Borneo Journal of Pharmacy
Vol 5 Issue 3 August 2022
Page 279 – 287

http://journal.umpalangkaraya.ac.id/index.php/bjop/article/view/3692
DOI: https://doi.org/10.33084/bjop.v5i3.3692
e-ISSN: 2621-4814

Research Article

Isolation of Endophytic Fungus from Leaves of *Uncaria cordata* (Lour.) Merr and Antibacterial Activity Against *Propionibacterium acnes* and *Escherichia coli*

Melzi Octaviani*
Winda Yusma Ameliah
Neni Frimayanti
Meiriza Djohari
Haiyul Fadhli

Department of Pharmacy, Sekolah Tinggi Ilmu Farmasi Riau, Pekanbaru, Riau, Indonesia

*email: melzioctaviani@stifar-riau.ac.id

Keywords:
Antibacterial
Endophyte fungi
Isolation
Uncaria cordata

Abstract

Uncaria cordata (Lour). Merr (*akar kaik-kaik*) is one of the medicinal plants used as antibacterial because it contains bioactive compounds that can inhibit the growth of microorganisms. The plant is one of the sources of endophyte fungal isolates that can be developed as an alternative to producing antibacterial compounds. This research aimed to isolate the endophytic fungus from the leaves of *U. cordata* and know the antibacterial activity against *Propionibacterium acnes* and *Escherichia coli* by disc diffusion. The Fungi that were isolated from the leaves of *U. cordata* were 17 isolates. The isolates were continued for antibacterial activity testing: IFED 1 (*Nigrospora* sp.), IFED 2 (*Aspergillus* sp.), IFED 3 (*Fusarium* sp.), and IFED 4, whose genus was unknown. The results obtained were fungal isolates IFED 1 to IFED 4 had activity in inhibiting the growth of *P. acnes* with moderate category (18.16 mm) and weak categories (6.21, 6.16, and 6.68 mm) and in *E. coli* with moderate category (14.56 mm) and weak categories (6.53, 6.71, and 7.23 mm). The results of One-Way ANOVA and Tukey's test showed a significant difference ($p < 0.05$) between the diameter of the inhibition zone with the type of endophytic fungus supernatant isolated from the leaves of *U. cordata*. The best isolate of endophytic fungi inhibiting *P. acnes* and *E. coli* bacteria was IFED 1 (*Nigrospora* sp.).

Received: June 29th, 2022
1st Revised: August 4th, 2022
Accepted: August 14th, 2022
Published: August 31th, 2022

INTRODUCTION

Infectious diseases are disorders caused by organisms such as bacteria, viruses, fungi, or parasites. Many organisms live in and on our bodies[1]. They are generally harmless or even helpful. However, certain organisms that may cause disease are called pathogens under certain conditions[2]. Pathogenic bacteria consist of Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*, while Gram-negative bacteria, for example, *Escherichia coli*, *Salmonella typhi*, and *Pseudomonas aeruginosa*[3,4].

Bacterial infections can be treated with antibiotics[5]. However, excessive use of antibiotics can cause drug side effects and even antibiotic resistance[6]. Therefore, people return to using natural materials such as medicinal plants[7]. Medicinal plants have certain parts that can be used, including roots, rhizomes, stems, leaves, and fruit[8]. The lack of side effects from natural ingredients is an alternative for treatment for the community[9].

Bioactive compounds from medicinal plants can be used by extracting certain parts[10]. However, this method can make the existence of plants even more scarce if bioactive compounds are taken directly from rare medicinal plants[11]. One efficient way to overcome this is to use endophytic fungi[12].

How to cite: Octaviani M, Ameliah WY, Frimayanti N, Djohari M, Fadhli H. Isolation of Endophytic Fungus from Leaves of Uncaria cordata (Lour.) Merr and Antibacterial Activity Against Propionibacterium acnes and Escherichia coli. Borneo J Pharm. 2022;5(3):279-87. doi:10.33084/bjop.v5i3.3692

Endophytic fungi are microorganisms with a living habitat in organs such as seeds, roots, stems, and leaves for a certain period, colonizing plant tissues without harming the host plant[13]. Endophytic fungi can live in symbiotic mutualism with their host plants and produce secondary metabolites that have bioactivity, such as antimicrobial, antifungal, anticancer, antiviral, and antiparasitic[14]. Endophytic fungi produce secondary metabolites similar to those of the host plant. Therefore, efforts have been made to identify endophytic fungi isolated from medicinal plants so that they do not have to be extracted from their host plants[15].

Based on the description above, in plants of the *Uncaria* genus, endophytic fungi have antibacterial activity. Therefore, researchers are interested in researching the leaves of *Uncaria cordata* (Lour.) Merr or *akar kaik-kaik* (Indonesian). This study aimed to isolate the endophytic fungus from the leaves of the *U. cordata* and determine the antibacterial activity against *P. acnes* and *E. coli* using the disc diffusion method.

MATERIALS AND METHODS

Materials

The materials used include 70% ethanol, sterile distilled water, 5.3% sodium hypochlorite, chloramphenicol antibiotic disc, Nutrient agar (NA) (Merck), Potato Dextrose agar (PDA) (Merck), Potato Dextrose yeast (PDY) (Merck), Potato Dextrose broth (PDB) (Himedia), 0.9% NaCl solution, 2N sulfuric acid, concentrated hydrochloric acid, 1% iron (III) chloride, chloroform, 0.005N ammonia chloroform, magnesium metal, activated carbon, Liebermann-Burchard reagent, Dragendorff's reagent, and Mayer's reagent. The main instruments used in this study were an autoclave (GEA Model YX-280B), oven (Memmert), incubator (Memmert), incubator shaker (Selecta), analytical balance (Shimadzu), microscope (Shimadzu), UV-Vis spectrophotometer (Shimadzu). The sample used in this study was the leaves of *U. cordata* taken in Special Purpose Forest Area (*Kawasan Hutan dengan Tujuan Khusus*; KHDTK) Bukit Suligi, Rokan Hulu Regency, Riau, Indonesia. Endophytic fungi isolated from leaves of *U. cordata*.

Methods

Identification of the sample

The sample was identified at the Botanical Laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Riau, Pekanbaru, with the letter 88/UN19.5.1.1.3-4.1/EP/2021. The *U. cordata* plants used in this study are shown in **Figure 1**.

Figure 1. *Uncaria cordata* plants.

Phytochemical screening

A phytochemical screening test was carried out on fresh leaf samples and endophytic fungus supernatants. The screening test included alkaloids, flavonoids, saponins, tannins, steroids, and terpenoids[16].

Isolation and purification of endophytic fungi

Endophytic fungi were isolated from the leaves of the *U. cordata*. The leaves were washed with running water to remove dirt on the surface and then cut into 1-1.5 cm sizes. The clean leaves were surface sterilized by soaking successively in 70% ethanol for a minute, 5.3% sodium hypochlorite for five minutes, and 70% ethanol for 30 seconds, then rinsed with distilled water three times. The sterilized leaves were dried on filter paper, and then the leaves were split using a sterile scalpel on a sterile slide, after that the pieces of the leaf halves were placed on PDA media that had been given 25 mg of chloramphenicol and then incubated at 28°C for seven days[17].

Purification of endophytic fungi

Purification of endophytic fungi was carried out to obtain pure endophytic fungal cultures. The medium used to purify endophytic fungi was the new Potato Dextrose agar chloramphenicol (PDAC) media. Endophytic fungi growing on PDAC media were purified each on new PDAC media, then incubated at 28ºC for seven days. After incubation, observations were made on the shape and color of the colonies on PDAC media. Each colony with different shapes and colors was subcultured again on new PDAC media.

Identification of fungus isolates

Identification of fungi was carried out by observing the morphological characteristics and characteristics macroscopically and microscopically from fungal colonies grown on PDAC media at 28°C. Macroscopically the observed characters included the color of the colony surface, the color at the bottom of the Petri dish, the shape of the surface, concentric circles, and diameter. Microscopic observations include septal hyphae or not. Hyphae are hyaline (colorless) or dark pigmented. Hyphae are spiral-shaped, nodular, or rhizoid in shape[18].

Fermentation of endophytic fungi

Fermentation of endophytic fungi was carried out by liquid fermentation using PDY media. Seven days old on PDAC media in a Petri dish, endophytic fungi were taken using a sterile scalpel. A total of four pieces of mushrooms were put into 50 mL of PDY liquid fermentation medium. Furthermore, shaken fermentation was carried out using an incubator shaker at a temperature of 28ºC at a speed of 100 rpm for 14 days. Centrifugation was carried out for 20 minutes at a speed of 2000 rpm to obtain the fermentation results. The endophytic fungal supernatant was filtered using Whatman No.1 filter paper. The supernatant obtained was used to test the antibacterial activity[19].

Antibacterial activity test of endophytic fungus

The method of testing the antibacterial activity of agar diffusion method using NA medium. Antibacterial activity test of the endophytic fungus supernatant against *P. acnes* and *E. coli* bacteria was carried out using the Kirby-Bauer test method using disc paper. Disc paper was made from Whatman filter paper with a pore size of 0.22 m by cutting with a paper punch to obtain a paper disc with a diameter of 6 mm.

The suspension of the test bacteria aged 24 hours was transferred to a Petri dish containing the test medium at a temperature of 40-50°C, then homogenized and allowed to solidify. Sterile disc paper was dripped aseptically with 20 L of endophytic fungus supernatant and allowed to stand for 15 minutes, and then the disc paper was placed aseptically on the surface of the media that had been inoculated with the test bacteria. Petri dishes were then incubated at 37°C for 24 hours. The diameter of the clear zone formed was measured using a caliper. Paper disc chloramphenicol 30 g was used as a positive control, while 10 L sterile distilled water was used as a negative control. The test was carried out with three repetitions[20].

Statistical analysis

The data were analyzed statistically using the One-Way ANOVA method with the SPSS program if the data distribution was normal and homogeneous. If the result of One-Way ANOVA is $p < 0.05$, there is a significant difference between each

endophytic fungal supernatant and the inhibition diameter of the tested bacteria. Further testing can be carried out using Tukey for One-Way ANOVA to find out which treatments are significantly different in providing inhibition.

RESULTS AND DISCUSSION

Phytochemical screening of fresh samples aims to determine the content of secondary metabolites contained in fresh samples. The secondary metabolites contained in the fresh samples were alkaloids, flavonoids, terpenoids, saponins, and phenolics. The results of the observations can be seen in **Table I**.

Table I. Results of phytochemical screening of fresh samples of leaves of *U. cordata*

Secondary metabolites	Reagent	Description	Result
Alkaloid	Mayer	White precipitate	+
Flavonoid	Mg + HCl	Red color	+
Phenolic	$FeCl_3$	Green color	+
Saponin	Foam test	Produce foam	+
Steroid	Liebermann-Burchard	No green color	-
Terpenoid	Liebermann-Burchard	Red color	+

The leaves of the *U. cordata* plant that have been taken are washed using running water until they are clean of stains attached to the leaf surface. Then the leaves were cut into small fragments with an area of 1 cm^2 using a sterile scalpel. Then the leaf pieces were sterilized using 5.3% NaOCl solution and 70% alcohol to kill and inhibit epiphytic microbes during the incubation of endophytic fungi. The sterile leaf pieces were then cut in half using a sterile scalpel, placed into a Petri dish containing PDAC media, and then incubated at 28ºC for seven days. The isolates of endophytic fungi from the leaves of *U. cordata* were purified and incubated for seven days at 28ºC to obtain pure isolates based on different morphological characteristics. The purification results obtained as many as 17 isolates of endophytic fungi that showed different macroscopic morphological characteristics with isolate codes IFEDs 1 to 17.

Macroscopic observation IFED 1 had the characteristics of culture growth within 3-4 days, the colonies were white and gray, the color at the bottom of the Petri dish was brown to black, the colony size was medium, the texture was rough, had concentric circles, and the growth diameter was 5 cm. Microscopic observation of hyphae insulated, conidia black. Based on the macroscopic and microscopic characteristics described and compared with the literature, IFED 1 belongs to the genus *Nigrospora*[21-23]. *Nigrospora* has white and then gray colonies; the color on the bottom of the Petri dish is brown to black; the hyphae are hyaline and insulated, and the conidiophores are simple and round.

Macroscopic observations of IFED 2 had the characteristics of colony growth within 3-5 days, white colonies then rapidly growing black, the color at the bottom of the Petri dish was black, the surface was downy, had concentric circles, and the growth diameter was 9 cm. Microscopic observations of hyphae are insulated and branched; conidiophores are hyaline in color, and conidia are round and oval. Based on the macroscopic and microscopic characteristics described and compared with the literature indicating that IFED 2 belongs to the genus *Aspergillus*[21-23]. *Aspergillus* has a white colony color, then quickly grows black and has concentric circles. Insulated hyphae and conidiophores are hyaline in color and round in shape. The mycelium is initially white, and then the sporangium becomes yellowish brown, green, or blackish.

Macroscopic observations of IFED 3 had colony growth characteristics within four days, and colonies were white like cotton and round in shape; the color at the bottom of the Petri dish was cream and had concentric circles and a growth diameter of 3.6 cm. Microscopic observations of hyphae are not insulated and are hyaline in color, branched conidiophores. Based on the macroscopic and microscopic characteristics that have been described and compared with the literature, IFED 3 is included in the genus *Fusarium* and has the characteristics of white colony color, short, simple, or branched conidiophores[21-23].

The observation results of the morphological characteristics of IFED 4 had the characteristics of white-green colonies. The color at the bottom of the Petri dish was dark green yellow, had concentric circles, and a growth diameter of 7.7 cm. Microscopic observations of the hyphae are hairline and insulated. The macroscopic and microscopic characteristics

described and compared with the literature indicate that IFED 4 is not yet known[21-23]. Isolates of endophytic fungus leaves of *U. cordata* can be seen in **Table II** and **Figure 2**.

Table II. Identification results of isolates of endophytic fungus of leaves of *U. cordata*

Fungal isolates code	IFED 1	IFED 2	IFED 3	IFED 4
Colony color	Grayish white	white	white	white
The color of the bottom of the Petri dish	Yellow dark brown	blackish green	White cream	White yellow
Diameter	5 cm	9 cm	3.6 cm	7.7 cm
Concentric circle	Yes	Yes	No	Yes
Texture	Coarse	Smooth	Cotton	Smooth
Hyphae	Insulated hyphae	Insulated hyphae	Insulated hyphae	Insulated hyphae
Hyphae color	Hyaline	Hyaline	Hyaline	Hyaline
Genus	*Nigrospora* sp.	*Aspergillus* sp.	*Fusarium* sp.	Unknown

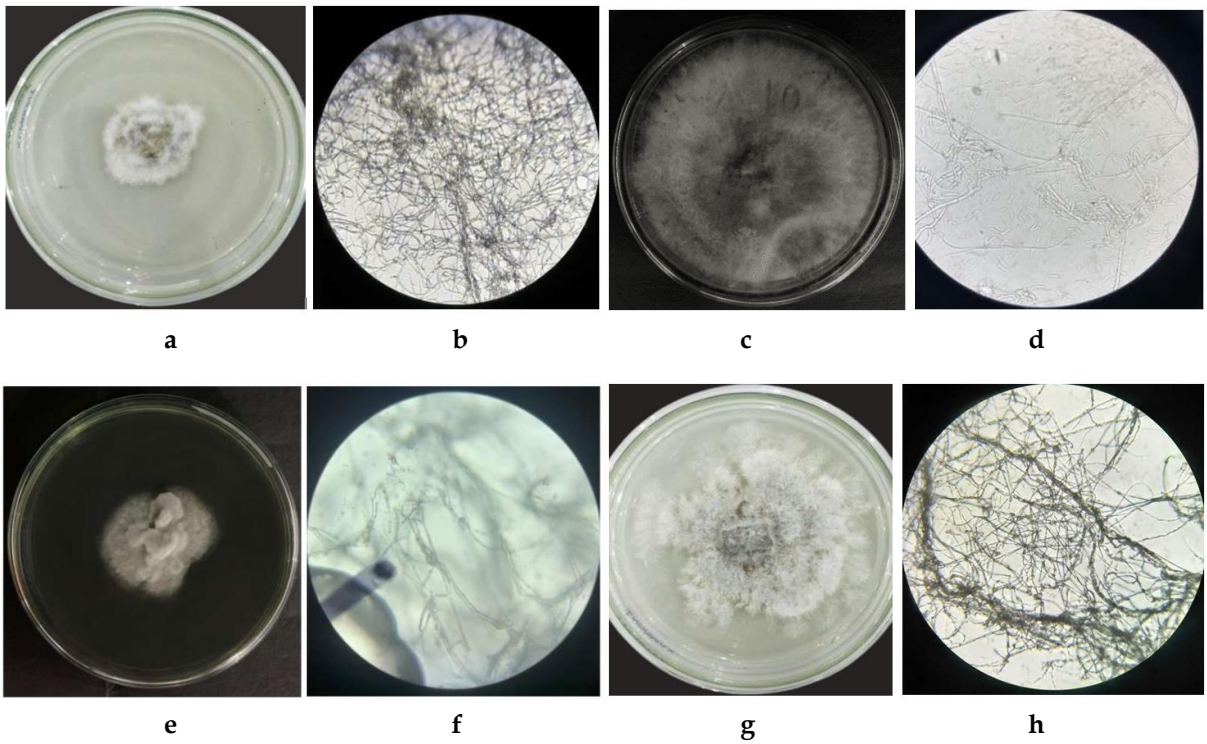


Figure 2. Isolates of endophytic fungus leaves of *U. cordata*. Respectively, the following are macroscopic and microscopic views of IFED 1 (**a** and **b**); IFED 2 (**c** and **d**); IFED 3 (**e** and **f**); and IFED 4 (**g** and **h**).

In this study, the IFEDs 1 to 4 has been identified by macroscopic and microscopic stages so that these isolates could be tested for antibacterial activity. As for the other isolates, the identification stages have not been carried out yet. The antibacterial activity test was used as supernatant of fermented endophytic fungi. Fermentation of endophytic fungi was carried out for 14 days by shaking using an incubator shaker. The fermentation process aims to remove secondary metabolites contained in endophytic fungal colonies. The formation of secondary metabolites occurs in the stationary phase, namely the phase when the rate of cell division and the rate of microbial death reach equilibrium, starting when the nutrients in the growth medium of microorganisms have been exhausted. The limitation of nutrients in the medium can cause the accumulation of secondary metabolite enzymes and secondary metabolite genes, which are thought to increase the production of secondary metabolites[24].

The fermentation process of endophytic fungi uses liquid media because fermentation with liquid media is more effective in producing biomass and bioactive compounds than fermentation in solid media. Fermentation of endophytic fungi using PDY media because this medium contains carbon from potatoes, dextrose, and yeast as nitrogen sources. Fermentation media must contain nutrients for growth, energy source, a constituent of cell substances, and biosynthesis of fermentation products. The most important media components are carbon and nitrogen sources because microbial cells and fermentation

products are mainly composed of carbon and nitrogen elements. In addition, it also contains organic salts as well as several vitamins and minerals[24].

The formation of microbial fermentation products can be influenced by several factors, such as substrates and nutrients. In fermentation, substrates are needed that are cheap, easy to obtain, and efficient in their use. Some substrates that can be used as carbon sources are molasses and starch. Meanwhile, ammonium salts, urea, nitrates, and soybean flour can be used as nitrogen sources. The second factor is pH; pH measurements are carried out to maintain the medium at the optimum pH during fermentation. Molds have an optimum pH between 5 and 7, and can grow in the pH range of 3-8.5[24,25].

The third factor is temperature; the fermentation temperature is carried out at a temperature where cell growth or metabolite production is highest. Most microorganisms can only grow in a temperature range of 20-30ºC. Based on the optimum growth temperature, the microorganisms used in the fermentation were classified as mesophiles with an optimum temperature of 20-45ºC and thermophiles with an optimum temperature of 45ºC. Microorganisms with a reasonable growth rate below 20ºC are classified as psychrophiles. The fourth factor is aeration and agitation; aeration aims to provide adequate oxygen supply, maintain aerobic conditions and remove carbon dioxide gas produced during fermentation. Agitation also aims to even out the spread of microorganisms, nutrients, and oxygen in the medium[24,26].

Phytochemical screening of the leaf endophytic fungus supernatant *U. cordata* using the TLC method. Phytochemical screening is a preliminary stage to provide an overview of the class of compounds contained in the endophytic fungal supernatant. Phytochemical screening of endophytic fungal supernatants was carried out using TLC, sprayed with staining reagent on the TLC plate, and observed the color changes that occurred on the TLC plate. The phytochemical screening found that secondary metabolites were contained in the endophytic fungal supernatant of the leaves of the *U. cordata*; IFED 1 contained alkaloids, flavonoids, phenolics, and terpenoids. The results of phytochemical screening of endophytic mushroom supernatants at IFED 2 were positive for phenolic compounds and flavonoids. In IFED 3, there are alkaloids, phenolic, and flavonoid compounds. Meanwhile, IFED 4 contains alkaloids and terpenoids. The results of the antibacterial activity of endophytic fungi against *P. acnes* and *E. coli*, endophytic fungi isolated from the leaves of the *U. cordata* showed different activities of each isolate in producing its antibacterial metabolites. Antibacterial activity of endophytic fungus leaves of *U. cordata* can be seen in **Table III** dan **Figure 3**.

Table III. Antibacterial activity of endophytic fungus of leaves of *U. cordata*

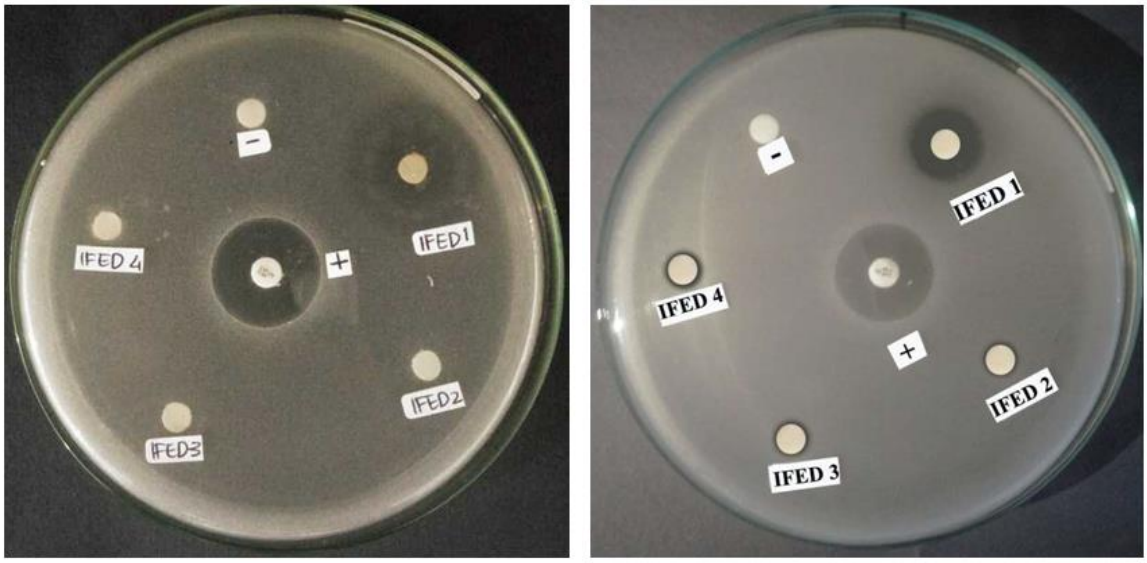
Bacteria	Treatment	Diameter of inhibition zone (mm)			Mean diameter of inhibition zone±SD (mm)
		I	II	III	
Propionibacterium acnes	K(-)	-	-	-	-
	K(+)	23.6	23.2	22.1	22.96±0.77
	IFED 1	17.6	18.7	18.2	18.16±0.55
	IFED 2	6.1	6.4	6.15	6.21±0.16
	IFED 3	6.1	6.25	6.15	6.16±0,07
	IFED 4	6.6	6.7	6.75	6.68±0.07
Escherichia coli	K(-)	-	-	-	-
	K(+)	24.5	25.1	23.3	24.3±0.91
	IFED 1	14.9	14.5	14.3	14.56±0.3
	IFED 2	7.1	6.4	6.1	6.53±0.51
	IFED 3	7.4	6.6	6.15	6.71±0.63
	IFED 4	6.3	7.5	7.9	7.23±0.83

Note: K(-): negative control; K(+): positive control

One-Way ANOVA analysis showed a significant difference ($p < 0.05$) between each endophytic fungal isolate with positive control and negative control on the diameter of the inhibition formed. Tukey's analysis showed that each endophytic fungal isolate differed significantly from *P. acnes* and *E. coli*. The best isolate of endophytic fungi inhibiting *P. acnes* and *E. coli* was IFED 1 (*Nigrospora* sp.).

This study showed that the inhibition zone formed in Gram-positive bacteria was more significant than that of Gram-negative bacteria. This is due to differences in the sensitivity of the bacteria. Gram-positive bacteria have a simpler cell wall than Gram-negative bacteria, which have a more complex cell wall. According to Harti[27], the cell wall structure of Gram-

negative bacteria is relatively more complex; the cell wall structure has two layers, namely the outer layer in the form of lipopolysaccharide and protein and the inner layer in the form of peptidoglycan. While the cell wall structure of Gram-positive bacteria is simple, it has one layer of peptidoglycan[28].



a **b**

Figure 3. Antibacterial activity of endophytic fungus leaves of *U. cordata* against *P. acnes* (**a**) and *E. coli* (**b**).

CONCLUSION

The fungi that were isolated from the leaves of *U. cordata* were 17 isolates. The isolates were continued for antibacterial activity test: IFED 1 (*Nigrospora* sp.); IFED 2 (*Aspergillus* sp.); IFED 3 (*Fusarium* sp.); and IFED 4, whose genus was unknown. The results were that fungal isolates IFED 1 to 4 had activity inhibiting the growth of *P. acnes* and *E. coli*. The results of One-Way ANOVA and Tukey's test showed a significant difference ($p < 0.05$) between the diameter of the inhibition zone with the type of endophytic fungus supernatant isolated from *U. cordata* leaves. The best isolate of endophytic fungi in inhibiting *P. acnes* and *E. coli* was IFED 1 (*Nigrospora* sp.).

ACKNOWLEDGMENT

We thank Sekolah Tinggi Ilmu Farmasi Riau for financing research funds through the Applied Research Grant year 2021 with grant number 12j.15.P3M.STIFAR.VIII.2021. This research was presented at the 2nd International Conference on Pharmacy Science and Practice (ICPSP) 2022 in Pekanbaru, Riau, Indonesia, on January 27th, 2022.

AUTHORS' CONTRIBUTION

Melzi Octaviani: conceptualization, funding acquisition, methodology, visualization, writing-original draft, writing-review & editing. **Winda Yusma Ameliah**: formal analysis, investigation, project administration, resources, writing-original draft. **Neni Frimayanti**: funding acquisition, methodology, supervision, validation. **Meiriza Djohari**: supervision, validation. **Haiyul Fadhli**: supervision, validation, writing-review & editing.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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