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Minimum Inhibitory and Fungicidal Concentration Tests Of Snakehead Fish (*Channa Striata*) Oil Extracts Against *Candida Albicans*: An In Vitro Study

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Abstract

***Introduction*:** *Candida albicans* (C. *Albicans*) is an oral cavity commensal organism that can shift into a pathogenic form under certain conditions. Nowadays, natural substances used as an alternative for a new antifungal agent. Snakehead fish oil extracts have been reported to have antifungal effect i.e; unsaturated fatty acids such as Omega-3 and Omega-6. The purpose of this study was to investigate the antifungal activity of snakehead fish oil extracts against *C. Albicans*. ***Materials and Methods*:** An in vitro laboratory study with a post-test control group design was established. To produce the snakehead fish oil extracts, wet rendering method was used. The snakehead fish oil extracts then tested with microdilution method to determine Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) against *C. Albicans*. An 0,062-32 µl/ml concentrations used for the microdilution test and 2-32 µl/ml for the disc-diffusion test. Nystatin (100 µg/ml) was used as the positive control. **Results:** There were visible growths and no inhibition zone in all concentrations of snakehead fish oil extracts. **Conclusion:** 0.062-32 µl/ml concentrations of Snakehead fish oil extracts did not have the abilities to decrease the growth of C. *albicans*.

Keywords: antifungal agents, *Candida albicans*, fish oil, fatty acid

Introduction

Candida albicans (*C. albicans*) is a normal microflora that commonly found in healthy individuals' oral cavities in the amount of 800 CFUs / ml.[1,2] It is an opportunistic microorganism and may change to pathogen form if the oral cavity environment conditions were changed.[2,4] These changes can result from prolonged broad-spectrum antibiotic therapies, impaired immune function, xerostomia, HIV infection, use of dentures, and uncomtrolled diabetes condition.[2]

The rise of Candida colonies can increase the risk of infection in the oral cavity; known as oral candidiasis.[5] About 50% of oral candidiasis are caused by this microorganism.[3,6] Treatments include elimination of the predisposing factors and the use of antifungal drugs.[7,8] Nowadays natural ingredients are becoming an alternative agents for producing a new antifungal drugs.[9] One of the natural substances that are thought to have an antifungal effect is snakehead fish oil.

Snakehead fish (*Channa striata*) lives in fresh water and is often found in Indonesia.[10] This fish contains protein (25.5%), minerals (1.3%), fat (5.7-11.9%), and carbohydrates (0.2%).[11,12] The oil derived from this fish is often used as medicine for wound healing, due to its amino acids and fatty acids bioactive agents.[13] The oil contains high Omega-3 and Omega-6. [14]

Previous studies have reported the potential of pure unsaturated fatty acids as antifungal agents. Omega-3, Omega-6, Omega-7, Omega-9, and their esters affect *C. Albicans*.[15,16] Unsaturated fatty acids may disturb the function of fungal cell membranes, resulting in the death of these cells. Thibane et al. (2012) reported that unsaturated fatty acids could also cause apoptosis in *C. albicans* cells due to condensation and nuclear fragmentation.[17]

Atif et al. reported the antifungal activity of snakehead fish extract, as did Jais et al. that showed the potential of snakehead fish extract as an antifungal agent, but further research is needed with purified extracts. This present study was done to analize the antifungal effect of snakehead fish oil extract against *C. albicans*.[18]

Methods

An in vitro laboratory experimental study with a post-test only control group design was established. The extraction of snakehead fishes oil were carried out at the Biochemistry Laboratory of the Faculty of Medicine, Universitas Sriwijaya. The antifungal effect of snakehead fishes oil extract on *Candida albicans* were tested at the Microbiology Laboratory of the Central Palembang Health Laboratory. Research ethical was obtain by the Research Ethics Commission of the Mohammad Hosein Central General Hospital, Palembang (Letter number: 033/kepkrsmhfkunsri/2020).

Six months old snakehead fishes that has been classified based on its morphological characteristics were used. A 700 to 1,000 grams/ each of Snakeheads fishes were harvested from the cork fish farming located in Palembang, South Sumatera. The organs used were skin, meat, bones, and fish heads of Snakeheads fishes. The scales and entrails were removed.

The snakehead fishes were washed and drained, cut into small pieces, and then boiled with distilled water. It was let stand for about 30 minutes, stirring it slowly. Then it was filtered using a separating funnel and a micropipette to separate the crude oil with the solids. Oil was stored in bottles that have been tightly closed and not be exposed to direct sunlight or air.[19]

Growth media such as Sabouraud Dextrose Agar (SDA) and nutrient broth (NB) were used. *C. albicans* were cultured on SDA media and incubated at room temperature for 24 hours. The candida colonies were picked up using a sterile inoculating loop and was suspended in a test tube containing 0.9 NaCl. The suspension in the test tube was made homogeneous. The turbidity level of the suspension was adjusted to the Mac Farland standard of 0.5 (1-5 x 106 CFU / ml).[20]

The minimum inhibitory concentration and minimum fungicidal concentration were tested by the microdilution method with 96 wells (8 rows and 12 columns). The first column was a control medium with 200 µl of nutrient broth (NB) growth media. No fungal growth should be found in the first column, since it was the medium control.[21] The second column is the column to see the growth of the fungal suspension (control) by pouring 100 µl of growth medium and 100 µl of *C. albicans* suspension. The third column first row was given an additional 100 µl of the test extract solution (snakeheads fishes oil) 32 µl/ml and mixed evenly. From the third column wells, 100 µl of the solution was taken and transferred to the fourth column. This was done repeatedly until the dilution process of the extract has been poured into the last well column (twelfth well). Furthermore, 100 µl of *C. Albicans* suspension was added to all test wells. The process of testing the snakehead fish oil extract was carried out three times.

For positive control, 100 µg/ml nystatin was filled in the third column well in the fourth row. The positive control function was to compare the antifungal inhibition of nystatin with the extract. The control group's solution was diluted in the same way as in the test extract solution, then in each well 100 µl of *C. albicans* suspension was added (Fig 1). Furthermore, the microplate incubated at a temperature of 28 ± 2 ° C ranging for 24 hours.

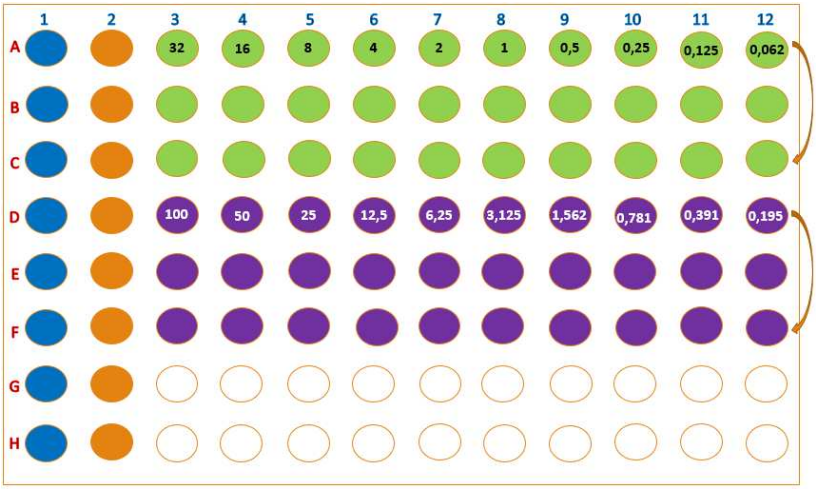

Figure 1. Illustration of Wells Filling on Microplates

: Media control
: Candidal suspension control
: Snakehead fish oil extract (µl/ml)
: Nystatin (µl/ml)

The Minimum Inhibitory Concentrations (MIC) was the lowest concentrations of the extracts at which no fungal growth was visible. It was determined visually by comparing the clarity level of the well. The Minimum Fungicidal Concentrations (MFC) was determined by subculturing wells that have an MIC value. Five microliters (µl) aliquots were taken from each well, then subcultured on agar media and incubated at 28 ± 2 ° C for 24 hours. Agar media that showed a clear appearance or the absence of fungus was considered as value of MFC.[21]

Inhibition test of snakehead fish oil extract in this study used the Disc-diffusion method with disc paper with a diameter of 6 mm.[22] The liquid SDA media was transferred to a petri dish (9 ml). After solidifying, the suspension of *C. albicans* inoculum was added as much as 200 µl. Disc papers soaked in 20 µl fish oil extract for about 15 minutes, then affixed to the media using tweezers. Similar steps were carried out on a positive control (nystatin) 100,000 IU and a negative control (Aquadest). Three repetitions were carried out in each of these treatments.[22] The measurement of the inhibition zone diameter is shown in the Figure 2.

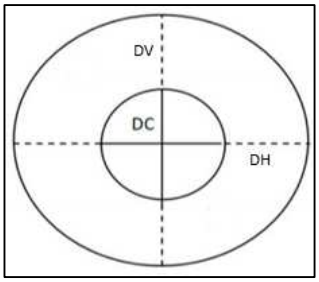

Figure 2. Illustration of The Inhibition Zone Diameter Measurement[66]

The diameter of the inhibition zone is then measured using the formula[66]:

$$\frac{(Dv - Dc) + (\mathrm{Dh} - \mathrm{Dc})}{2}$$

The Shapiro-Wilk test was used to check the normality of the data, while the Levene test was to ensure data homogeneity. Based on these tests, it was known that the data were not normally distributed homogeneous, thus the Kruskal-Wallis test was used. The result was $p < 0.05$. This means was a significant if there were a difference between the inhibition zone

diameter of the treatment groups and the positive control group. Post-hoc follow-up test was not carried out because the results of the data obtained were the same for each group of snakehead fish oil extract with the negative control group.

Results

MIC and MFC Tests

MIC testing was done on 0.062 µl/ml to 32 µl/ml concentrations of snakehead fish oil extracts against *Candida albicans*. Visual observation on the tested microplates which had previously been incubated for 48 hours was carried out. 0.062 µl/ml, 0.0125 µl/ml, 0.25 µl/ml, 0.05 µl/ml, and 1 µl/ml concentration concentrations of snakeheads fishes oil extracts did not show any visual clarities, whilst 2 µl/ml to 16 µl/ml concentrations did show clarities. The 32 µl/ml (highest) concentration of the extract was observed clear when compared to other wells eventhough there was still a little turbidity in the third row (Fig 3).

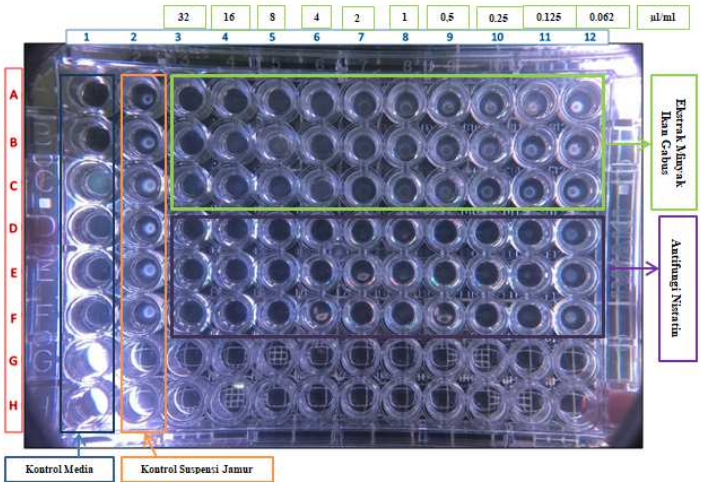

Figure 3. The result of MIC

MIC value are shown in Table 1.

Table 1. Test Results for Determination of MIC Value

Concentration of Test Extract	I	II	III
Media control	-	-	-
Candida albicans suspension	+	+	+
0.062 µl/ml	+	+	+
0.125 µl/ml	+	+	+
0.25 µl/ml	+	+	+
0.5 µl/ml	+	+	+
1 µl/ml	+	+	+
2 µl/ml	±	±	±
4 µl/ml	±	±	±
8 µl/ml	±	±	±
16 µl/ml	±	±	±
32 µl/ml	±	±	±

Annotation: +: turbid, -: clear, ±: slightly turbid

2-32 µl/ml concentrations of snakehead fish oil extract which began to show clarities in the microplates wells were subcultured to ensure the growth of *C. albicans*. The results showed the presence of *C. albicans* growth in all concentrations used in this study (Fig. 4).

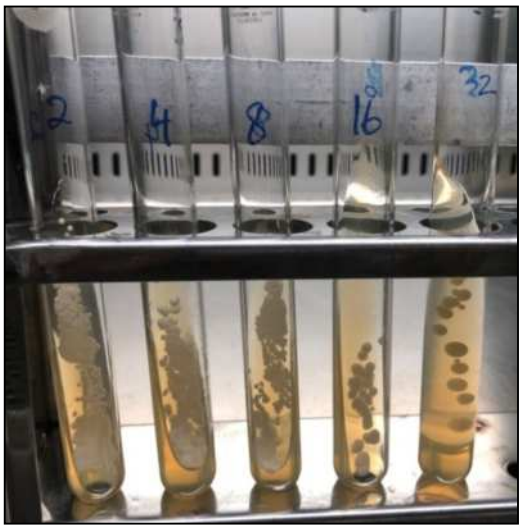

Figure 4. Subculture results of *C. albicans* on Snake Fish Oil Extract with a Concentration of 2-32 µl / ml

Inhibition Test with the Disc Diffusion Method

Calipers in mm (millimeters) was used to measure the inhibition zones. Zones of inhibition formed on the disc can be seen in the Figure 5. Based on the test results, the inhibition zones

formed were in the positive control group only. The inhibition zones were not formed in all the tested groups and also the positive control group (Table 2).

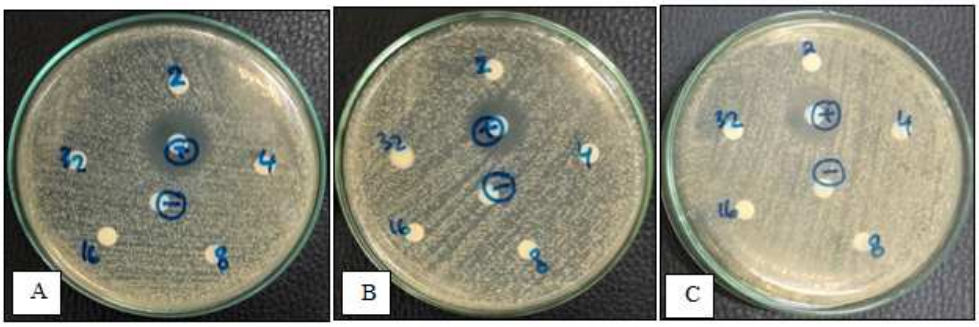

Figure 5. Disc Diffusion Test Results (a) Repetition 1 (b) Repetition 2 (c) Repetition 3

Table 2. Measurement Results of the Inhibition Zone Diameter

Test groups	Diameter (mm)			Mean	P value
	I	II	III		
2 µl/ml	0	0	0	0	0,03
4 µl/ml	0	0	0	0	
8 µl/ml	0	0	0	0	
16 µl/ml	0	0	0	0	
32 µl/ml	0	0	0	0	
Negative Control	0	10,75	11,80	11,83	
Positive Control	11,95				

Discussions

Omega-3 unsaturated fatty acids (DHA, EPA) and Omega-6 (ARA) found in snakehead fish oil were reported to have an antifungal potential agent against *Candida albicans*.[16,17,23] The absence of the inhibitory strength of the snakehead fish oil extract in *C. albicans* in this study could be due to the snakehead fish oil extract was harden at room temperature, so it must be heated repeatedly during the research process.

The high content of unsaturated fatty acids, especially Omega-3 in fish oil, causes fish oil to be easily oxidized and unstable. Oxidation increases through the presence of oxygen, light, and heat. The repeated heating process of snakehead fish oil extract can cause oxidation and degradation of Omega-3 content that leads to the decrease of the oil quality.[24-26] Heating

fish oil to a temperature of 50° C can also result in the degradation of unsaturated fatty acids of omega-3 EPA and DHA.[26]

The length of time the fish oil was stored also reported to have a decreasing effect on the fatty acid on it. This was due to the absence of substances that inhibit the oxidation process so that during the storage process there would be damage to the fatty acid content.[27] Zuta et al. reported that oxidation products will form in the fish oil emulsion during storage. This will cause oxidative damage which results in the double bond chain of fatty acids in fish oil broken.[28]

To maintain the quality of the Omega-3 unsaturated fatty acids in fish oil, urea crystallization techniques could be used to make Omega-3 unsaturated fatty acid concentrates. The concentrate was made to obtain a higher DHA/EPA content, by adding urea so that its properties became more stable. This was expected to increase the antifungal potential of snakehead fish oil.[29] In addition, prevention of oxidative damage to fish oil could be prevented by mixing antioxidants into fish oil.[30]

In this study, snakehead fish oil extract did not show antifungal properties. The 32 µl / ml snakehead fish oil extract concentration used as the highest concentration in this study was based on a similar study using oil extract, namely essential oils made from the plant *Zataria multiflora*.[31]. There has been no previous research examining the antifungal inhibition effect of fish oil on fungi.

It cannot be stated that snakehead fish oil extract does not have antifungal potential against *C. albicans*. Further research was needed to select the optimum concentration and modify methods in the process of making snakehead fish oil extract which tends to be unstable so that during the research process it does not experience the re-oxidation process.

Conclusion

0.062 µl/ml, 0.125 µl/ml, 0.25 µl/ml, 0.5 µl/ml, 1 µl/ml, 2 µl/ml, 4 µl/ml, 8 µl/ml, 16 µl/ml, and 32 µl/ml concentrations of snakehead fish oil (*Channa striata*) extracts did not have the abilities to decrease the growth of *Candida albicans*.

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