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Research Article

Antibacterial Activity and Bioautography of the Chloroform Fraction of Morel Berry (*Physalis angulata* L.) Root Against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*

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Abstract
The urgency of finding novel sources of active compounds to overcome infectious diseases is encouraged. Morel berry (*Physalis angulata* L.) is a traditional herbal plant that can be used as an antimicrobial because of its unique chemical content. This study aims to find compounds that have antibacterial activity from the chloroform fraction of morel berry roots. This study used the Kirby -Bauer method with concentrations of 20%, 40%, 60%, 80%, and 100% against *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* bacteria. Determination of antibacterial compounds and their functional groups was performed using thin-layer chromatography (TLC) technique and reagent spray test, TLC-Bioautography and Fourier transforms infrared (FTIR) studies. The chloroform fraction of morel berry showed a zone of inhibition with the highest diameter of 11.50 and 12.00 mm against *S. epidermidis* and *P. aeruginosa*, respectively. Phytochemical screening revealed the presence of alkaloids, flavonoids, tannins, and saponins in the chloroform fraction. The relative retention stain of 0.54 on the TLC plate inhibited the growth of the two tested bacteria and indicated the presence of functional groups O-H phenol, C-H aliphatic, C=O ester, C=C aromatic, C-OH alcohol, and C-H aromatic. This study found that tannins in the roots of morel berries could be used as a natural antibacterial agent to treat *S. epidermidis* and *P. aeruginosa*.

Keywords: *Physalis angulata* L, bioautography, tannin, antibacterial, chloroform fraction

1. INTRODUCTION

The use of natural materials derived from plants as traditional medicine has long been practised to treat various health problems in tropical countries including Indonesia. This ethnomedical technique is quite profitable because the raw materials are easily obtained or cultivated, relatively cheap, can be prepared at home, have low toxicity, efficient, and are rarely accompanied by side effects [1]. Plants that have been selected for medicinal use for thousands of years are the most obvious starting point for effective new medicines. Recently, the use of medicinal plants has increased in spite of advances in synthetic drugs. There is also an increasing use of medicinal plants in industrialized countries for galenic preparations and herbal remedies [2].

The morel berry plant (*Physalis angulata* L.) belongs to the Solanaceae family, known in Indonesia as "ceplukan" or "ciplukan" (Figure 1). *P. angulata* L. is known to be rich in benefits as medicinal plants and has been shown to have antiparasitic, anti-inflammatory, antinociceptive, antimicrobial, antimalarial, antileishmanial, immunosuppressive, antiasthmatic, diuretic, and anticancer properties [3]. This ability is inseparable from the active compounds present in morel berry plants. Morel berry contains bioactive compounds like flavonoids (leaves and shoots), alkaloids (roots), tannins (fruit), saponins (shoots), polyphenols and physalin (leaves), and vitamin A and vitamin C (fruit) [4]. Other studies stated that this plant also contains flavonoids, saponins, fisalin A and B, witafisalin A and B, and also terpenes [3]. It is known that the composition of the active compounds in morel berry leaves is rich in flavonoids, alkaloids and physalin B, which has been conducted several times *in vitro* and *in vivo* studies showing the biological and pharmacological activities of flavonoid compounds are very diverse, one of which is having antibacterial activity [5].

Various research results state that morel berry root has shown antibacterial activity. The ethanol extract of the morel berry root was screened for phytochemicals positive for flavonoids, alkaloids and saponins and showed bactericidal ability against *Pseudomonas aeruginosa* bacteria at a concentration of 20% with an inhibition zone

Figure 1. Morel berry plant (*Physalis angulata* L.)

diameter of 10.01 mm [6]. The *n*-hexane, ethyl acetate and water fractions of morel berry herbal have antibacterial activity against *Staphylococcus epidermidis* [7]. The essential oil of the aerial and root parts of morel berry has antibacterial and antifungal activity with a MIC value of 4 mg/mL [8].

The results of the phytochemical screening showed that the ethanol extract of morel berry contained flavonoids, saponins, tannins, and alkaloids [9]. However, the ethanol extract still contains chemical compounds which have not been separated based on their polarity. Therefore, fractionation is carried out to separate the active compounds based on their polarity level. The solvent used for the morel berry root fraction is chloroform because the use of chloroform facilitates the process of separating compounds because only compounds that are semi-polar to those that are polar can dissolve in chloroform solvent. This is adapted to the basic principle of extraction, namely a compound will be dissolved only with a solvent that is relatively the same in polarity and besides that chloroform has a relatively low boiling point so that it is easily evaporated [10].

The bioautography method can be used as a detection method to find an antimicrobial compound that has not been identified by localizing the antimicrobial activity on a chromatogram resulting from thin-layer chromatography (TLC). The basis of this method is the agar diffusion technique, and the antibacterial compounds transfer from the TLC plate to the agar medium which already contains bacteria [11]. So far, the method for detecting antibacterial from morel berry plants uses the agar diffusion method. So, this study was done to see if the chloroform fraction of morel berry roots could suppress the growth of *S. epidermidis* and *P. aeruginosa* bacteria using bioautography methods and to find out what secondary metabolites were in the chloroform fraction of morel berry roots.

2. MATERIALS AND METHODS

2.1. Materials

Morel berry roots (*P. angulata* L.) were collected from Bandar Lampung, Lampung, Indonesia in March 2019. The two test bacteria used were *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Meanwhile, *Nutrient Agar* (NA), *Nutrient Broth* (NB), amikacin, aquadest (H_2O), ethanol 70% (C_2H_6O), ethanol pa, chloroform pa ($CHCl_3$), *n*-hexane pa ($CH_3(CH_2)_4CH_3$), methanol, $FeCl_3$, ammonia (NH_3), Liebermann-Burchard, and Bourchadat reagents were purchased and used without further purification.

2.2. Methods

2.2.1. Morel Berry Root Extract

Fresh morel berry roots, as much as 3 kg, were cleaned with flowing water to remove impurities. The roots were cut and dried without direct sunlight to obtain simplicia. A total of 500 g of simplicia morel berry roots were macerated with 70% ethanol. The solvent was changed every 24 hours until the solvent was clear (six times). Then, 250 mL of liquid extract was made by letting the extract evaporate in a rotary evaporator.

2.2.2. Morel Berry Root Fraction

A total of 100 mL of liquid extract of morel berry root was fractionated using a separating funnel and added to 100 mL of *n*-hexane, then shaken slowly and allowed to stand until the ethanol and *n*-hexane fractions were separated. The *n*-hexane fraction was separated and then repeated up to three times. The *n*-hexane fraction was collected. Next, 100 mL of chloroform was added to the ethanol fraction and shaken slowly and then allowed to stand until a layer of the ethanol fraction and chloroform fraction was obtained. The chloroform fraction was separated and then

repeated up to 3 times. The chloroform fraction was collected and evaporated with a rotary evaporator and then the obtained fraction was stored in the refrigerator.

2.2.3. Antibacterial Analysis

An antibacterial activity test using the agar well diffusion method. In a sterile petri dish, a 100 µL suspension of *P. aeruginosa* and *S. epidermidis* bacteria was added and followed by the addition of 25 mL of unsolidified NA medium. The mixture was homogenized and allowed to solidify. Several holes were made in the substrate using a 6 mm corkborer. The chloroform fraction of morel berry roots was injected with concentrations of 20%, 40%, 60%, 80%, 100%, as well as amikacin as a positive control and DMSO 1% as a negative control. Each hole received up to 100 µL of chloroform fraction of morel berry root, amikacin, and distilled water. All petri dishes were incubated for 24 hours at 37 °C. Furthermore, observations and measurements of the inhibition zone formed around the diffusion hole were carried out using a caliper. According to the classification of antimicrobial activity, it is classified into three levels: strong activity (DIZ > 20 mm), moderate activity (12 mm < DIZ < 20 mm), and weak activity (DIZ < 12 mm) [12].

2.2.4. Thin Layer Chromatography

The separation of compounds from the chloroform fraction was carried out using a silica gel plate $G_{60}F_{254}$ with a size of 7 × 2 cm. Next, a line was marked on the top and bottom edges of the plate with a distance of 1 cm to indicate the initial position of the spots and the top edge as a boundary sign of the elution process. Furthermore, the plate was activated by heating at a temperature of 100 °C for 1 hour to remove the water content on the TLC plate. Prior to elution, the eluent used was chloroform fractionate was left to be saturated first. Afterward, each mobile phase mixture was inserted into the chamber and then tightly closed and saturated using filter paper for 10 minutes. This saturation was done to equalize the vapour pressure in the entire vessel.

The chloroform fraction was spotted on the TLC plate at a distance of 5 cm from the bottom edge of the plate using a capillary tube and then dried by aeration. Then, it was put into a saturated chamber and allowed to elute up to the specified chromatogram plate limit. The plate was removed from the chamber and observed with a 254 nm and a 366 nm UV lamp. Visible stains were detected with Bouchardat spray reagent for alkaloids (brown color), ammonia for flavonoids (yellow brown/ green/brown/pink color), $FeCl_3$ for tannins (strong green, red, purple, blue or black color) and

Table 1. Inhibition zone of morel berry root chloroform fraction against *S. epidermidis* and *P. aeruginosa* bacteria

Concentration	Inhibitor zone diameter (mm)	
	S. epidermidis	***P. aeruginosa***
K. 20%	$0^{a} \pm 0.00$	$0^{a} \pm 0.00$
K. 40%	$8.04^{c} \pm 0.65$	$8.56^{c} \pm 1.45$
K. 60%	$8.76^{cd} \pm 0.28$	$8.82^{cd} \pm 0.98$
K. 80%	$9.94^{cd} \pm 0.92$	$9.95^{cd} \pm 1.20$
K. 100%	$11.50^{de} \pm 0.63$	$12.00^{de} \pm 0.88$
K -	$0^{a} \pm 0.00$	$0^{a} \pm 0.00$
K +	$30.03^{b} \pm 0.38$	$30.16^{b} \pm 0.38$

** Numbers followed by the same letter in the same column were not significantly different based on the 5% LSD test.*
*** K -: sterile distilled water; K +: Amikacin*

Liebermann-Burchard for saponins (purple color) [6]. Furthermore, the measurement of the retention factor (Rf) spots in the chromatogram from the TLC results was carried out.

2.2.5. Bioautography Test

As much as 20 mL of nutrient agar was poured into petridishes and left to solidify. On solid media, 100 µL suspensions of *S. epidermidis* and *P. aeruginosa* bacteria were spread. The chromatogram of the compounds separated by TLC was placed on the medium. The media was allowed to stand for 3 hours in the refrigerator, The chromatogram plate was removed from the medium. It was then incubated for 24 hours at 37 °C. The inhibition zone formed was calculated for its Rf value. Rf with a value equal to or close to the Rf value in TLC was thought to be the most effective active compound as an antibacterial.

2.2.6. Functional Group Characterization with FTIR Spectrophotometer

Identification of functional groups was carried out by scraping the TLC plate which had the same Rf as TLC-Bioautography from the chloroform fraction of morel berry roots. Subsequently identified using an FTIR spectrophotometer (Agilent/Cary 630) with the Inhouse method.

3. RESULTS AND DISCUSSIONS

3.1. Extraction and Fractionation

In this study, 3 kg of fresh morel berry roots were used and processed to obtain 500 g of simplicia. The drying process was done to reduce the amount of water in simplicia. The extraction of the active compounds contained in the morel berry roots was carried out by the maceration method. This method is included in the cold extraction method which can be carried out without using heating to avoid damage to the active substance. In the extraction process, 250 mL of morel berry root liquid extract was obtained. Ethanol 70% was selected as a solvent because the sample tested is a dry material so 30% of the water content serves to open the pores of the simplicia which facilitates the process of withdrawing the compound at the time of extraction [13].

Soaking the sample in maceration can make the cell wall of the sample break and make the compounds present in the sample in the cytoplasm attracted by the solvent. The cell wall breaks due to the difference in concentration inside and outside the cell. The concentration outside the cell is higher than the concentration inside the cell which is low enough that the cell wall breaks because it cannot withstand the pressure of the difference in

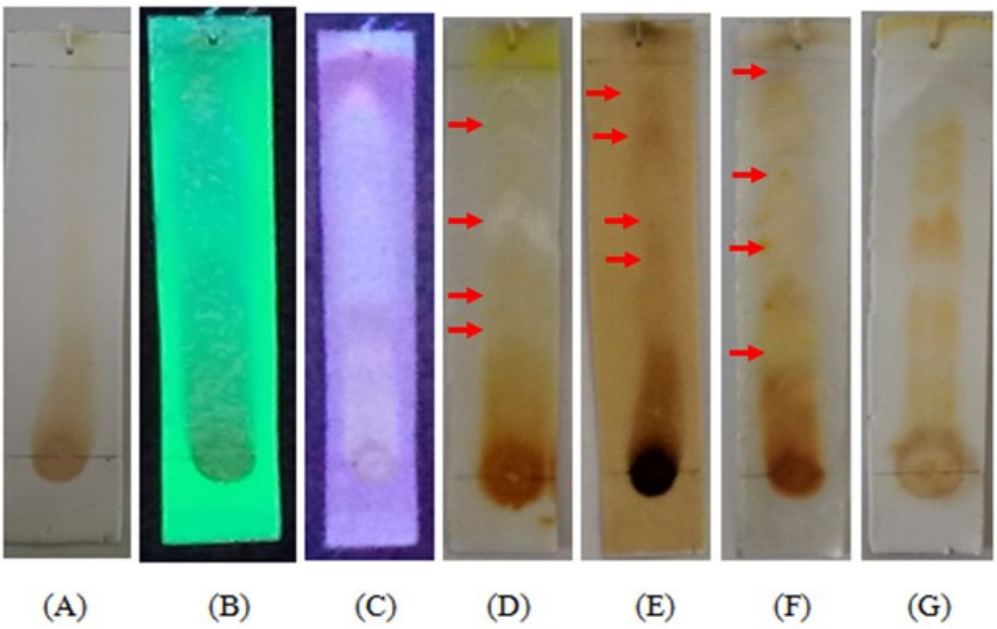

Figure 2. The cross-sectional results of the TLC test of the chloroform fraction of morel berry roots with the mobile phase of chloroform (5) (v)). A: Visible Light, B: UV 254, C: UV 366, D: Ammonia, E: $FeCl_3$, F: Bouchardat reagent and G: Liebermann-Burchard reagent.

concentration [14]. The sample was re-macerated by changing the solvent until the solvent was colorless which indicated that the maceration was completed as the maceration is more efficient to do repeatedly than just once [15].

The maceration results are then filtered and concentrated. Concentration is carried out with the help of a rotary evaporator at a temperature of 60 °C. Substances that are thermolabile must be concentrated at a temperature below their boiling point so that the content of secondary metabolites contained in the solvent is not damaged by high temperatures [16]. In this study, fractionation was carried out by the liquid-liquid fractionation method, which is a separation method using two immiscible liquids. The solvent with a higher density is in the lower layer while the solvent with lower density is in the upper layer. The fraction that was found was dried out with a rotary evaporator and then used for the test of antibacterial activity.

3.2 Antibacterial Analysis

Testing the antibacterial activity of the chloroform fraction using the agar diffusion method (well diffusion). The test bacteria used were *S. epidermidis* representing gram-positive bacteria and *P. aeruginosa* representing gram-negative bacteria. The use of these bacteria was aimed at determining whether the chloroform fraction of morel berry roots had specific or broad-spectrum antibacterial activity.

Antibacterial compounds will diffuse into solid media inoculated by bacteria and inhibit bacterial growth which is indicated by the formation of a clear area around the hole [11]. This method was chosen because it was simple, easy to work with, accurate, does not require special equipment, was versatile for all fast-growing pathogenic bacteria, and was often used in antibiotic susceptibility testing in quality control programs. The results indicated the presence of an inhibition zone around the well which is marked by a clear zone. This shows that the tested bacteria were sensitive to the chloroform faction of the morel berry roots and the antibiotics that were used as positive controls.

In this test, it was carried out at various concentrations of 20%, 40% 60%, 80% and 100% to determine the effect of giving different fraction concentrations to the test bacteria. The results of the average diameter measurement of the antibacterial power of the chloroform fraction against the test bacteria *S. epidermidis* and *P. aeruginosa* can be seen in Table 1.

In this study, the chloroform fraction was more effective in inhibiting the growth of *P. aeruginosa* bacteria than *S. epidermidis* bacteria. The different responses of these two bacteria to antibacterial compounds were caused by differences in the composition and structure of their cell walls. Differences in the cell wall structure of Gram-negative bacteria and Gram-positive bacteria affect the sensitivity to bacteria. The cell wall of Gram-positive bacteria consists of about 40 layers of peptidoglycan so that peptidoglycan makes up 70% of the dry mass of the cell wall. This causes the cell walls to become thick and stiff. In contrast to gram-negative bacteria, the cell wall of peptidoglycan is only about 10% of the dry mass of the cell wall,

Table 2. TLC test results chloroform fraction of morel berry root

Compound	Detection	Positive result	Research result	Commentary	Rf
Flavonoid	Ammonia	Brownish yellow, orange, red	Brownish yellow	+	0,36; 0,44; 0,60; 0,84
Tannin	$FeCl_3$	Green, red, purple, blue or black	Black	+	0,54; 0,68; 0,82; 0,92
Alkaloid	Bouchardat reagent	Brown	Brown	+	0,3; 0,58; 0,72; 0,96
Saponin	Lieberman Burchard	Purple	No changes	–	-
	Water 50 °C	Foam lasts 10 minutes	Foam was formed for 10 minutes	+	-

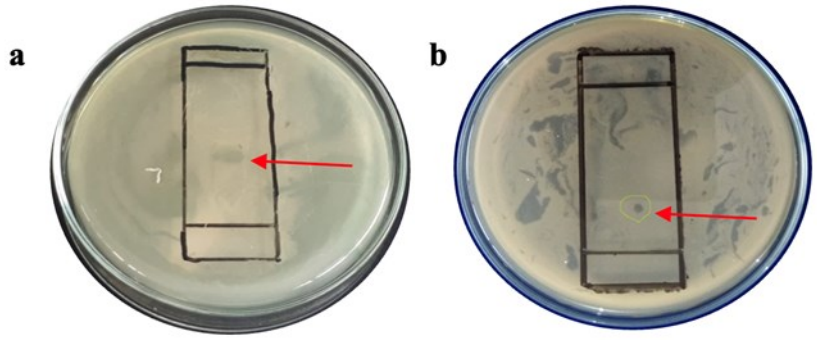

Figure 3. Results of bioautography of the chloroform fraction of morel berry roots with chloroform 5 (v/v) mobile phase against a.) *Staphylococcus epidermidis* and b.) *Pseudomonas aeruginosa.*

thus causing the cell wall of Gram-negative bacteria to be thinner [17]. Gram-negative bacteria have a large lipid content and have a protein porin that acts as a channel for the entry of active substances into bacterial cells. The entry of these active substances impairs enzyme activity in cells and causes cell damage. When there are a lot of lipids in cells, it makes it easier for active substances to get into cells [18].

The results obtained from the average inhibition zone measurement showed that the chloroform fraction of morel berry roots was not active against *S. epidermidis* and *P. aeruginosa*. These fractions with concentrations of 40, 60% and 80% against the test bacteria *S. epidermidis* and *P. aeruginosa* were classified as weak category. For a concentration of 100%, it is classified as moderate activity against *P. aeruginosa* bacteria. Based on the results, it shows that the antibacterial properties of the compounds in the chloroform fraction of morel berry roots are broad-spectrum. This means that they work against both Gram-positive and Gram-negative bacteria.

The ability of an antibacterial agent to eliminate the ability of living organisms depends on the concentration of the material used [14]. Based on the results of the antibacterial activity test in Table 1, the diameter of the inhibition zone for the growth of *S. epidermidis* and *P. aeruginosa* bacteria formed at a concentration of 100% was greater than the concentrations of 40, 60%, and 80%. The higher concentration of an antibacterial agent, the greater the antibacterial activity as the extract has more phytochemical compounds with antibacterial properties.

3.2. Phytochemical Screening by TLC

Morel berry roots were subjected to TLC analysis aiming to determine the compound content of the chloroform fraction. The mobile phase used for the chloroform fraction, namely chloroform (5) (v)), was chosen because it was adapted to the solubility properties of the compound being analyzed because it is semipolar [19]. The selection of this eluent is based on the results of the orientation of the eluent that has been carried out which in this eluent produces the best separation with the highest number of stains after detection of spots on UV lamps at 254 and 366 nm. According to Saifudin (2014) [20], the characteristics of a good eluent are marked by the number of spots that appear, the spots formed are tailless, and the distance between one spot and another is clear.

The results of the cross-sectional test of the chloroform fraction of morel berry roots on the TLC plate showed that there were flavonoids, tannins, and alkaloids (Figure 2 and Table 2). The saponin test based on Liebermen-Burchard's reagent did not show positive results, but with detection with warm water, a long-lasting foam was formed which proved that the chloroform fraction of morel berry roots was positive for saponins.

Morel berry roots contain active compounds including alkaloids, flavonoids, and saponins [6]. Spots on the chloroform fraction of morel berry roots by looking at the results of the ammonia spray test can be stated to contain flavonoid compounds with Rf 0.36, 0.44, 0.60, and 0.84. One of the stains on this fraction Rf 0.44 has the same Rf as group flavonoids so the ethanol fraction of morel berry roots may contain flavonoid compounds. Previous research on the identification of flavonoids in Alchemilla species showed the presence of

flavonoid compounds that had the same Rf value as flavonoids and rutin with Rf of 0.72 and 0.44, respectively [21]. Phytochemical results and TLC analysis from other studies showed that the results of spraying using $FeCl_3$ on the *E. hirta* L. extract produced a black color in the tannins [22].

The cross-sectional results of TLC of the chloroform fraction of morel berry roots showed positive results containing tannins after being sprayed with $FeCl_3$ which was indicated by the formation of a black color on the plate. The formation of a black color after being added to $FeCl_3$ is due to the fact that tannins will form complex compounds with Fe^{3+} ions [23]. The chloroform fraction of morel berry roots produced an Rf of 0.54, 0.68, 0.82, and 0.92. One of the spots from this fraction with an Rf of 0.54 is almost the same as the Rf in general, so it can be said that the chloroform fraction of the plant roots contains tannin compounds. Research on the polar fraction of *Murraya panicullata* L. Jack leaf extract stated a Rf value of 0.55 which is a tannin compound [24]. Phytochemical results and TLC analysis from other studies showed that the results of spraying using $FeCl_3$ on the *E. hirta* L. extract produced a black color in the tannins [22].

The cross-sectional results of TLC of the chloroform fraction of morel berry roots showed positive results containing alkaloids after being sprayed with Bouchardat reagent which was indicated by the formation of a brown color on the plate. In the manufacture of Bouchardat reagent, iodine reacts with I^- ions from potassium iodide to produce I^{3-} ions which are brown in color. In the Bouchardat test, metal ions K^+ will bind with nitrogen in the alkaloids to form a precipitated potassium-alkaloid complex [23]. The most common Rf values for alkaloids were 0.07–0.62 and the chloroform fraction of morel berry roots produced Rf values of 0.30, 0.58, 0.72, and 0.96, respectively. The spots with Rf 0.30 and 0.58 for the chloroform fraction were in accordance with the Rf in general so it can be said that the chloroform fraction of morel berry roots contained alkaloid compounds [25].

3.3. Bioautography Analysis

The bioautography test was carried out by TLC method. This test aims to determine the natural compounds contained in the chloroform fraction of morel berry roots on the chromatogram by looking at the inhibition zone formed on the agar medium. In this study, the contact bioautography method was used due to its simplicity of the work and the results are more clearly visible. The eluted plates were contacted on the surface of solid media containing *S. epidermidis* and *P. aeruginosa* bacteria inoculated for 3 hours, respectively. The plate was then removed from the medium and the agar medium was incubated, in order to obtain a zone of

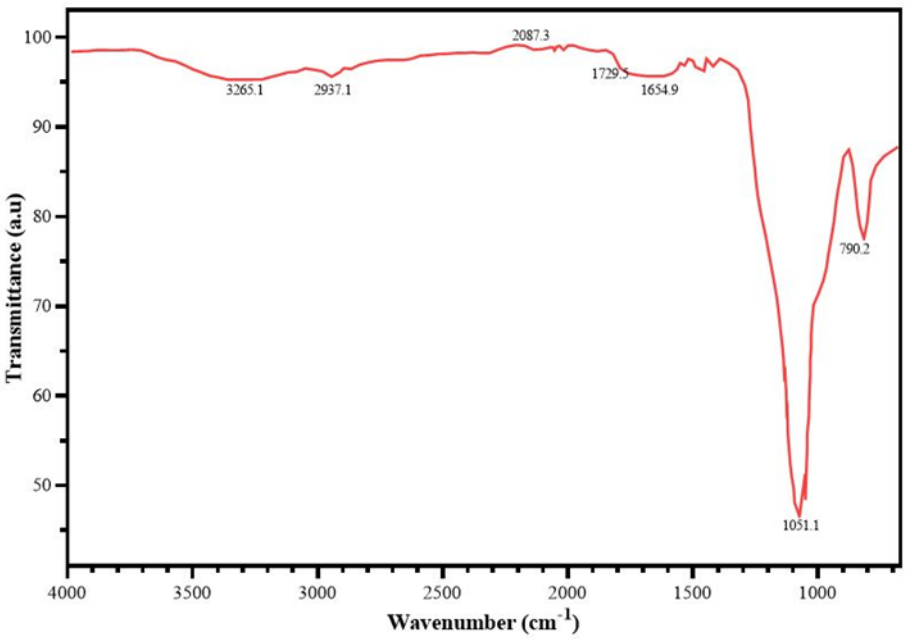

Figure 4. Results of the FTIR spectrum for the chloroform fraction of morel berry roots

Table 3. FTIR absorption pattern of morel berry root chloroform fraction

Wave number (cm^{-1})	Functional groups
3265.1	O-H phenol
2937.1	C-H aliphatic
1729.5	C=O ester
1654.9	C=C aromatic
1051.1	C-O alcohol
790.2	C-H aromatic

inhibition on the surface of the media where the plates were contacted indicating the location of the compound responsible for the antibacterial activity.

The results of TLC-Bioautography showed that there was an inhibition zone in the two tests (Figure 3). The largest inhibition zone that appeared was in the spots with an Rf value of 0.54 against both *S. epidermidis* and *P. aeruginosa*. From the results of the TLC test, the spots on the chloroform fraction Rf 0.54 were tannins, indicating that these active compounds had the most role as antibacterial agents.

3.4. FTIR

The functional groups of antibacterial compounds were identified to elucidate which functional groups that exhibit antibacterial activity against *S. epidermidis* and *P. aeruginosa* bacteria. The chloroform fraction of morel berry roots was re-eluted on the TLC plate with chloroform 5 (v/v) as eluent and the stain with a Rf value of 0.54 was scraped off. The results of the scrapings were analyzed by FTIR and shown in Figure 4.

The results of the FTIR analysis (Table 3) showed that the O-H group with a broad but not sharp band at a wavelength of 3265.1 cm^{-1} is a phenol group. This assumption was confirmed by absorption with a wavelength of 1051.1 cm^{-1}, indicating the presence of a C-O alcohol group. Absorption at a wavelength of 2937.1 cm^{-1} indicates the presence of a C-H aliphatic group. The absorption at a wavelength of 1729.5 cm^{-1} indicates the presence of a C=O ester group. Absorption at a wavelength of 1654.9 cm^{-1} indicates the presence of a C=C aromatic group. This assumption is reinforced by absorption at a wavelength of 790.2 cm^{-1} which indicates the presence of an aromatic C-H group [26].

The Rf stain of 0.54 from the chloroform fraction of morel berry roots showed the presence of functional groups O-H phenol, C-H aliphatic, C=O ester, C=C aromatic, C-O alcohol, and C-H aromatic. This functional group leads to the tannin group. Characterization of the bark of *Ceriops tagal* proved that the functional groups of O-H phenol, C-H aliphatic, C=C aromatic, C-O alcohol and C-H aromatic belong to the condensed tannin compounds [27].

4. CONCLUSIONS

The chloroform fraction of morel berry (*Physalis angulata* L.) root was proven to inhibit the growth of *S. epidermidis* and *P. aeruginosa* bacteria while the extract was only able to inhibit the growth of *P. aeruginosa*. The chloroform fraction of morel berry root contains potential bioactive substances including flavonoids, alkaloids, saponins, and tannins. The Rf stain 0.54 from the chloroform fraction of morel berry roots showed the presence of functional groups O-H phenol, C-H aliphatic, C=O ester, C=C aromatic, C-O alcohol, and C-H aromatic. Tannins with an Rf value of 0.54 at TLC in morel berry roots have been shown to have antibacterial activity against *S. epidermidis* and *P. aeruginosa*. From that perspective, they contain broad-spectrum antimicrobial agents that can be developed as an effective antibacterial treatment to combat typical antibiotic-resistant pathogens.

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Conflicts of Interest

The author(s) declared no conflict of interest

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