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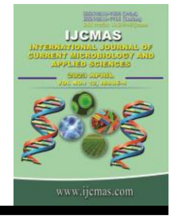

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Antibacterial Activities of Endophytic Fungi Liquid Cultures from *Michelia champaca* L. Plant

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A B S T R A C T

Endophytic fungi can produce secondary metabolites containing antibacterial compound**.** Some species of endophytic fungi have been isolated from *Michelia champaca* L. plant, those are: *Colletotrichum alienum*, *Geotrichum candidum*, *Rhizoctonia sp*, *Aspergillus ochraceus*, *Mycellia sterilia* 1, *Papulosporasp*, *Mycellia sterilia* 2, *Mycellia sterilia* 3, *Curvularia lunata*, *Colletotrichum kahawae*, dan *Aspergillus parasiticus*. The aims of this research are: 1) To examine the antibacterial activity of each endophytic fungi species against *Escherichia coli*, *Bacillus subtilis*, and *Propioni bacterium acnes*, 2) to determine the endophytic fungi species that has the highest antibacterial activity. Each endophytic fungi isolate is inoculated on Potato Dextrose Agar plate medium, and incubated in 27^{o}C during 7x24 hours, then cut with a sterile scalpel and inoculated into Potato Dextrose Broth (PDB) medium and shaken at 120 rpm for 7x24 hours. Then the liquid culture was centrifuged at 3000 rpm for 10 minutes. Furthermore, the supernatant from each endophytic fungi was treated to *E.coli*, *B. subtilis* and *P. acnes* to examine the antibacterial activity by agar diffusion method. The research results showed that: 1) each species of endophytic fungi have antibacterial activity against *E.coli*, *B. subtilis* and *P. acnes*, 2) the *Rhizoctonia* sp. has the highest antibacterial activity against *E. coli*, *Aspergillus parasiticus* has the highest antibacterial activity towards *B. subtilis*, and *Curvularia lunata* has the highest antibacterial activity towards *P. acnes*.

Keywords

Endophytic fungi, *Michelia champaca* L. plant, flower petal

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Introduction

Some bacteria species have been resistant towards some antibiotics. Some studies have been done to find eco-friendly natural antibiotics. Some medicinal plants can produce secondary metabolites, i,e: flavonoid, alkaloid, tannin which are potential to be used as a source of antibiotics (Ciocan and Bara, 2007). Medicinal plant parts i.e: rhizome, leaves, twig, bark, and flower petal could be use in this research. Most of the medicinal plants live in a symbiotic mutualism with endophytic fungi that live in their host plant tissues (Nair and Padmavathy, 2014). The endophytic fungi are able to produce antimicrobial secondary metabolites, that's why they can protect the host plants by inhibit the plant from microbial attack that cause diseases (Mwanga *et al.*, 2019; Mousa and Raizada, 2013). That's why some medicinal plants could stay healthy and are rarely attacked by microbes that cause diseases. The results of the preliminary study showed that there were 11 species of endophytic fungi isolated from *Michelia champaca* L. Each endophytic fungi species are able to produce: alkaloid, flavonoid, tannin, saponin, and terpenoid with different content. The five secondary metabolites could be extracted from the leaves, twigs, and flower petal. It is important to examine the antibacterial activity of 11 the endophytic fungi species liquid cultures towards *E.coli*, *B.subtilis*, and *P.acnes*. If the research result could prove that antibacterial potential of each endophytic fungi liquid cultures from *Michelia champaca* L. plant parts, so it can be used as an alternative for using the endophytic fungi liquid culture for eco-friendly natural antibiotic.

This research was aimed to: 1) To examine the antibacterial activity of each endophytic fungi liquid cultures species against *Escherichia coli*, *Bacillus subtilis*, *Propioni bacterium acnes*, 2) To determine the endophytic fungi species that has the highest antibacterial activity.

Materials and Methods

Materials : endophytic fungi isolates (*Colletotrichum alienum*, *Geotrichum candidum*, *Rhizoctonia sp*., *Aspergillus ochraceus*, *Mycellia sterilia* 1, *Papulospora sp*., *Mycellia sterilia* 2, *Mycellia sterilia* 3, *Curvularia lunata*, *Colletotrichum kahawae*, and *Aspergillus parasiticus*), cork borer, Potato Dextrose Agar (PDA) medium from MERCK brand, Potato Dextrose Broth (PDB) medium (consist of potato tuber essence, dextrose from MERCK and aquadest), Nutrient Broth (NB) medium (consist of beef extract for microbiology from MERCK, pepton from meat from MERCK, and distilled water)), Agar Nutrient medium from MERCK, chloramphenicol, and 70% alcohol.The antibacterial examination method was Agar Diffusion Method. The procedures are described as follows.

Endophytic Fungi Liquid Culture Preparation

The endophytic fungi liquid culture was prepare to obtain the secondary metabolites produced by each species of endophytic fungi (Guo *et al.*, 2008). Each endophytic fungi isolate was inoculated on PDA plate medium containing chloramphenicol (100mg/L) and then incubated at ± 27^{o}C for 7x24 hours. Then each endophytic fungi culture were cut into five pieces 1x1 cm in size. Each endophytic fungi pieces were inoculated into 100 mL PDB medium and shaken at 120 rpm incubated in 27^{o}C for 7x24 hours. Furthermore, the endophytic fungi liquid culture was filtered and centrifugated at the rate of 3000 rpm for 10 minutes. Each endophytic fungi species supernatant as much in 20 µLvolume was used for antibacterial examination in vitro (Siqueira *et al.*, 2011).

The Antibacterial Effect Examination of Endophytic Fungi Liquid Culture

The antibacterial activity examination of each endophytic fungi species liquid cultures supernatant was done against *Eschericia coli*, *Bacillus subtilis*, and *Propioni bacterium acnes* used agar diffusion method. Each bacterial species was inoculated into NB medium, standardized by McFarland 0.5 ($1.5x10^{8}$ cfu/mL) and incubated in 37^{o}C for 18 hours. The bacterial cultures were inoculated on NA plate medium and cut with a sterile cork borer to make wells in the medium then incubated in 37 ^{o}C for 1x24 hours.

Furthermore, the wells were filled with 20µL of supernatant for each endophytic fungi species, then incubated at 37^{o}C for 1x24 hours. The positive control used 5mg/mL chloramphenicol and negative control used PDB medium. The antimicrobial effect of each endophytic fungi species against *E. coli*, *B. subtilis*, and *P. acnes* was determined by measuring the growth inhibition zone around the well on NA medium. The data were analyzed with Anova and continued with Duncan's test to determine which species of endophytic fungi have the highest antibacterial activity compared to another species of endophytic fungi based on the growth inhibition zone diameter.

Secondary Metabolite Compound Detection of Each Endophytic Fungi Species

The flavonoid, alkaloid, tannin, saponin, and terpenoid compound in each endophytic fungi species liquid culture was analyzed by spectrophotometric methods.

Results and Discussion

The antibacterial activity of endophytic fungi liquid cultures can be determined based on the results of measuring diameter of the bacteria growth inhibition zone around the wells that have been filled with endophytic fungi species liquid cultures on NA plate medium. The NA plate medium was inoculated with the bacteria cultures: *E. coli*, *B. subtilis,* and *P. acnes*. The results showed that each endophytic fungi liquid cultures isolated from the *Michelia champaca* plant have antibacterial activity against that bacteria species. The antibacterial activity of endophytic fungi *Rhizoctonia* sp. towards three bacteria species is shown in Fig 3.1.

The *Rhizoctonia* sp. liquid culture was the most effective against *E. coli*, the *Aspergillus parasiticus* liquid culture was the most effective against *B. subtilis*, while the *Curvularia lunata* liquid culture was the most effective against *P. acnes* based on the growth inhibition zone diameter. The clear zone around the well contained with endophytic fungi liquid culture which is containing secondary metabolites proved that there was inhibitory activity on the growth of the bacteria (Fig 3.1). This proves that endophytic fungi secondary metabolites in the wells diffuse into the medium and inhibit bacterial growth. The results showed that each endophytic fungi species could produce the five antibacterial compounds, although the endophytic fungi species has been isolated from *Michelia champaca* plant as its host plant which could also produce similar secondary metabolites as produced by each endophytic fungi researched species.

The data were analyzed by Anova and followed by Duncan's test 5% to determine which endophytic fungi liquid cultures that have the highest antibacterial activity against *E.coli, B.subtilis*, and *P.acnes*. There was a difference in growth inhibition zone diameter between each endophytic fungi species towards the three bacteria species. The *Rhizoctonia* sp. liquid culture produced the largest growth inhibition zone diameter against *E.coli*, the *Aspergillus parasiticus* liquid culture produced the largest growth inhibition zone diameter against *B. subtilis*, and the *Curvularia lunata* liquid culture produced the largest growth inhibition zone diameter against *P. acnes.* The data are shown in Table 3.1.

Each endophytic fungi species liquid cultures isolated from *Michelia champaca* plant contained antibacterial compounds secondary metabolites. The antibacterial compounds that have been put into the well diffuse around it, so the bacteria that grow around the well are exposed by active compounds i.e: flavonoids, alkaloids, tannins and saponins, so it caused the death of the bacteria cells and formed clear zone called bacteria growth inhibition zone (Guo *et al.,* 2008; Yu *et al.,* 2010).

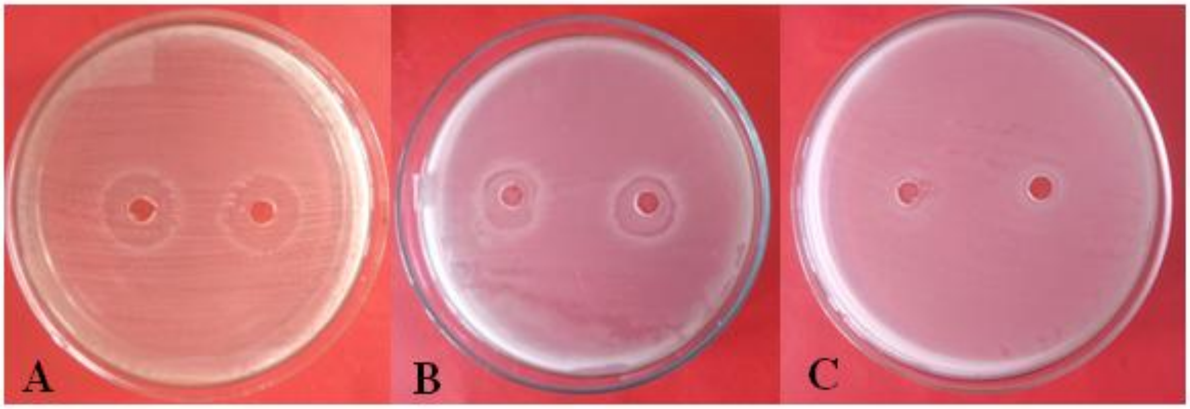
Alkaloids are one of the secondary metabolites active compounds which have antibacteria activity (Khameneh *et al.,* 2019). The antibacterial activity of alkaloid has been proven through various research results. Alkaloids are able to inhibit the formation of peptidoglycan in the bacteria cell wall so that the bacteria cell wall layer is not fully formed. The bacteria cell wall is a protector to the entire cell contents.

Table.1 Duncan's Test of Antibacterial Activity of Each Liquid Culture of Endophytic Fungi against Test Bacteria of *E. coli*, *P. acnes*, and *B. subtilis*

Treatment Endophytic Fungi Spesies	Test Bacteria	Average (mm)	Notation
Colletotrichum alienum	*E. coli*	0	a
Colletotrichum alienum	*P. acnes*	0	a
Colletotrichum alienum	*B. subtilis*	0	a
Geotrichum candidum	*E. coli*	0	a
Aspergillus ochraceus	*E. coli*	0	a
Aspergillus ochraceus	*P. acnes*	0	a
Aspergillus ochraceus	*B. subtilis*	0	a
Mycelia sterilia 1	*E. coli*	0	a
Mycelia sterilia 1	*P. acnes*	0	a
Mycelia sterilia 1	*B. subtilis*	0	a
***Papulospora* sp.**	*E. coli*	0	a
***Papulospora* sp.**	*P. acnes*	0	a
***Papulospora* sp.**	*B. subtilis*	0	a
Mycelia sterilia 2	*E.coli*	0	a
Mycelia sterilia 2	*B. subtilis*	0	a
Mycelia sterilia 3	*P. acnes*	0	a
Mycelia sterilia 3	*B. subtilis*	0	a
Curvularia lunata	*E. coli*	0	a
Colletotrichum kahawae	*E. coli*	0	a
Colletotrichum kahawae	*P. acnes*	0	a
Colletotrichum kahawae	*B. subtilis*	0	a
Aspergillus parasiticus	*E. coli*	0	a
Aspergillus parasiticus	*P. acnes*	0	a
K-	*E. coli*	0	a
K-	*P. acnes*	0	a
K-	*B. subtilis*	0	a
Mycelia sterilia 3	*E. coli*	1,075	a
Mycelia sterilia 2	*P. acnes*	2,2	a
Geotrichum candidum	*P. acnes*	2,34	a
***Rhizoctonia* sp.**	*B. subtilis*	2,525	a
***Rhizoctonia* sp.**	*P. acnes*	2,875	a
Curvularia lunata	*P. acnes*	2,98	a
Geotrichum candidum	*B. subtilis*	3,205	a
Curvularia lunata	*B. subtilis*	4,125	a
Aspergillus parasiticus	*B. subtilis*	4,34	a
***Rhizoctonia* sp.**	*E. coli*	14,44	b
K+	*B. subtilis*	18,45	b
K+	*E. coli*	18,76	b
K+	*P. acnes*	20,02	b

Explanation: K^+ = Chloramphenicol; K^- = PDB medium

Fig.1 Antibacterial activity of Endophytic Fungi *Rhizoctonia*sp against *E. coli*, *B. subtilis*, *P.acnes*. Description: A. *Rhizoctonia* sp. liquid culture against *E.coli*; B. *Rhizoctonia* sp. liquid culture against *B. subtilis*; C. *Rhizoctonia* sp. liquid culture against *P. acnes*.



The inhibition of cell wall formation could cause cell membranes susceptible to damage, so it can reduce cell wall semipermeability. Furthermore, the cell contents will come out from the cytoplasm and cause cellular metabolism inhibition. Furthermore, it caused inhibition in ATP formation, so the bacteria colony growth inhibited and followed by bacteria cell death (Sudrajat *et al.,* 2012).

Flavonoids are known as antibacterial agent against various pathogenic microorganisms (Xie *et al.,* 2015; Lamb and Cushnie, 2005). Flavonoid which have antibacteria activity can bind extracellular protein on the bacterial cell membrane. This can cause changes in the extracellular proteins structure. Furthermore, there will be a decrease in semipermeability of the cell membrane, thereby reducing cell metabolism. It can also cause inhibition of bacterial colony growth (Konate *et al.,* 2012). The OH- ion in the phenol component can bind to H+ ion in hydrogen bonds on the structural protein cell wall and cause protein denaturation (Zainab, 2013). Furthermore, it causes damage to the bacteria cell wall and cell membrane.

Tanninis a group of polyphenol that is commonly found in nature, besides that tannin is secondary metabolites result that can take a role as antimicrobia substance (Abbas, 2014; Kumar and Goel, 2019). Tannin can dissolve the fat layer on the bacterial cell wall and also denature structural protein on the bacteria cell membrane (Al-Ani *et al.,* 2008; Kaczmarek, 2020). This causes the cell wall structural damage and reduces the semipermeability of the cell membrane. Furthermore, it causes nutrient and enzymescome out of the cell, so that will cause cellular metabolism and colony growth inhibition.

Saponins are one of the secondary metabolites produced by endophytic fungi and have antimicrobial potential (Sadananda *et al.,* 2011; Jin *et al.,* 2017). Saponins can reduce surface tension, so it can increase in cell wall permeability. This causes intracellular compounds are released from the cytoplasm, so it could inhibit the cellular metabolism and cause bacteria cell death (Warganegara and Restina, 2016).

Terpenoid is an active chemical compound that can inhibit the growth of pathogenic bacteria (Abdel-Rahman *et al.,* 2019). Terpenoid can bind with purine, a transmembrane protein on the outer layer bacteria cell wall, to form a strong polymeric bonds that cause damage to purine. The cell wall damage causes the cell membrane will be susceptible to damage and then cause decrease in the cell membrane semipermeability. This facts causes the nutrients in the cytoplasm come out, and it cause an obstacle in cellular metabolism. Furthermore, the ATP produced by cellular metabolism will be decreases, so it caused bacteria colony forming inhibition (Salni *et al.,* 2011; Sathya *et al.,* 2012).

The research results showed that the liquid cultures of 11 species of endophytic fungi isolated from *Michelia champaca* are able to produce secondary metabolites containing antibacteria compounds, those are flavonoid, alkaloid, tannin, saponin and terpenoid. The 11 endophytic fungi species liquid culture isolated from *Michelia champaca* plant could inhibit *E. coli*, *B. subtilis*, and *P. acnes* colony growth.

The research result can be used as the basic for using liquid cultures of these endophytic fungi as the base material for eco-friendly natural antibiotics. The use of this liquid cultures useful as an another alternative for picking activity on *Michelia champaca* plant parts in order to take as a natural medicine.

Rhizoctonia sp. is an endophytic fungi species isolated from *Michelia champaca* plant, which is able to produce antibacterial active compounds, with have the highest antibacterial effect against *E.coli* compared to 10 other endophytic fungi species. *Aspergilus parasiticus* was able to produce antibacterial active compounds with the highest antibacterial activity against *B. subtilis*. *Curvularia lunata* was able to produce active compounds, which is antibacterial, with the highest antibacterial effect against *P.acnes*, compared to 10 other species of endophytic fungi.

The conclusion of this research are:

Each endophytic fungi species isolated from *Michelia champaca* L. have antibacterial activity against *E. coli*, *B. subtilis*, *P. acnes*;

Secondary metabolite in *Rhizoctonia* sp. liquid culture has the highest antibacterial activity towards the three bacterial species. The *Curvularia lunata* liquid culture is the most effective against *P.acnes*, the *Aspergillus parasiticus* liquid culture is the most effective against *B. subtilis*, and *Rhizoctonia* sp. liquid culture is the most effective against *E.coli*.

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