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Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Extracts from sponge-associated fungus *Trichoderma longibrachiatum* have been studied and contain antibacterial compounds which can inhibit several pathogenic multidrug-resistant organisms. This study aims to determine the active fraction of the extract which is antibacterial against the gram-negative Multi Drug-Resistant *Klebsiella. pneumoniae* pathogen and gram-positive Methicillin-Resistant *Staphylococcus aureus.* In this study, the fungus was cultivated using solid media of malt extract agar (MEA) for 6-9 days (24 hours dark, static, pH 5.6, 60 ‰ salinity, and 27 ^{o}C). The mycelia and media were macerated by methanol and then partitioned using ethyl acetate. Active fraction tracing was carried out using the bioautography method and then isolated by the open column chromatography method. Antibacterial activity testing was done using the Broth Dilution method to determine the Minimum Inhibitory Concentration (MIC). The results of the study showed that ethyl acetate extract contained one active fraction (R_f value = 0.14), which has reactive characteristics on vanillin reagent and absorbed ultraviolet light (λ 375.5 nm absorbance peak). The active fraction was able to inhibit the growth of MDR *K. pneumoniae* and MRSA bacteria at the same MIC value, i.e. 256 µg mL^{-1}. In conclusion, an active fraction of *T. longibrachiatum* can be developed as an antibacterial against MDR *K. pneumoniae* and MRSA.

Keywords: Sponge-Associated Fungus, Active Fraction, Antibacterial, Minimum Inhibitory Concentration

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic pathogen which can be categorized in gram-negative group, non-motile, facultatively anaerobic, and rod-shaped bacterium. These bacteria produce Extended Spectrum β-Lactamases (ESBL) which can degrade certain antibiotics (β-lactam group), such as penicillin and cephalosporin, so they become inactive (Farhat *et al*., 2009). Also, it is protected by a capsule (composed of polysaccharides), both of which will further increase its pathogenicity. The infectious diseases caused by them are such as liver abscess, bacteremia, lung infection, acute leukemia, meningitis, and the bacteria may even cause death (Turton *et al*., 2010; Adwan *et al*., 2020)). Hospitalized patients with weak immunity are the main target for this bacterial attack. In current conditions, there is a tendency to increase the prevalence of infection caused by *K. pneumonia* along with the decrease in sensitivity to antibiotics used to treat the infection (Li *et al.*, 2014; Santana *et al*., 2016). According to Adwan *et al.* (2020), the prevalence of capsular polysaccharide genes among *K. pneumoniae* and high level of drug resistance will make bacterial infections are increasingly widespread, both in the hospital environment and community which leads to failure of treatment.

Staphylococcus aureus (*S. aureus*) is another cause of some dangerous infection. *S. aureus* is gram-positive, coccus-shaped, non-spore-forming, non-motile, facultative anaerobes, and forms a biofilm. In particular, biofilm formation by Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection makes difficult treatment and causes a hard prognosis (Sato *et al*., 2019). For a long time, the infection has been treated by such semi-synthetic penicillin antibiotics as methicillin, and it is considered the first representative of multidrug-resistant bacteria (MDR) since 1961s (Gajdács, 2019). MRSA incidence and prevalence are more likely to occur in health care/hospital settings area than in the community environment. These bacteria produce enterotoxins (exotoxins), ESBL enzymes, and immune modulators (Abbas *et al*., 2015).

According to Narendran and Kathiresan (2016) as well as Basiriya *et al.* (2017), some species of *Trichoderma* sp. have been screened and eventually have the ability to synthesis some antibacterial compounds. Moreover, some studies reported that ethyl acetate extract of *Trichoderma* sp. has antibacterial activity against pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, and *S. aureus*,). These fungi isolated from the

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mangrove rhizosphere could be used as a producer of secondary metabolites to be developed into a new antibiotic against resistant bacteria. This was also stated by Synytsya *et al.* (2017) who investigated antibacterial compounds derived from ethanol and petroleum ether extracts. Some other researchers stated that the potential of antibacterial compounds produced by *Trichoderma* sp., such as *trichodin* A and B, pyridoxatin have antibacterial activity against *Staphylococcus epidermidis* and *S. aureus* (Wu *et al.*, 2014; Wang *et al*., 2020), and trichodermaquinone to be antibacterial compound against MRSA (Khamthong *et al*., 2012). There are many classes of secondary metabolite considered as antibacterial compounds from marine fungi, such as glycopeptides, peptides, proteins, lipopeptides, aminolipopeptides, polyketides, polybrominated biphenyl ether, cyclic depsipeptides, terpenes, pentaketides, alkaloids, diketopiperazins, anthraquinones, chromones, steroids, lactones, quinolone derivatives, trisindole derivatives, macrolactam, and phenol derivatives (Thomas *et al*., 2010; Nalini *et al*., 2018; Wang *et al*., 2020).

A review by Li *et al.* (2019) showed that *Trichoderma* spp. can produce many metabolites with different bioactivities. These fungi are commonly distributed in many ecosystems, including the sea. The investigation by Sedjati *et al.* (2020) proved that ethyl acetate extract of sponge-association fungus *T. longibrachiatum* contains compounds that have antibacterial activity against MRSA and *K. pneumoniae*. Based on these findings, this study aims to determine the active fraction in ethyl acetate extract of *T. longibrachiatum* using bioautographical methods by the guidelines of its bioactivity test results.

2. Materials and Methods

2.1. Fungus Isolate

The sample used in the study was from the sponge-associated fungus obtained from Falajava Beach, Ternate Island, North Maluku, Indonesia (00°47'09.12" N; 127°23'21.76" E coordinates) with TE-PF-03.1 code. The fungi have been identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence, and confirmed as *T. longibrachiatum* macro and microscopically (Sedjati *et al*., 2020).

2.2. Bacterial Pathogen

The test bacteria used in this experimental study were MDR *K. pneumoniae* obtained from Microbiology Laboratory, Diponegoro National Hospital, and MRSA from the University of Indonesia. Before being used for antibacterial tests, pathogenic bacteria were recultured first. The process was done by taking bacterial stock colonies and transferring them into Mueller-Hinton Broth (MHB; Oxoid) and further incubated at 37°C for 24 hours.

2.3. Fungus Cultivation

Fungus cultivation according to the method by Sedjati *et al*. (2020). *T. longibrachiatum* isolate coded TE-PF-03.1 was subcultured using Malt Extract Agar (MEA; Merck). Then, the mycelia were taken about 2 mm in diameter and cultivated on new MEA media. The treatment of cultivation periods was carried out at 6,7,8, and 9 days (static, 24 hours in dark, pH 5.6, salinity 60‰, temperature 27°C). MEA preparation was conducted by using sterile seawater (solid, 20 mL media/Petri dish).

2.4. Extraction and Determining Extract Weight

After the cultivation period finished, the media and the mycelia were cut into small pieces and then macerated with methanol (1:1v/v), filtered using Whatman paper no. 42 and the filtrate were evaporated at a rotary evaporator with 40°C and low pressure. Furthermore, partitioning of the fungal extract was done using methanol-distilled water (50%) and ethyl acetate (1:1v/v). Moreover, each fraction was evaporated using rota vapor to be methanol and ethyl acetate extracts, and then these were weighed.

2.5. Profiling of Secondary Metabolites

The Thin Layer Chromatography (TLC) method (Harborne, 1984) was used for profiling chemical extracts. There were in total 10 µl of extract solution in methanol (1 mg mL^{-1}) was spotted on the baseline of the TLC plate (Merck, silica gel 60 F254). The mobile phases used were sequential based on polarity levels, i.e. a mixture of n-hexane and ethyl acetate (4:1; 3:2; 2:3; 1:4, and 0:5). Spot identification using the value of R_f (Retention Factor) and spraying with staining reagents. After the elution process, TLC was visualized by UV light (365 nm), 2% vanillin-H_2SO_4, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (Harborne, 1984; Sen *et al*., 2012; Trianto *et al*., 2019). Furthermore, the TLC plate was heated at 110 °C for 2-3 minutes. The same method was used to detect the active antibacterial fraction after the isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

2.6. Antibacterial Activity Test

The antibacterial activity test was conducted using a disc diffusion assay method to determine the inhibition zone against pathogen growth. Pathogenic bacteria were cultured on Mueller-Hinton Agar (MHA; Oxoid) with an initial density equivalent to 0.5 McFarland (1.5 $x10^8$ CFU mL^{-1}). Extract in the dimethylsulfoxide (DMSO) solvent was tested against pathogenic bacteria. 10 µL of extract solution was dropped onto the sterile disc paper (6 mm diameter; Oxoid) with 500 µg $disc^{-1}$ concentration. The negative control used was DMSO, while the positive control used was chloramphenicol (30µg $disc^{-1}$; Oxoid). The inhibition zone was measured after 24 hours of incubation at 37 °C (Trianto *et al*., 2017).

2.7. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured on MHA media with 1.5 $x10^8$ CFU mL^{-1} initial density. The extract in ethyl acetate solvent (10 µL, 10 mg mL^{-1} concentration) was spotted on the TLC surface baseline and eluted with a suitable mobile phase to produce perfectly separated spots. The TLC plate was applied with silica surface attached to the MHA media surface (facing downward) and left for 60 minutes. Furthermore, the TLC plate was removed from the test bacteria medium and the Petri dishes were closed. All processes were carried out in a laminar flow cabinet. The incubation was carried out for 24 hours at 37 °C and the formation of the inhibition zone around the TLC spot was

carefully observed. The spots around which the next inhibition zone appears were called an active fraction.

2.8. Active Fraction Isolation

The active fractions found were isolated using the open column chromatography method with the appropriate mobile phase (referring to the TLC profile). The column was filled with silica gel (60–120 mesh) mixed with n-hexane: ethyl acetate (2:3) solvent. The extract was dissolved in the solvent and was slowly being loaded on the top surface of the silica gel. The extract was then eluted using a solvent sequence based on the polarity increase. Furthermore, the eluate coming out was collected using a test tube (every 10 mL of eluate), checked again using the TLC method, and the same eluates were put together. The eluates containing active fraction were concentrated at a rotary evaporator for further testing.

2.9. Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was conducted based on the Broth Dilution method. MIC determination refers to the method proposed by Sowjanya *et al.* (2015) and Fajarningsih *et al.* (2018) using 96-well microplates with resazurin (Sigma-Aldrich) as an indicator of the viability of the test bacteria (REMA assay). A total of 100 µL of the extract solution in the DMSO solvent with the highest concentration (2,048 µg mL^{-1}) was filled in the first well in certain rows. The next well was filled with 50 µL of sterile MHB nutrients. The 50 µL test material was transferred from the first well to the next well to reach serial dilution (at wells no. 1-10). Then, 30 µl of resazurin solution (0.02% in distilled water) was added to each well. At last, 10 µl of the bacterial suspension (1.5 $x10^8$ CFU mL^{-1}) was added to each well. Chloramphenicol was used as a positive control (the highest concentration was at 64 µg mL^{-1}) and DMSO as a negative control (at well no. 11). The well contained MHB without extract as growth media control (at well no. 12). The microplate was incubated at 37 ^oC for 24 hours. After the incubation period, the well functioning as growth control would appear pink. The MIC value was determined based on the lowest concentration which could inhibit the growth of the tested bacteria.

3. Results and Discussion

3.1. Assessment of Antibacterial Potential

T. longibrachiatum species is one of the fungi species which are easy to cultivate. It can grow well in MEA and modified media. The modified media is prepared by replacing malt extract with a cheaper material, namely fish and cassava extracts. All of them produce secondary metabolites that have antibacterial activities, but the best is obtained from ethyl acetate extract of fungus cultivated with MEA. The best antibacterial potential achieved is against pathogens *K. pneumoniae* and MRSA (Sedjati *et al.*, 2020). Data from the results of this study indicated that the secondary metabolite product of *T. longibrachiatum* cultivated in MEA was mostly in the form of methanol extract (polar compound) and only a small part of them was ethyl acetate extract (semi-polar to non-polar compound). The weight of fungal extract is based on the polarity of its secondary metabolites after 6-9 days cultivation periods described in (Figure 1a). Ethyl acetate extract was produced in only small amounts. On the other hand, the ethyl acetate extract has antibacterial activity against these two pathogens as shown in (Figure 1b). The greatest potential as an antibacterial was seen in the ethyl acetate extract from the fungus which had been cultivated for 9 days.

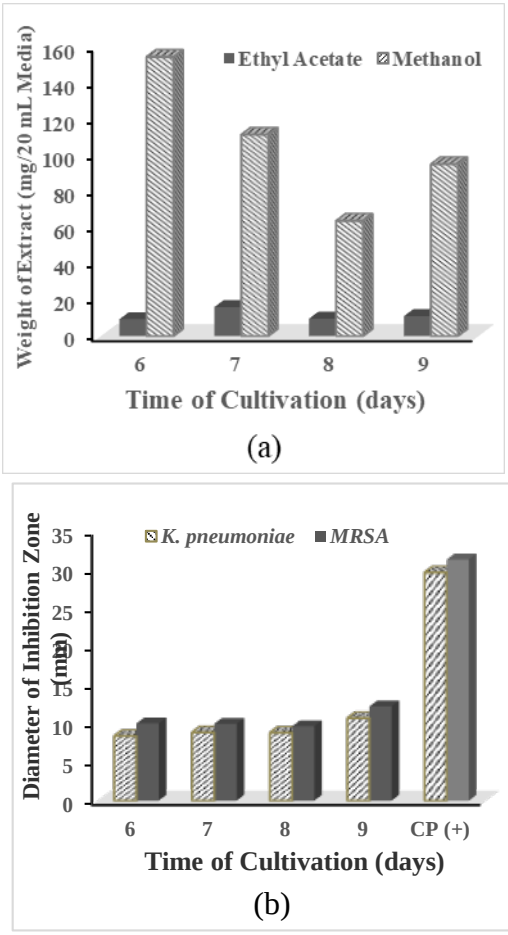

Figure 1. Characteristics of *T. longibrachiatum* extracts: (a) Extract weight on polarity basis; (b) Antibacterial potential of ethyl acetate extract against MDR pathogens *K. pneumoniae* and MRSA at a concentration of 500 µg $disc^{-1}$ (note: CP= Chloramphenicol, at a concentration of 30 µg $disc^{-1}$)

Some synthesized fungal secondary metabolites are only in small amounts because they are not for the main energy supply needed by the fungus and are only made at suboptimal conditions as a response to environmental pressure (Nielsen and Nielsen, 2017). The peak of secondary metabolite production in this study occurs when the fungal life cycle was in a stationary phase. This statement conforms to several research results stating that the fungus has entered a stationary period on day 6 to 9 after being cultivated (Gliseida *et al.*, 2013; Arumugam *et al.*, 2015). Methanol extract seemed to predominate over the extract of *T. longibraciatum.* However, when tested for antagonists against *K. pneumoniae* and MRSA at a concentration of 500 µg $disc^{-1}$, they did not show antibacterial activity. This fact is similar to the research result of Leylaie and Zafari (2018). In general, the ethyl acetate extract metabolite of the *T. longibrachiatum* is more likely to be antibacterial than its methanol extract. According to the statement of Chamekh *et al.* (2019), methanol extract is presumed to contain enzymes

synthesized by *T. longibrachiatum* for external digestion, along with several units of saccharides, amines/peptides, fatty acids/glycerol which are hydrolysis results of organic compounds in the media. Polar metabolites dissolved in methanol consist of enzymes (such as amylase, protease, and lipase) which are synthesized by fungi to degrade the nutrients in the media. Based on the research of Massadeh *et al*. (2010), the fungi can utilize a variety of carbon sources and produce various ligninolytic and cellulolytic enzymes. Added by Muthulakshmi *et al*. (2011), protease is produced by fungi from the first day of cultivation and reaches its peak on the 7th day (wheat bran as a media, pH 5.0, temperature 30^{0}C)

3.2. Secondary Metabolite of Fungal Extract

Chemical compounds contained in ethyl acetate extract of *T. longibraciatum* which was cultivated for 9 days can be traced based on its TLC profile as shown in Figure 2. Only ethyl acetate extract was used for the next stage of research since methanol extract is not potentially antibacterial. The best spot separation was seen in the results of TLC with a mobile phase of n-hexane and ethyl acetate (2:3) as seen in Figure 2. Based on the number of spots that appeared, at least 5 compounds were detected with R_f values: 0.14, 0.26, 0.57, 0.71, and 0.89. As congenial with the order of R_f values, the compound with the smallest R_f value is relatively the most polar, while the largest R_f is the most non-polar.

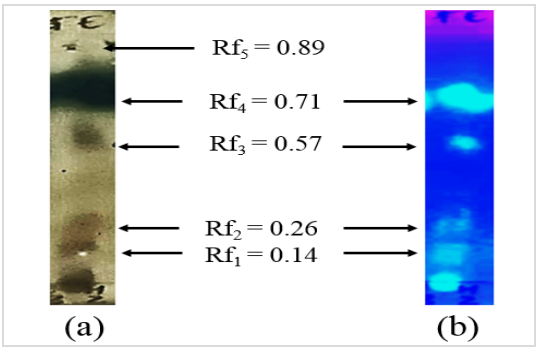

Figure 2. TLC Profile of ethyl acetate extract using mobile phase of n-hexane and ethyl acetate (2:3) : (a) Visualization results with 2% vanillin-H_2SO_4; (b) Visualization results with 365 nm UV light

Compound prediction in ethyl acetate extract of *T. longibrachiatum* was traced based on previous studies' references. The spot looks fluorescent blue when exposed to UV light indicating that the organic compound has a double bond (polyene or conjugated compound). The increased wavelength of the UVs (200-400 nm) absorbed indicates that the number of double bonds also increased (Hamilton-Miller, 1973; Mohammed, 2018). Besides, compounds reacting positively with the vanillin indicates the presence of carbonyl functional groups that contain a carbon-oxygen double bond (aldehydes, ketones). Accordingly, these compounds probably are from terpenoids, fatty acids/essential oils, steroids, flavonoids, or phenolic groups. A compound that reacts negatively to ninhydrin shows that it is not a nitrogen compound or its derivative. In contrast, negative to ferric (III) chloride indicates that the compound does not have a phenol functional group (Harborne, 1984; Jork, 1990). In this study, several spots in the TLC profile of ethyl acetate extract reacted positively to 365 nm UV light and vanillin reagent, but all of them reacted negatively to ninhydrin and ferric (III) chloride (as shown in Figure 2).

3.3. 3.3. Active Fraction as Antibacterial Against K. pneumoniae and MRSA

After an bioautography test was conducted on *K. pneumoniae* and MRSA pathogens, it was found that the spot with the smallest R_f (0.14) was the active fraction as antibacterial. The results of the bioautography test will help detect the presence of antibacterial compounds by the formation of an inhibition zone around the active spot as shown in Figure 3.

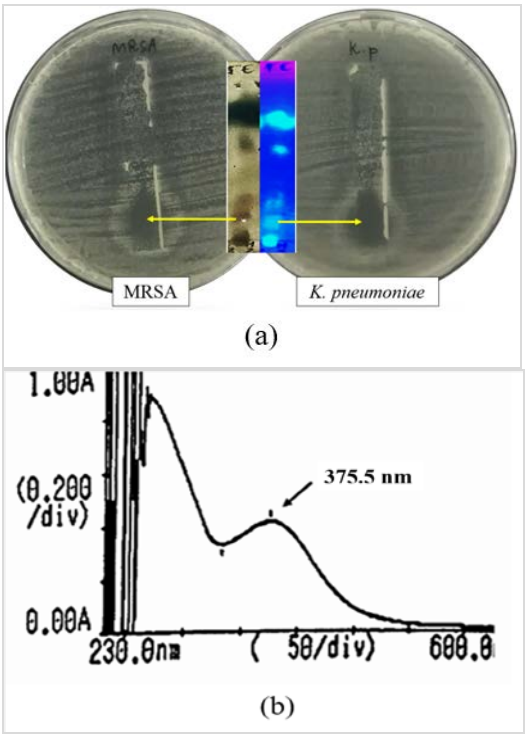

Figure 3. Determination of active fraction as antibacterial **:** (a) Bioautographical results of ethyl acetate extract against MRSA and MDR *K. pneumoniae*, (b) Characteristic of antibacterial active fraction based on spectra patterns towards UV light absorbance

The active fraction was reactive to vanillin and 365 nm UV light on TLC visualization results and was strengthened by the active fraction spectra pattern using a UV-Vis spectrophotometer which has λ 375.5 nm absorption peak (illustrated in Figure 3b). Based on the description of this characteristic, the active fraction is likely thought to have a carbonyl group and contain conjugated double bonds.

3.4. Minimum Inhibitory Concentration of Active Fraction

The cell wall of gram-negative bacteria is thinner, composed of peptidoglycan, 2 layers of phospholipids, and is protected by a lipopolysaccharide capsule. Gram-positive bacteria have thicker walls composed of peptidoglycan and lipoteichoic acid, and 1 layer of phospholipids (Lima *et al*., 2013). Antibacterial activity of the active fraction against MDR *K. pneumoniae* and MRSA pathogens resulted in a similar MIC value, i.e. at 256 µg mL^{-1}. *K. pneumoniae* bacteria are categorized as gram-negative bacteria, while MRSA is gram-positive. Both bacteria are still sensitive to chloramphenicol

antibacterial since their MIC value is less than 8 μg mL^{-1} (CLSI, 2017).

Chloramphenicol is a commercial broad-spectrum antibacterial. Moreover, chloramphenicol can damage important metabolic pathogens by binding the 50S ribosome subunit and blocking essential ribosomal function. The interaction of the nitrobenzyl functional group from chloramphenicol and the bacterial RNA nitrogen base may interfere with the formation of peptides during the process of protein biosynthesis done by bacteria (Kostopoulou *et al*., 2011).

The active fraction resulted from this study had a carbonyl group and also alternating double bonds (conjugation). The aldehyde and ketone carbonyl groups are highly polarized because carbon is less electronegative than oxygen. Carbon contains a partial positive charge (δ^+), while oxygen has a partial negative charge (δ). Hence, the carbonyl group can function as a nucleophile and an electrophile. The conjugation of a double bond to the carbonyl group will transmit the electrophilic character of the carbonyl to the beta-carbon of the other double bonds, or popularly called charge delocalization (Sarker and Nahar, 2007). Charged compounds ions will make them easier to interact with bacterial cell wall so that they can penetrate the cytoplasm membrane.

The mechanism of action of the active fraction as an antibacterial is assumed to be related to its ability to form electrophile sites, i.e. C with δ^+ partial charge which will electrostatically interact with the phospholipid head (PO_4^-) on the surface of the bacterial cell wall. According to Malanovic and Lohner (2016), a positive charge is essential for the initial binding to the surface of the bacterial membrane with a negative charge, which allows it to enter the bacterial cell membrane. Furthermore, these active compounds can affect the metabolic activity of bacterial cells which will eventually cause growth retardation or even death of pathogens.

T. longibrachiatum fungus does not only live in association with sponges in the sea. However, it has also been previously found in soft corals from the water of Panjang Island, Central Java. In addition, its ethyl acetate extract was able to inhibit the growth of MDR-*S. haemolyticus* and produced a 12.2 mm inhibition zone at a concentration of 300 μg $disc^{-1}$ (Sabdaningsih *et al*., 2017). The secondary metabolite from the same fungus has been published by Sperry *et al.* (1998). The ethyl acetate extract of *T. longibrachiatum* is associated with *Haliclona sp.* sponge from Sulawesi water containing an epoxysorbicillinol ($C_{14}H_{16}O_5$), is a member of sorbicillinoids (vertinoids) polyketide compounds. According to Harned and Volp (2011); Meng *et al*. (2016); Salo *et al*. (2016), sorbillinoids are secondary metabolites of hexaketide that undergo cyclization at the carboxylate terminus. Its chemical structure has several double bonds and carbonyl groups. The results of a study from Corral *et al*. (2018) showed that some of these have antibacterial activity, such as sorbicillin ($C_{14}H_{16}O_3$), sorbicillinol ($C_{14}H_{16}O_4$), dihydrosorbicillin ($C_{14}H_{18}O_4$), oxosorbicillinol ($C_{14}H_{16}O_5$), bisvertinol ($C_{28}H_{34}O_8$), and bisvertinolone ($C_{28}H_{32}O_9$). These compounds can inhibit pathogens *Acinetobacter baumannii*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*.

4. Conclusion

T. longibrachiatum fungi extracts contain an active fraction that can be developed as an antibacterial against gram-negative pathogens *MDR K. pneumoniae* and gram-positive MRSA. The active fraction is assumed to contain a carbonyl functional group and a conjugated double bond. The mechanism of its antibacterial action is related to the formation of electrophile sites on carbon. Thus, electrostatic interactions occur with negative charges on the cell walls of both gram-positive and gram-negative bacteria making it possible to penetrate the cytoplasmic wall. The active fraction of ethyl acetate extract was antibacterial against pathogens MDR *K. pneumoniae* and MRSA with the same MIC value, i.e. 256 μg mL^{-1}.

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Table 1. MIC value of active fraction and chloramphenicol against MDR *K. pneumoniae* and MRSA pathogens

Tested bacteria	Value of MIC (μg mL^{-1})	
	Active fraction	Chloramphenicol (positive control)
MRSA	256	4
K. pneumoniae	256	4