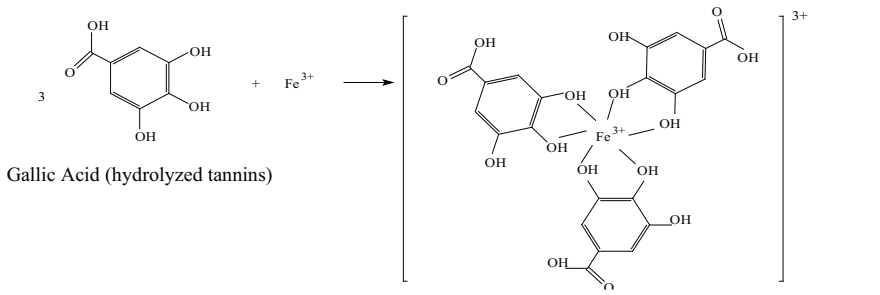

Figure 4: Complex compound formation reaction between Tannin and $FeCl_3$ proposed [19].

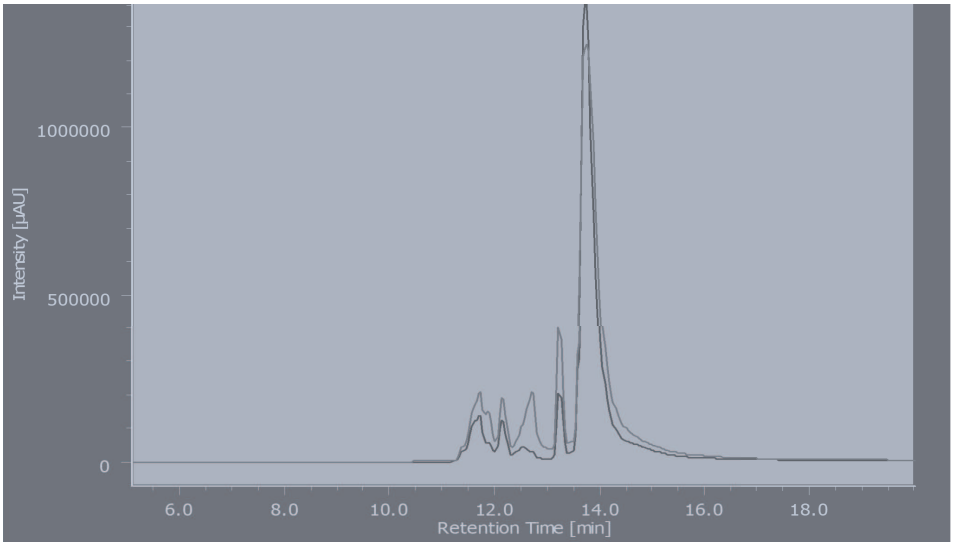

Figure 5: Preparative HPLC chromatogram of FE Isolate.

To determine whether the isolate was condensed or hydrolyzed tannin, the isolate was added with 3% formaldehyde: 1N HCl (2:1) and then heated on a water bath at 90 °C for 5 minutes. If a pink precipitate was formed, then the tannin was condensed tannin (catechol). The FE isolate did not form a pink red precipitate, so the filtrate was heated again and then sodium acetate and 1% $FeCl_3$ were added. The FE isolate formed a blue-black color which indicated that the isolate contained hydrolyzed tannins (gallotanin or ellagitanin).

Based on the phytochemical and antifungal test, it can be said that the isolate was hydrolyzed tannin that can inhibit the growth of *Fusarium solani*, pathogenic fungal on dragon fruits, with the inhibition of 35.50 mm in diameter. Li *et al*. [20] investigated the inhibitory effects of gallic acid on fungal strains. Gallic acid has a broad spectrum of antifungal activity against dermatophyte strains. Gallic acid was also active against three *Candida* strains. Congyi *et al*. [21] reported that tannins reduced the disease

severity of green mold on citrus fruit by 70%. Mechanism study showed that the cell wall of the pathogen (*Penicillium digitatum*) was the target for tannins. Mailoa *et al*. [22] reported the antimicrobial activity of tannins extract on guava leaves against pathogenic microbes. The results showed that the inhibitory activity of tannins on five microbial pathogens was different. This was because the composition of the microbial cell walls of the five microbes was different.

Isolates resulting from preparative HPLC were analyzed by infrared spectrophotometer to determine the functional groups of active isolates. The spectra of the infrared analysis are presented in Figure 6. Based on the infrared spectra, the isolates were suspected to contain the -OH functional group with wide absorption at a wave number of 3294.42 cm^{-1}, which was thought to have hydrogen bonding. According to Sastrohamidjojo [23], the free -OH group has a sharp absorption of around 3600 cm^{-1}, and if it undergoes intramolecular hydrogen bonding, the absorption looks wider and shifts towards a lower wavelength (between 3500 and 3200 cm^{-1}). The uptake supports the structure of the phenol-containing tannins. The -OH group was supported by the presence of -C-O absorption at a wavelength of 1070.49 cm^{-1}.

The stretching absorption at wave number 2938 cm^{-1} indicated the presence of an aliphatic C-H group which was supported by a bending absorption at 1307.74 cm^{-1} which is a $-CH_3$ group. In the absence of aromatic –CH absorption, it was assumed that all C atoms in the aromatic ring have been substituted. The absorption at 1620.21 cm^{-1} showed an aromatic –C=C group. The wave number of 2083.12 cm^{-1} showed the C-H absorption peak of out-of-plane deformation. In this spectrum, it can be seen that there was a small carbonyl absorption band in the 1716.65 cm^{-1} region, but there was a slightly widened absorption band at the wave number 1620.21 cm-1 which was probably a combined band of C=O stretching and C=C aromatic double bond absorption. . This was due to the strong resonance effect of the carbonyl group with the aromatic ring. Due to resonance, the electron density at C=O decreases and gives a single bond character. As a result, the absorption shifts to a lower frequency [20].

The suspicion of tannin compounds was confirmed by the presence of a substituted aromatic ring at the ortho position which was indicated by absorption peaks at wave numbers 748.38 and 669.30 cm^{-1}. These specific peaks were the specific peaks of the tannin compound, thus strengthening the assumption that the FE isolates were tannins. The interpretation of the IR spectra of FE isolates is presented in Table 8.

Analysis of FE isolate with UV-vis spectrophotometer showed that the isolates absorb UV light at a wavelength of 264.90 nm. This shows that there is a transition from π to π^* or n to π^* (Figure 6).

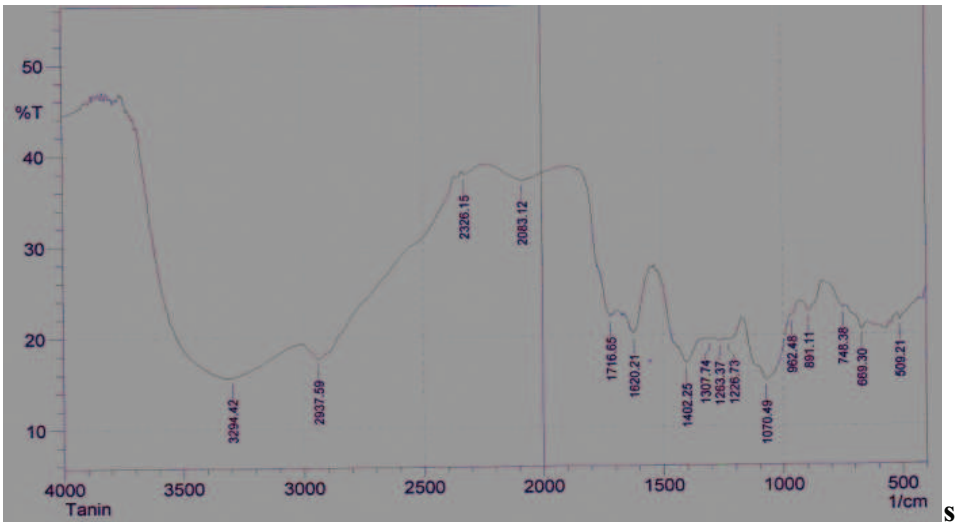

Figure 6: Infrared Spectra of FE Isolate.

TABLE 8: Interpretation of infrared spectra of FE isolates.

Peak	Wave Number (cm^{-1})		Vibration Type	Intensity
	FE isolate	References [23]		
1	3294.42	3500-3200	OH stretching phenol (hydrogen bonds)	*m-s*
2	2937.59	3000-2850	-CH stretching (alkena)	*m-w*
3	2326.15	-	CO_2 (air)	*w*
4	2083,12	-	-CH deformation	*w*
5	1716.65	1725-1700	-C=O	*s*
6	1620.21	1680-1600	-C=C aromatic	*m-w*
7	1402.25; 1307.4	1450; 1375	$-CH_3$ bending	*m-s*
8	1263.37; 1226.73; 1070.49	1300-1000	-C-O	*s*
9	962.48; 891.11; 669.3	900-420	-CH *out of plane*, p- benzene substitution	*m-w*
10	748.38	900-650	-OH *out of plane*, o-benzene substitution	*m-w*

Note: vs = *very strong*; s = *strong*; m = *medium*; w = *weak*

5. Conclusion

Based on the research results obtained, it can be concluded that acetone extract was able to inhibit the growth of *Fusarium solani* with a diameter inhibition of 25.50 mm. The

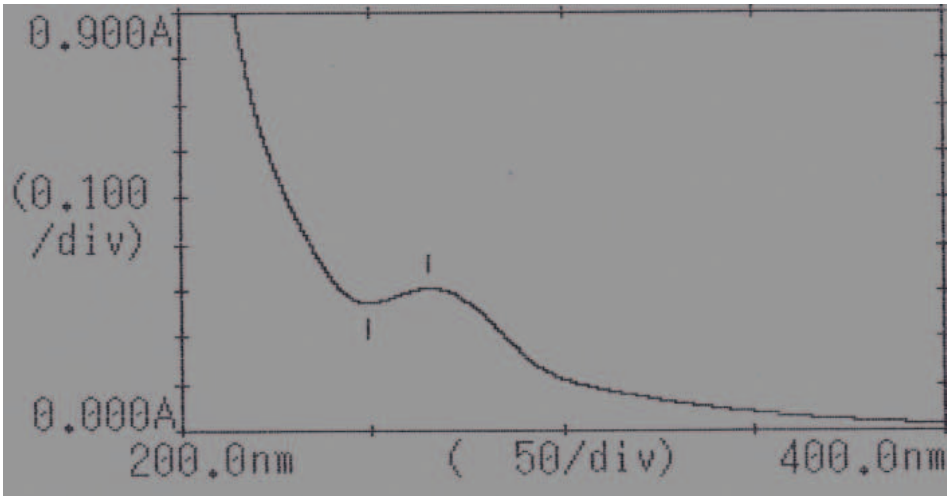

Figure 7: UV-vis spectrum of FE isolate.

most active fraction very stongly inhibited the growth of the fungus with an inhibition of 35.50 mm.

Phytochemical test of isolate from separation with HPLC showed that the isolate was a hydrolyzed tannin compound that absorbed UV-vis light at a wavelength of 264.90 nm. Based on infrared spectrophotometer analysis, isolated tannins contained –OH (phenol), C-H aliphatic, -C=O, -C=C aromatic, and -C-O groups.

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