

ANTIFUNGAL ACTIVITY OF EXTRACT AND FRACTION OF *AURICULARIA AURICULAR* ON *CANDIDA ALBICANS*, *MICROSPORUM GYPSEUM*, AND *ASPERGILLUS FLAVUS*

IKA KURNIA SUKMAWATI*, ARI YUNIARTO, DIAH RAKHMAWATI

Department of Pharmacy Bandung School of Pharmacy, Jalan Sukarno Hatta No 754, Bandung, Indonesia. Email: Ika.kurnia@stfb.ac.id

Received: 05 December 2017, Revised: 8 February 2018 Accepted: 14 February 2018

ABSTRACT

Objective: Indonesia with high humidity and rainfall strongly supports the growth of fungi. The incidence of fungal infections has increased dramatically over the past three decades. Several factors including microbiological resistance led to the failure of antifungal therapy. This research was to evaluate the antifungal activity of extract and fraction of *Auricularia auricula* against the test fungus.

Methods: Antifungal activity was performed by microdilution method using extract and n-hexane fraction, ethyl acetate fraction, methanol, and water fraction of *A. auricula*. MIC was determined by observing the turbidity compared with the control solution. Minimum fungicidal concentration (MFC) was determined by the presence of fungi colonies growth on medium after incubation for 24 h.

Results: The fractions of *A. auricular* have antifungal activity against *Candida albicans*, with MIC 512 µg/ml and MFC 512 µg/ml; *Microsporium gypseum* with MIC 256 µg/ml in the extract; MIC 512 µg/ml and MFC 512 µg/ml of ethyl acetate fraction; MIC 256 µg/ml and MFC 512 µg/ml in n-hexane fraction; and *Aspergillus flavus* with MIC 2048 µg/ml and MFC 2048 µg/ml in the extract and ethyl acetate fraction. The most potent activity on *C. albicans* was exhibited by ethyl acetate fraction with MIC and MFC 512 µg/ml. Scanning electron microscopy showed changes in the structure of fungi cells after contact with the test solution.

Conclusion: The best antifungal activity of *A. auricula* was ethyl acetate fraction against *C. albicans*.

Keywords: Antifungal activity, *Auricularia auricula*, *Candida albicans*, *Microsporium gypseum* and *Aspergillus flavus*

© 2018 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2018.v11s1.26591>

INTRODUCTION

Infectious diseases are the highest contributor to morbidity and mortality rates in developing countries including Indonesia [1]. Indonesia with high humidity and rainfall strongly supports the growth of fungi. The incidence of fungal infections has increased dramatically over the past three decades. Several factors including microbiological resistance led to the failure of antifungal therapy [2].

Indonesia Health Profile 2010 which shows that skin diseases and subcutaneous tissue are ranked third of the top 10 diseases in outpatients in hospitals throughout Indonesia based on the number of visits that are 192,414 visits and 122,076 visits are new cases [3].

An alternative antifungal therapy is needed, one of them from *A. auricula*. *A. auricula* is a food ingredient that contains nutrients and minerals such as protein, fat, carbohydrates, fiber, ash, and vitamins such as thiamin 0.015 mg; riboflavin 0.844 mg; niacin 6.267 mg; pantothenic acid 0.481 mg; Vitamin B6 0.112 mg; and 38 mcg of folate. In addition, this plant has been used for traditional treatment as an antidote in the body, prevent cancer, cure heart disease and stroke, and blood circulation [4].

According to the journal antimicrobial activity of some local mushrooms on pathogenic isolates, it is mentioned that ethanol extract from mushroom *A. auricula* has antifungal activity against *Candida albicans* with the inhibition zone of 4.89 mm and MIC and MBC value of 50 mg [5].

METHODS

Chemical and reagents

The main materials used in this study were *A. auricula*, potato dextrose agar (PDA) MERCK, nystatin, 96% ethanol solvent, n-hexane, ethyl acetate, methanol, aquades, DMSO solvent MERCK, sodium hydroxide,

gelatin, aliquot, Liebermann reagent Bouchardat, Dragendorff's reagent, reagent Mayer, Mg powders, H₂SO₄, FeCl, NaCl, BaCl, and paper discs.

Method

Collection of test plants, determination, extraction, and fractionation Mushroom *A. auricula* wet obtained from Caringin Market, Bandung, West Java. Furthermore, the determination was made in the Department of Biology Faculty of Mathematics and Natural Sciences Padjadjaran University. Peak mushroom processing is wet sorting, washing, dry sorting, chopping to become the small part, drying in direct sun heat, and milling to become powder simplicia. The *A. auricula* that has been treated is extracted by reflux using 96% ethanol. A total of 100 g of sample were put into a round bottom flask and 96% ethanol was added as much as 500 mL. The extract was carried out for 4 h. The liquid ethanol extract obtained was fed to a rotary evaporator and then evaporated until a viscous extract was obtained. The condensed extract was then screened for phytochemical and fractionation.

The ethanol extract was dissolved in a methanol-water mixture (2:8) and placed in a separating funnel, into which an n-hexane solvent was added with a ratio of 1:1, then shaken gently until mixed, then sterilized to a proper separating into two phases comprising phase n-hexane and water phase. The n-hexane phase is separated and the water phase is fractionated back up to 3 times. The collected n-hexane phase was concentrated using a rotary evaporating device and a water bath. The water phase is fractionated back using a 1:1 ethyl acetate solvent, this fractionation process is carried out 3 times until the phase of ethyl acetate and water phase, each of which is concentrated by a rotary evaporating device and a water bath. From fractionation obtained a fraction of n-hexane, ethyl acetate, and water [1].

Test fungus

C. albicans and *Microsporium gypseum* fungi were obtained from the Laboratory of Microbiology, School of Pharmacy, ITB. The *Aspergillus*

flavus fungus was obtained from the Microbiology Laboratory, UNPAD.

Media creation

PDA of 39 g dissolved in 1 L aquades, and potato dextrose broth of 24 g in 1 L of aquades and heated to complete dissolved. Before use, all media were sterilized with an autoclave at 121°C for 15 min.

Making mushroom suspension

Microbes were suspended into a liquid medium and incubated 18–24 h at 25°C. The suspension is shaken and turbidity adjusted to a standard of 0.5 McF turbidity. The turbidity standard of 0.5 McF contains bacteria of 108 CFU/mL, yielding absorbances at 530 nm

(optical density wavelengths should be between 0.08 and 0.10). McFarland's half standard is equivalent to 1–5×10⁶ yeast/mL (density recommended by CLSI for mushroom inoculum stock) [6].

Test antifungal activity

A 100 µL gross domestic product (GDP) is inserted in the microplate in the first column (as a negative control). 5 µL mushroom suspension added to 10 mL of GDP is then stirred with a vortex. A total of 100 µL of the mixture are inserted in the microplate on the second to the 12th column. In the 12th column, 100 µL of an antibiotic solution/extract was added with a certain concentration and homogenized. From the 12th column, taken 100 µL, then moved the 11th column. Dilution continues until the third column has the smallest concentration. The plates were incubated at 25°C for 3×24 h, then observed clear parts (no microbial growth). The smallest concentration in which no visible microbial growth was observed is defined as MIC. A 5 µL aliquot of each clear part was removed in a PDA and incubated at 25°C for 3×24 h was then observed. The lowest concentration in which no microbial growth was observed was defined as the minimum fungicidal concentration [7].

Bioautographic test

The 15 mL PDA medium mixed with 0.2 mL of *C. albicans* suspension was poured into a sterile Petri dish and allowed to solidify. After the media solidifies, the eluted thin-layer chromatography (TLC) plate is placed on the surface of the agar medium. After 30 min, the plates were removed and then the medium which had been occupied by TLC plate incubated at 25°C for 24 h, observed the constricted zone [8].

Test of antifungal equality

The equivalent activity of the test substance with comparative antifungals was performed by agar diffusion method. The antifungal used as a comparison is nystatin. Various comparative antifungal concentrations were prepared using a suitable solvent such as those listed in Pharmacopoeia Indonesia IV edition. Based on the measurement of antibiotic inhibitory diameter, a line equation between concentration logarithm and drag diameter is made. The obtained equation is used to see the equality of activity between the test extract and the antibiotic comparison [9].

Scanning electron microscopy (SEM)

The preparation of SEM testing was initiated by experimental culturing on PDA media incubated at 25°C for 24 h. The result of rejuvenation was taken and inserted on Eppendorf tube filled with NaCl, then centrifugation was done for 15 min with speed 3000 rpm. Sedimentation of centrifugation result was taken and test substance was added to 8X KHM concentration for 4 h. After contact with the

test substance, then centrifugation was done for 15 min with a speed of 3000 rpm again to separate the sediment and test substances. The precipitate was removed and 10% formalin was stored in the refrigerator temperature. For testing with SEM device, the formalin solution is removed, the precipitate is placed on the holder specimen and dried on the desiccator for 15 min, then coating with gold for 4 min. Subsequent specimen holder was inserted into chamber specimen and a sample was analyzed by SEM.

RESULTS

The result of processing showed the dry *A. auricula* simplicia obtained was 1018.72 g. The extraction result obtained rendement as much as 2.314%. Phytochemical screening is performed to determine the presence of classes of compounds in a plant, this is done as initial information in knowing any chemical compounds contained in a plant. The results of phytochemical screening showed on *A. auricula* contain an alkaloid, steroid/triterpenoid, and tannin. Preparation of the fraction is carried out by means of liquid-liquid extraction (ECC) with n-hexane, ethyl acetate, and methanol:water (2:8) solvents. It was found that n-hexane fraction rendement was 22.2%, ethyl acetate fraction was 1.8%, and methanol fraction: 37.6% water.

Test antifungal activity

Test of antifungal activity against *C. albicans*, *M. gypseum*, and *A. flavus* was done in microdilution with the test substance in the extract of fungus with nystatin as a comparison. The microdilution method is expected to know the value of minimum inhibitory concentration (KHM) and fungicidal minimum (KFM) concentration of the sample to be tested. In testing, the antifungal activity was first made extract and fraction solution with a concentration of 1024 µg/mL. The concentration can facilitate the calculation of dilution in the next stages. The solution of extract and fraction will be diluted in liquid medium to concentration of 1 µg/mL. Different concentrations will show the strength and number of compounds contained in the solution of extracts and fractions having antifungal activity, at this stage in the search for the smallest concentration that can inhibit the growth of the test fungus with the turbidity indicator of the test media added the test mushroom with the extract and fraction solution which is compared with the positive control of the media plus the test fungus and the negative control of the media only, if the media is cloudy, then it is assumed to be the growth of test fungi and if the clear media such as negative control, hence, assumed the growth of test fungus is inhibited by extract solution and added fraction. The results of antifungal activity test can be seen in Table 1.

Equality test

In this test, the equivalence test was carried out between ethyl acetate fraction and nystatin on *C. albicans* fungus. The equivalent test is done by diffusion method that is paper disc method with several series of solution concentration. Selected paper disk method is easy because of its procedure and requires a little volume of solution. The solution of solution concentration is based on the best KHM value of microdilution test. Concentrations made from the ear fungus ethyl acetate fraction are 100, 500, 1000, 5000, 10,000, and 50,000 µg/mL. Nystatin as a compound was made with concentrations of 10, 25, 50, 100, 250, and 500 µg/mL. From the results of the antifungal equivalence test shows the existence of a clear zone around the disc of paper which means the concentration used has antifungal activity. The equivalence test results of the fungal and ethyl acetate fraction of *C. albicans* can be seen in Tables 2 and 3.

Table 1: Antifungal activity test results

Fungus Test	E		FMA		FE		FNH		N	
	KHM (µg/mL)	KFM (µg/mL)	KHM (µg/mL)	KFM (µg/mL)	KHM (µg/mL)	KFM (µg/mL)	KHM (µg/mL)	KFM (µg/mL)	KHM (µg/mL)	KFM (µg/mL)
<i>C. albicans</i>	-	-	512	512	512	512	512	512	1	1
<i>M. gypseum</i>	256	-	-	-	512	512	256	512	4	4
<i>A. flavus</i>	2048	2048	-	-	2048	2048	-	-	4	4

A. flavus: *Aspergillus flavus*, *C. albicans*: *Candida albicans*, *M. gypseum*: *Microsporium gypseum*

From the table of nystatin inhibitory diameters on *Candida albicans*, a graph of line equation is then made to determine the equivalence value of the ethyl acetate fraction of the fungus *Auricularia auricula* with nystatin. The graph of the line equation can be seen in Fig. 1.

The value of equality test was obtained by taking one concentration data and the inhibitory diameter of the fungus ethyl acetate fraction. The result of testing of nystatin inhibition diameter, then made calibration curve with drag diameter (mm) on y-axis and logarithm of comparative antifungal concentration the x-axis, so that we can get the equation of a line. The best inhibitory diameter, substituted into the equation $y=bx+a$ as the y value, so that the value of x will be obtained, then the value of x is antilog. The result of inferential antifungal inhibitory diameter was obtained by linear regression equation $y=4.2143x+2.8333$ and $R^2=0.9845$. Ethyl acetate fungus fraction against *C. albicans* at the concentration of 100 $\mu\text{g/mL}$ yielded a diameter of 7.25. Selected at a concentration of 100 $\mu\text{g/mL}$ because it produces a resistor diameter that is almost equal to one of the inhibitory diameters of nystatin. The value is then substituted on the equation $y=4.2143x+2.8333$ as the value of y. Obtained value 1.048 and then in antilog and obtained value which is equivalent with comparative that is 11.16 $\mu\text{g/mL}$. The equilibrium count of the ethyl acetate fraction to nystatin was 0.1 mg/mL of the fungal ethyl acetate fraction equivalent to 0.01116 mg/mL nystatin.

Bioautographic test

Bioautographic tests are used to estimate the class of compounds that have activity as an antifungal. Bioautographic tests were performed using contact bioautography method on the solid medium of Potato Dextrosa Agar. Observations were made by looking at inhibitory diameter compared with the previous monitoring results can be seen in Fig. 2.

After incubation, there was a clear zone with Rf values of 0.90 and 0.854 on the medium, then matched with the monitoring results of ethyl acetate fungus fraction on TLC. Bioautographic test results are estimated to a class of active compounds as antifungals are a group of steroids having Rf value of 0.863.

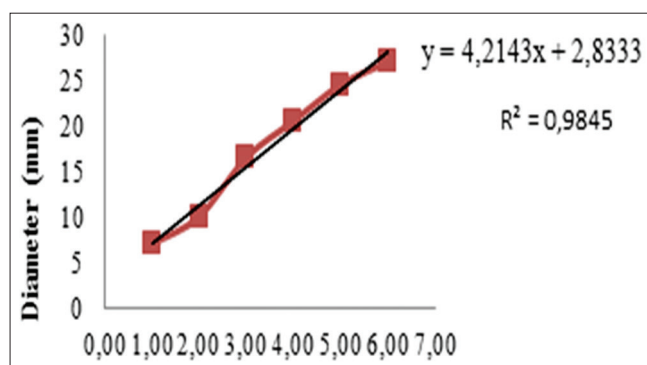


Fig. 1: Graph of nystatin line equations

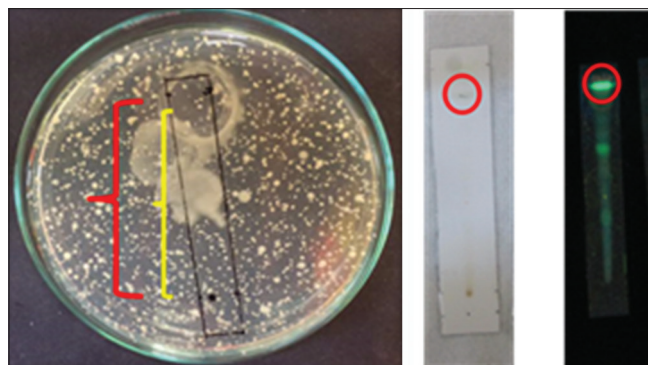


Fig. 2: Bioautographic test results

Test SEM

Samples tested were *C. albicans* on ethyl acetate fraction, n-hexane fraction, methanol fraction:water, and *M. gypseum* on extract and n-hexane fraction. This test is based on KHM results from the activity test that has been done. In the SEM test, the test fungus is contacted with an extract/fraction solution of 8 X KHM. Changes in the morphology of the SEM test can be seen in Fig. 3.

- a - Normal *Candida albicans* cells (controls),
- b - *C. albicans* cells after exposure to ethyl acetate fraction,
- c - *C. albicans* cells after exposure to the n-hexane fraction,
- d - *C. albicans* cells after exposure to the methanol fraction:water,
- e - Normal *Microsporium gypseum* cell (control),
- f - *M. gypseum* cell after exposure to the extract,
- g - *M. gypseum* cells after exposure to the n-hexane fraction

The results of SEM on *C. albicans* fungus seen at $\times 20,000$ magnification, cell morphology changes after exposure with ethyl acetate fraction in the form of cell to dent, formed a dimple in the middle of the mushroom cell (Fig. 3b). In fungal cells exposed to the n-hexane fraction, the morphological changes of the cell become dents and there is a bulge on one side of the cell (Fig. 3c). In fungal cells are contacted with the methanol fraction:morphological water in the form of fungal cells becomes shrunken (Fig. 3d). Morphological changes occurring in the *M. gypseum* fungus seen at $\times 1500$ magnification of fungus cells exposed to extracts become shrunken (F image), whereas in the fraction of n-hexane in the form of spots on cells (Fig. 3g).

DISCUSSION

Determination results showed that the plant that was determined by *A. auricular*. Extraction is used reflux method because the process is easy, simple, fast, and more effective because of continuous saving so it is more efficient solvent. The use of ethanol solvent because it is more selective, neutral, in higher concentrations can prevent the growth of fungus on the extract. Fractionation is used selected Liquid-liquid extraction (ECC) method because it is simple, easy, and fast. Selection of three types of solvent to attract compounds that are non-polar, semipolar, and polar.

Test of antifungal activity against *C. albicans*, *M. gypseum*, and *A. flavus* was done in microdilution with test substance in the extract of fungus with nystatin as the comparison. The microdilution method is expected to know the value of minimum inhibitory concentration (KHM) and fungicidal minimum (KFM) concentration of the sample to be tested.

From the results of antifungal activity test on *C. albicans* fungus, *M. gypseum*, and *A. flavus*, the fraction of water-methanol, ethyl acetate fraction, and n-hexane fungus fraction is active test substances in inhibiting the growth of *C. albicans* fungus. This can be seen from the value of KHM and KFM of 512 $\mu\text{g/mL}$ and 512 $\mu\text{g/mL}$. In the most active *M. gypseum* fungus inhibited the n-hexane fraction with KHM and KFM values of 256 $\mu\text{g/mL}$ and 512 $\mu\text{g/mL}$. While in the active *A. flavus* mushroom inhibits the extract and fraction of ethyl acetate with KHM and KFM value of 2048 $\mu\text{g/mL}$ and 2048 $\mu\text{g/mL}$. From this antifungal activity test data, one of the best results was chosen for further activity test which included equality test and bioautography. The best activity test data selected were the ethyl acetate fraction in the *C. albicans* test fungus. The reason for this data was chosen because in triploid test KHM and KFM values were always consistent at 512 $\mu\text{g/mL}$ while in *M. gypseum* and *A. flavus* fungi only on

Table 2: Diameter of ethyl acetate fraction in *C. albicans*

Concentration ($\mu\text{g/mL}$)	(mm \pm SD)*
100	7.25 \pm 0.90
500	8.17 \pm 0.29
1000	8.17 \pm 0.29
5000	
10,000	8.50 \pm 2.18
50,000	8.92 \pm 3.32

C. albicans: *Candida albicans*, SD: Standard deviation

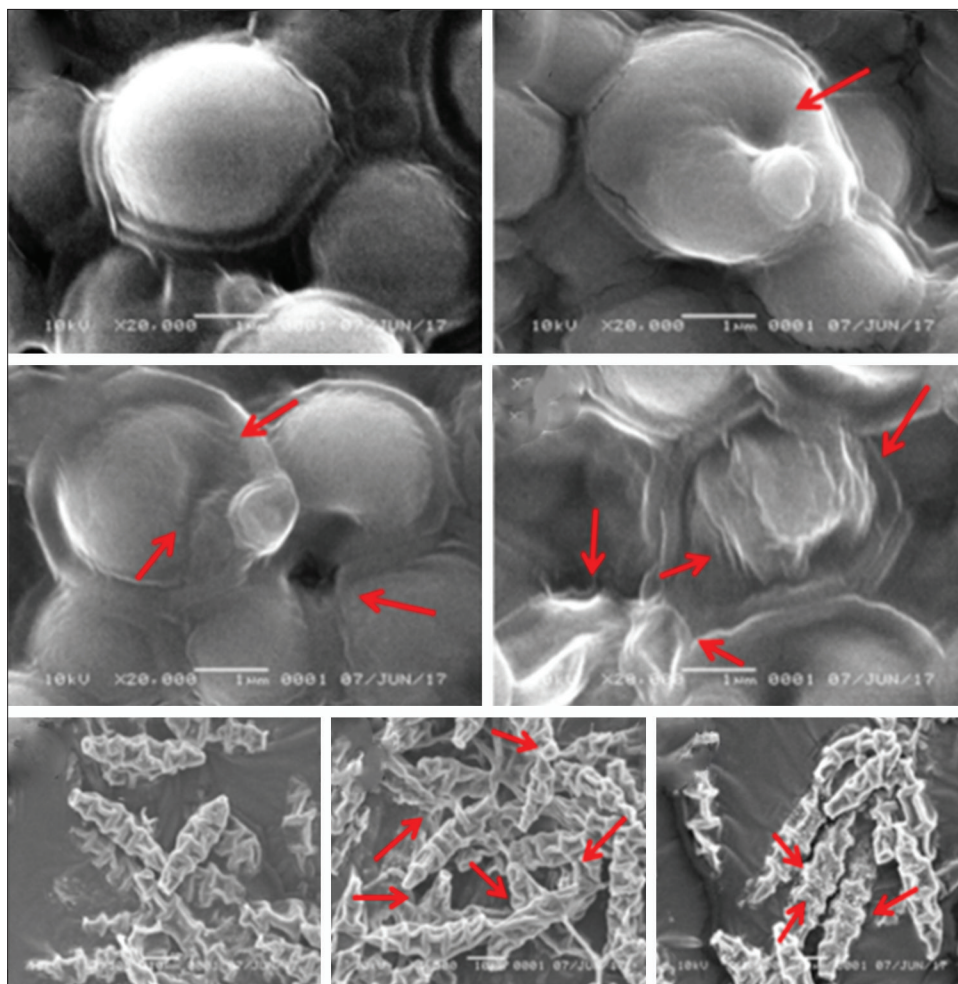


Fig. 3: Scanning electron microscopy test results information. A - Normal *Candida albicans* cells (controls), B - *C. albicans* cells after exposure to ethyl acetate fraction, C - *C. albicans* cells after exposure to the n-hexane fraction, D - *C. albicans* cells after exposure to the methanol fraction:water, E - Normal *Microsporium gypseum* cell (control), F - *M. gypseum* cell after exposure to the extract, G - *M. gypseum* cells after exposure to the n-hexane fraction

Table 3: Diameter of nystatin in *C. albicans*

Concentration ($\mu\text{g/mL}$)	Log concentration ($\mu\text{g/mL}$) (x)	Inhibitory diameter ($\text{mm}\pm\text{SD}$)*(y)
10	1.00	7.0 \pm 0.16
25	1.40	10.0 \pm 0.50
50	1.70	16.5 \pm 0.44
100	2.00	20.5 \pm 0.14
250	2.40	24.5 \pm 0.14
500	2.70	27.0 \pm 0.38

C. albicans: *Candida albicans*, SD: Standard deviation

one column showing the best activity of the triple test.

The equality test is done to determine the equivalence of the best extract/fractions with the comparison drug. The equilibrium count of the ethyl acetate fraction to nystatin was 0.1 mg/mL of the fungal ethyl acetate fraction equivalent to 0.01116 mg/mL nystatin.

Bioautographic tests are used to estimate the class of compounds that have activity as an antifungal. Bioautographic tests were performed using contact bioautography method on the solid medium of Potato Dextrosa Agar. Bioautographic test results are estimated to a class of active compounds as antifungals are a group of steroids having Rf value of 0.863. Steroid compounds inhibit the growth of fungi, either through the cytoplasm or interfere with the growth and development of fungal spores. Steroids may act as antifungals because the lipophilic properties possessed by steroids can inhibit the spore germination of fungi [10].

The SEM test was performed to see the morphological changes of fungal cells both before and after contact with the extract/fraction. Morphological changes that occur in *C. albicans* and *M. gypseum* cells are suspected due to secondary metabolite activity contained in the extracts and fungus fungi. The formation of small protrusions on microbial cells due to the inability of peptidoglycan cells damaged by antimicrobial compounds that withstand high intracellular pressure, so the cytoplasm exits and this bulge usually appears in regions attenuated by compounds antimicrobials [1].

CONCLUSION

The results showed that extract and fracture of *A. auricula* had antifungal activity against the test fungi with the best KHM and KFM value on the ethyl acetate fraction on *C. albicans* at concentration of 512 $\mu\text{g/mL}$, equivalence between ethyl acetate fraction of the ear mushroom

to nystatin was 0.1 mg/mL and ethyl acetate fungus fraction equivalent to 0.01116 mg/mL nystatin, SEM showed morphological changes in the test fungus after the extract and fracture of the ear mushroom were compared with the control.

ACKNOWLEDGMENT

The author thanks to Bandung School of Pharmacy for providing financial assistance and P3M STFB.

REFERENCES

1. Yani M, Sukandar Y, Adnyana E, Ketut I. Study of anti-bacterial activity of ethanol extract and singayan fungal fraction (*Petiveria alliacea*) against resistant bacteria. Pharm Mag Indones 2011;22:293-9.
2. Illnait-Zaragozi MT, Martinez RE, Ferrar JI, Audreu CM, Machin GF, Lancha MR, et al. *In vitro* antifungal activity of crude hydro-alcoholic extract of *Petiveria alliacea* L on clinical candidia isolates. Clin Microbial 2014;3:159.
3. Kemenkes RI. Riskerdasnas 2010. Jakarta: Center for Health Development Research; 2011.
4. Misaki A, Kakuta M, Sasaki T, Tanaka M, Miyaji H. Studies on interrelation of structure and antitumor effects of polysaccharides: antitumor action of periodate-modified, branched (1→3)-β-D-glucan of *Auricularia auricula-judae*, and other polysaccharides containing (1→3)-glycosidic linkages. Carbohydr Res 1981;92:115-29.
5. Eijikeme N, Uzoeto HO. Antimicrobial activity of some local mushrooms on pathogenic isolates. Int J Curr Res 2011;3:1-5.
6. Indriyanti N, Adnyana IK, Sukandar EY. Activity of ethanol extract and root fruit of singawalang (*Petiveria alliacea* L.) against mushroom causes of dandruff with broth microdilution method. J Trop Pharm Chem 2013;2:113-7.
7. CLSI. M38-A2 Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Approved Standard. 2nd ed. Wayne, Pennsylvania: Clinical and Laboratory Standard Institute; 2008. p. 89-96.
8. Akhyar R. Power Test Barrier and TLC Analysis Bioautography Extract Roots and Mangroves (*Rhizophora stylosa* Griff.) Against Vibrio Harveyi. Thesis Pharmacy Program Faculty of Pharmacy Hasanuddin University; 2010. p. 35-8.
9. Rostinawati T. Antimicrobial Activity of Tespong Herba Extract (*Oenanthe javavica* D.C) Against *Eschericia coli*, *Staphylococcus aureus* and *Candida albicans*. Jatinangor: Mandiri Research; 2010. p. 23-6.
10. Alfiah RR, Rieska R, Khotimah S, Turnip M. Effectiveness of metanol extract of rilled leaves (*Mikania micrantha* Kunth) against *Candida albicans* mushroom growth. Protobiont Biol Study 2015;5:1.