Antibacterial Activities of Secondary Metabolites in Endophytic Fungi from Bark of Beringin (*Ficus benjamina* L .) Tree *In Vitro*

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ABSTRACT

Aim: This study aims to isolate endophytic fungi and to elaborate the bacterial activity of endophytic microbes from bark of Beringin (*Ficus benjamina* L.) tree.
Methods: Isolation is carried out from the inside of the the bark of Beringin tree, with the surface sterilization method and direct planting. Isolated were fermented, the fermented supernatant was extracted partially with *n*-hexane, ethyl acetate, and *n*-butanol solvent. The extract was then concentrated and tested for antibacterial activity by agar diffusion method.
Results: Six endophytic fungi colonies were isolated from bark of beringin tree from this study, ethyl acetate and n-butanol extracts from the six isolates showed, in the ethyl acetate phase KB 4.1 isolates provide the greatest inhibitory power against pathogenic microbes compared to other isolates.
Conclusion: It was concluded that the six isolates from the bark of beringin tree had antibacterial activity.

Keywords: antibacterial, secondary metabolite, endophytic fungi, bark of *Ficus bejamina* L. tree

INTRODUCTION

Antimicrobials are substances that are often used to treat infections disease caused by pathogenic bacteria and fungi. The use of various types of plants is considered as one of the solutions to obtain alternative new drugs that are efficacious as antimicrobials. However, if the plant is used in large quantities, it does not rule out the possibility of extinction in the future[1].
Beringin tree or waringin (*Ficus benjamina* L.) is one of the most widely woody plants in Indonesia. *Ficus benjamina* L. contains various chemical compounds such as naringenin and quercetin amino acids, phenols, flavonoids[2]. Which can inhibit bacterial growth. Endophytes are defined as microbes that live in the internal tissues of living plants without causing negative effects on the host plant[3]. Endophytic

microbes can be isolated from a medicinal plant and produce secondary metabolites which similiar to the host plant. Using endophytic microbes do not need to cut down the original plants which may cause environmental condition. Particulary for the plant require long growing time or limited availability in nature [4]. The current study focussed on isolation of the endophytic microbes and their bacterial activity of secondary metabolite.

MATERIALS AND METHODS

Materials

Bark of Beringin tree (*Ficus benjamina* L.) obtained from BALITRO research garden, Department of Agriculture, Cimanggu, Bogor, West Java.

Solvents : The solvents used for extracts were *n*-hexane, ethyl acetate, and *n*-butanol.

Microbial Test:

Gram positive bacteria: *Bacillus subtilis* and *Staphylococcus aureus* ATCC 6538

Gram negative bacteria: *Escherichia coli* ATCC 8739 and *Klebsiella pneumoniae*

Media: PDA (Potato Dextrose Agar) chloramphenicol, Fermentation media using PDY (Potato Dextrose Yeast) media, NA (Nutrient Agar), Nystatin.

Isolation of Endophytic Fungi

Surface sterilization method

The bark of *Ficus benjamina* L. are washed under running water for 10 minutes, then cut into small pieces with a size of 2-3 cm in length. Then using surface sterilization by soaking the sample in 75% ethanol for 1 min, 5% NaOCl for 5 minutes, 75% ethanol for 30 seconds and finally rinsed with sterile aquadest for 1 minute. Furthermore, the pieces of the bark twigs were split longitudinally into two equal parts and the inner surface of the twigs were cultured directly on PDA (Potato Dextrose Agar) medium sterile which has been added with 0.005% chloramphenicol, and then incubated at room temperature (27-30^{o}C) for 5-7 days. The grown endophytic fungi were purified by several subcultures on the same medium until a single isolate was obtained [4].

Morphological Observation of Endophytic Fungi

Single isolates of endophytic fungi from the bark of a *Ficus benjamina* L. were observed macroscopically (visually) and microscopically using a light microscope[5].

Fermentation of Endophytic using Shake methods

Single isolates of endophytic fungi that have been grown for 7 days are taken as much as 3-5 pieces using *cork borer* (1 cm in diameter). Then put into PDY liquid medium (Potato Dextrosed Yeast) 50 mL in 250 mL Erlenmeyer.

Fermentation was carried out by shaking methos using an orbital shacker incubator for 5 days at a speed of 120 rpm at room temperature (27-30^{o}C) (starter). 10 mL starter was transferred to PDY 190 mL medium which had been added $CaCO_3$ in Erlenmeyer 1 L, then fermented again for 7 days with shaking at a speed of 120 rpm and room temperature (27-30^{o}C).

The fermentation results were centrifuged at 2000 rpm for 20 minutes at room temperature (27-30^{o}C) to separate the biomass from the supernatant[6].

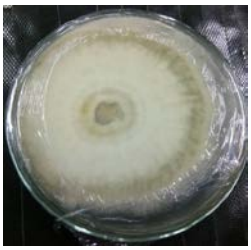
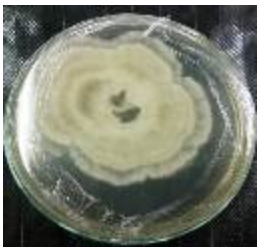
Endophytic Fungi Fermentation Extraction
Supernatants obtained from the endophytic fungi fermentation were extracted using organic solvents, *n*-hexane, ethyl acetate, and *n*-butanol.

Testing of antimicrobial activity
The suspension of the test bacteria which has been synchronized with Mc Farland 0.5 turbidity was used. Spread onto nutrient agar (NA) evenly using sterile cotton bud in a Petri dish until all surface was covered. The disc paper was saturated with *n*-hexane, ethyl acetate, and *n*-butanol extract, negative control, and positive control (chloramphenicol). Paper disc that has been saturated is placed on the NA media. Then incubated for 18-24 hours at 37 ° C. Clear zone around a the disc demonstrated microbe inhibition zone area and was measured in millimeter (mm) scale [7].

Phytochemical Screening
The phytochemical screening test used the filtered supernatant from each isolate to determine the chemical compounds of each isolate.
Phytochemical screening included:
Identification of Alkaloids, Identification of Flavonoid groups, Identification of Saponin groups, Identification of Tanin groups, Identification of quinone groups, Identification of steroid and triterpenoid groups, Identification of essential oil groups, Identification of coumarin groups[8].

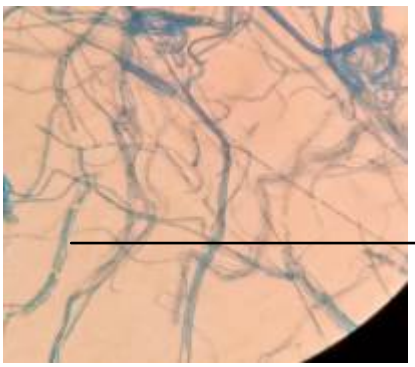
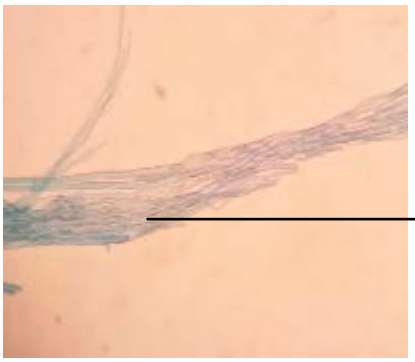
RESULTS AND DISCUSSION
The results from isolation the barks of *Ficus benjamina* L obtained 6 isolates. Macroscopic observation of endophytic fungi isolates of barks of (*Fiscus benjamina* L.) was shown in Figure 1.

Isolate code	Front appearance	Back appearance
KB 1.1	 The shape of the colony is round, the surface of the colony is convex and the colony surface is cotton likes. White, the edges of the colony are flat.	 The white color in all colonies

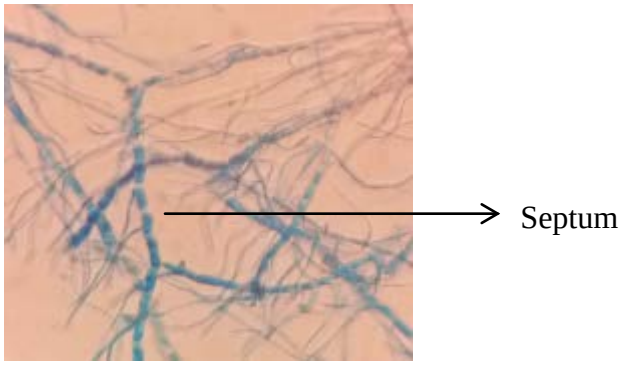
KB 2.1			The shape of the colony is round, the surface of the colony is convex and, white, the edges of the colony are flat, forming a concentric circles and have radial lines	The edge of the colony is flat with the green color, then white, and in the center of the colony is green
KB 3.1			The shape of the colony is round, the surface of the colony is wavy and smooth, the edge of the colony is wavy, the colony white	The edge of the colony is flat with white color, the center of the colony is yellowish green
KB 3.2			The shape of the colony is round, the surface of the colony is smooth the edge of the colony is wavy, forming concentric circles	The edge of a yellowish colony, the center of a dark green colony
KB 4.1			The shape of the colony is round, the surface of the colony is smooth the edge of the colony is wavy, forming concentric circles	Greenish yellow colonies and green colony centers

KB 5.2		The shape of the colony is round the surface of the colony is rough and flat. The dge of the colony is flat , with the white color, forming concentric.circle	 White colony edge and orange colony center

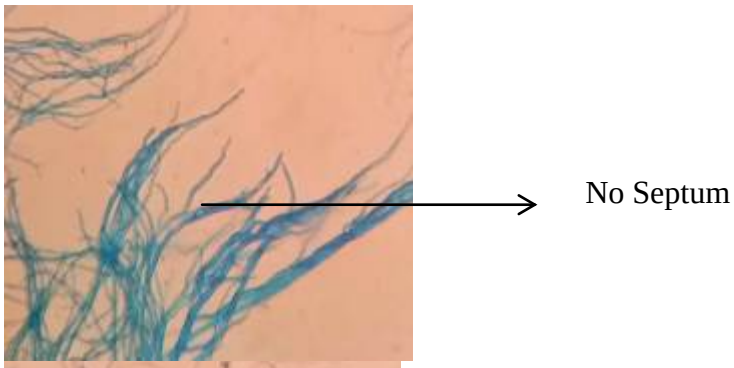
Figure 1. Macroscopic observation of endophytic fungi isolates of barks of *Ficus benjamina* L

Isolate code	Microscopic observation 400x Magnification
KB 1.1	 Septum
KB 2.1	 No Septum

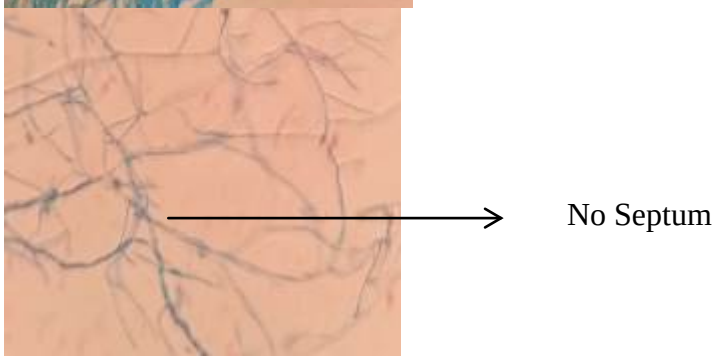
KB 3.1



KB 3.2



KB 4.1



KB 5.2

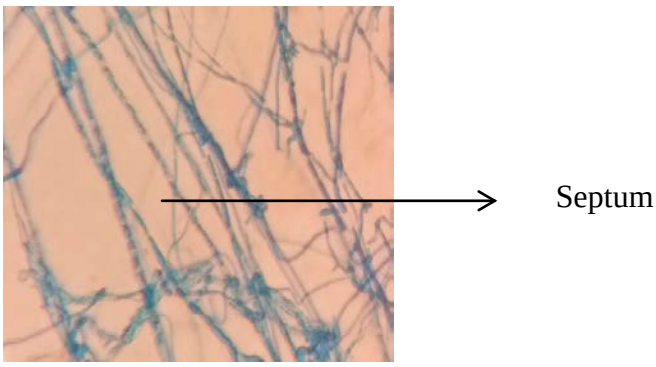


Figure 2. Microscopic observation of endophytic fungi isolates of *Ficus benjamina* L

Table 1. Phytochemical Screening of Supernatant Isolated from Endophytic Fungi Bark of *Ficus benjamina* L.

NO	Phytochemical screening	Endophytic isolate					
		KB 1.1	KB 2.1	KB 3.1	KB 3.2	KB 4.1	KB 5.2
1	Alkaloid	-	-	-	-	-	-
2	Flavonoid	+	+	+	+	+	+
3	Saponin	+	+	-	-	+	+
4	Tannin	-	-	-	-	-	-
5	Quinone	-	-	-	-	-	-
6	Steroid/Triterpenoid	-/+	-/+	-/+	-/+	-/+	-/+
7	Coumarin	-	-	-	-	-	-
8	Volatile oil	-	-	-	-	-	-

\+ : positive reaction

\- : negative reaction

Phytochemical screening of n-hexane, ethyl acetate, and n-butanol phase extracts from isolates of endophytic fungi isolated from bark of *Ficus benjamina* L. was shown in Table2.

Table 2[NMS1]. Phytochemical Screening of *n*-Hexane, Ethyl Acetate, And *n*-Butanol Phase Extracts from Isolates of Endophytic Fungi Isolated From Bark of *Ficus benjamina* L.

n- HEXANE						
Phytochemical screening	Isolate of Endophytic Fungi					
	KB 1.1	KB 2.1	KB 3.1	KB 3.2	KB 4.1	KB 5.2
Alkaloid	-	-	-	-	-	-
Flavonoid	-	-	-	-	-	-
Saponin	-	-	-	-	-	-
Tannin[NMS2]	-	-	-	-	-	-
Quinon[NMS3]e	-	-	-	-	-	-
Steroid/ Triterpenoid [NMS4]	-/+	-/+	-/+	-/+	-/+	-/+
Coumarin	-	-	-	-	-	-
Volatile oil	-	-	-	-	-	-
ETHYL ACETATE						
Phytochemical screening	Isolate of Endophytic Fungi					
	KB 1.1	KB 2.1	KB 3.1	KB 3.2	KB 4.1	KB 5.2
Alkaloid	-	-	-	-	-	-
Flavonoid	+	+	+	+	+	+
Saponin	-	-	-	-	-	-
Tannin	-	-	-	-	-	-

Quinone	-	-	-	-	-	-
Steroid/ Triterpenoid	-/+	-/+	-/+	-/+	-/+	-/+
Coumarin	-	-	-	-	-	-
Volatile oil	-	-	-	-	-	-
***n*-BUTANOL**						
Phytochemical screening	Isolate of Endophytic Fungi					
	KB 1.1	KB 2.1	KB 3.1	KB 3.2	KB 4.1	KB 5.2
Alkaloid	-	-	-	-	-	-
Flavonoid	+	+	+	+	+	+
Saponin	-	-	-	-	-	-
Tannin	-	-	-	-	-	-
Quinone	-	-	-	-	-	-
Steroid/ Triterpenoid	-/-	-/-	-/-	-/-	-/-	-/-
Coumarin	-	-	-	-	-	-
Volatile oil	-	-	-	-	-	-

\+ : positive reaction

\- : negative reaction

Table[NMS5] 3. Inhibition zone diameter extract *n*-butanol, ethyl acetate and *n*-butanol phase against pathogenic microns

***n*-Hexane**								
Microbial test	**Diameter of Inhibition (mm)**						**Negative control**	**Positive control**
	KB 1.1	**KB 2.1**	**KB 3.1**	**KB 3.2**	**KB 4.1**	**KB 5.2**		
E.coli	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**27.37±1**
S.aureus	**7.1±0.72**	**6.82±0.21**	**6.95±0.44**	**0±0**	**7.27±0.51**	**6.15±0.05**	**0±0**	**26.7±3.5**
K.pneumoniae	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**0±0**	**28.3±1**
B.subtilis	**6.8±0.4**	**6.62±0.3**	**0±0**	**0±00**	**7.95±**	**6.77±**	**0±0**	**30.3±1.8**
Ethyl Acetate								
Test microbe	**Isolate code**						**Negative control**	**Positive control**
	KB 1.1	**KB 2.1**	**KB 3.1**	**KB 3.2**	**KB 4.1**	**KB 5.2**		
E.coli	**7.45±0.22**	**7.7±0.35**	**8.08±0.08**	**8.03±0.08**	**8.15±0.13**	**8.03±0.25**	**0±0**	**17.75±0.5**
S.aureus	**8.92±0.69**	**8.75±0.23**	**8.45±0.44**	**7.88±0.06**	**11.35±0.96**	**7.37±0.43**	**0±0**	**26.9±2.45**
K.pneumoniae	**8.07±0.12**	**9.1±0.57**	**7.98±0.47**	**8±0.5**	**16.08±2.11**	**8±0.2**	**0±0**	**28±0.41**
B.subtilis	**8.43±0.14**	**10.73±0.6**	**9±0.93**	**8.82±0.54**	**13.42±0.45**	**9.63±1.42**	**0±0**	**33.2±21.4**
***n*-Butanol**								
Test microbe	**Isolate code**						**Negative control**	**Positive control**
	KB 1.1	**KB 2.1**	**KB 3.1**	**KB 3.2**	**KB 4.1**	**KB 5.2**		
E.coli	**10.37±0.12**	**10.1±0.3**	**10.95±1.06**	**10.63±0.25**	**10.67±0.76**	**11.53±0.28**	**0.2±0.02**	**22.4±1.89**

S.aureus	**11.65±0.48**	**9.72±0.58**	**11.23±0.13**	**11.78±0.16**	**11.43±0.08**	**10.62±0.54**	**0.25±0.03**	**30.8±0.65**
K.pneumoniae	**10.13±0.08**	**11.22±0.6**	**11.32±0.24**	**11.28±0.08**	**12.55±0.3**	**10.13±0.1**	**0.19±0.01**	**28.52±1.1**
B.subtilis	**12.52±1**	**12.98±0.9**	**12.82±1.7**	**14.07±1.23**	**13.03±1.21**	**11.13±0.76**	**0.23±0.02**	**28.8±1.13**

Surface sterilization was a stage to eliminate epiphytic microorganisms that were on the surface of the sample by immersing the sample using 75% ethanol for 1 minute, 5.3% NaOCl for 5 minutes, 75% ethanol for 30 seconds and rinsing with sterile aquadest. Ethanol was a phenol derivative that could denaturate and coagulate bacterial cell proteins, ethanol containing water had antibacterial activity which was stronger than absolute ethanol. Sodium hypochlorite (NaOCl) was a disinfectant of sulfhydryl (-SH) which could an interfere with the enzymatic reactions of microorganism cells irreversibly. NaOCl when dissolved in water, salt the hypochlorite will form HClO compounds, which were compounds that could cause DNA damage which would eventually cause bacterial cell death [9]. The use of two types of sterile material could increase the mortality of microorganisms compared to using one type of sterilized material which was less effective in reducing contamination.

The fermentation process was carried out to obtain secondary metabolites produced by each endophytic fungi isolate. The fermentation method used in this study was fermented shake using an orbital shaker. The presence of a wobble allows sufficient oxygen supply to maintain aerobic conditions and remove the carbon dioxide gas produced during fermentation, in addition to flattening the spread of microorganisms, nutrients and oxygen in the medium [10].

Each fermented endophytic fungus isolate was a culture that had been single, without contaminants, and had reached the age of 7 days since rejuvenation. The first stage of the fermentation process was the making of a starter. Starter was a microbial population in number and physiological conditions that were ready to be inoculated on fermentation medium so that microbes could grow quickly and fermentation could occur immediately. The preparation of the starter began with inserting 5 pieces of endophytic mold isolates with a size of 1 cm diameter, using cork bore into 50 mL of fermented medium that has been sterile is then shaken out with an orbital shaker with a speed of 120 rpm for 5 days at room temperature (27-30^{o}C). The starter results were transferred as much as 10 mL into 190 mL of PDY medium and then shaken out at room temperature for 7 days. Then centrifuged to separate the supernatant and biomass. All fermentation processes were carried out under aseptic conditions to prevent contaminants from being fermented.

The fermentation medium used was PDY liquid medium which was added with $CaCO_3$ as a pH fermentation stabilizer so that the medium pH was in the range of pH 6-7. Fermentation using liquid media was easier to work aseptically and made it easier to control during the fermentation process. The fermentation medium must provide all the nutrients needed by microbes.

In general, microbes need water, energy, carbon, nitrogen and mineral sources as well as oxygen adequacy during the fermentation process so as to optimize cell formation and the production of secondary metabolites produced. Medium PDY was a medium commonly used for fungi fermentation, this medium consists of PDB and

Yeast Extract. PDB contains potato juice and dextrose which is a source of carbon and energy, while yeast extract helps fermentation by acting as a nitrogen source needed by microbes during the fermentation process [5].

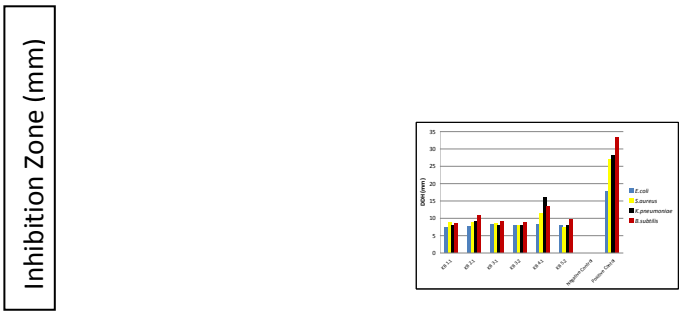


Figure 3. The average diameter of *n*-hexane, ethyl acetate, and *n*-butanol inhibitory area (secondary) and secondary metabolites isolate endophytic fungi

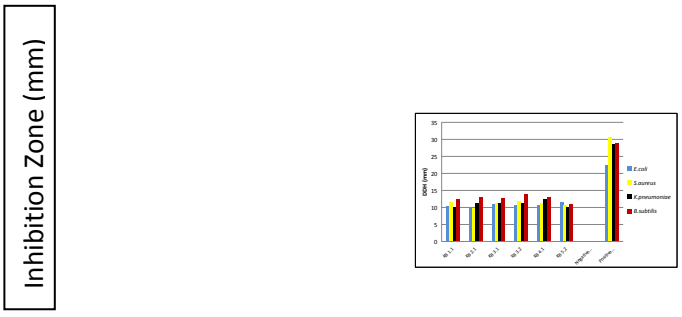


Figure 4. Inhibitory Diameter extracts n-butanol phase against pathogenic microbes

The test results of n-hexane extract isolate endophytic fungi of *Ficus benjamina* L**.** bark showed isolates KB 1.1, KB 2.1, KB 4.1, and KB 5.2 can inhibit the growth of Gram positive bacteria, which are 7.10 mm, 6.82 mm, 7.28 mm, and 6.15 mm respectively *Staphylococcus aureus* bacteria and against bacteria *Bacillus subtilis* respectively 6.80 mm, 6.62 mm, 7.95 mm, and 6.67 mm, while n-hexane extract isolates KB 3.1 can only inhibit *Staphylococcus aureus* bacteria by 6.95 mm. All n-hexane extracts isolated from *Ficus benjamina* L. bark endophytic fungi cannot inhibit the growth of Gram-negative bacteria, *Escherichia coli* and *Klebsiella pneumoniae*. Negative control that is n-hexane solvent does not provide inhibitory power to all test microbes, this indicates that the negative solvent control used does not give effect to the inhibitory power produced by each extract in the n-hexane phase layers (outer layer, middle layer, and inner layer), whereas in Gram positive bacteria only has a single layer on the cell wall structure. The cell wall structure of Gram negative bacteria is relatively more complex frame consisting of crystalline, chitin and β-glucan polysaccharides [11]. Further research is needed, molecular identification of fungi isolates to identify to the species level and determination of the class of compounds contained in the bark of *Ficus benjamina* L.

CONCLUSION

The results from isolation barks of *Ficus benjamina* L obtained 6 isolates KB 1.1, KB 2.1, KB 3.1, KB 3.2, KB 4.1, and KB 5.2. All isolates were able to inhibit Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*), Gram negative bacteria (*Escherichia coli* and *Klebsiella pneumoniae*), but KB isolates 3.1 phase of n-butanol showed the greatest inhibitory against *Escherichia coli*. KB phase 3.2 isolate n-butanol gave the greatest inhibitory effect on *Staphylococcus aureus*, *Bacillus subtilis*. KB 4.1 isolates in the ethyl acetate phase showed the greatest inhibitory effect on *Klebsiella pneumoniae*.

ACKNOWLEDGEMENT

We thank for the Microbiology laboratory in Medical faculty, University of Indonesia for the Microbial test in this study.

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