The Antifungal Inhibitory Concentration Effectiveness Test From Ethanol Seed Arabica Coffee (Coffea arabica) Extract Against The Growth Of Candida albicans Patient Isolate With In Vitro Method

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Abstract-Candida albicans are the most frequent cause of Vulvovaginalis Candidiasis infection. Its treatment using antifungal drugs, are oftenly caused side effects. The reduction of C.albicans growth and the reduction of antifungal drugs side effect, were our main purposed. Our study objective is determine the effectiveness of inhibitory power of arabica coffee seed ethanol extract on the growth of C.albicans patient isolates. The type of this research is experimental research. Kirby-bauer method with the Saboraud Dextrose Agar (SDA) media was used in this experiment. Inhibitory zone was observed around the disc, to determine the inhibitory power. The results showed that the inhibitory zone was formed on arabica coffee seed ethanol extract on 10%, 20%, 40%, and 80% concentration. Kruskal-Wallis test results (p<0.05) showed that there was a significant difference in mean between the concentration groups tested against the treatment group. The inhibitory zone was formed because of biochemical compound in arabica coffee seed such as caffeine, phenol, alkaloids, flavonoids, and saponins. Inhibitory zone in C.albicans patient isolates were smaller compared with C.albicans ATCC 90028 as gold standard. This showed that the virulence of C.albicans from patients isolates were higher. We concluded that arabica coffee seed ethanol extract could inhibiting the growth of C.albicans patient isolates. Optimization of coffee seed ethanol extract to obtain maximum active ingredients still needs to be done. This knowledge is expected to be used for the beginning manufacturer antifungal drug from natural product.

1. Introduction

Infectious diseases caused by fungus are known as mycosis and the highest incidence of mycosis is Candidiasis. Candidiasis is a fungal infection caused by Candida species. There are more than 200 species of Candida and the most pathogenic Candida is Candida albicans.¹ One of the most common types of Candidiasis is vulvovaginalis Candidiasis. Vulvovaginalis Candidiasis is the most common disease in the Dermatology and Venereology clinic RSUD Dr. Soetomo Surabaya with 65.4%.² In 2013, as many as 13 patients (56.5%) of 23 pregnant women at Haji Adam Malik Hospital Medan found cases of vulvovaginalis Candidiasis.³

Various natural ingredients that exist in the community turned out to have an useful alternative as a herbal treatment against C.albicans.⁴ One of the natural ingredients known to have an ability to inhibit the growth of C.albicans is coffee beans. Coffee beans could be selected because they were widely used in the community, their side effects are relatively lower than synthetic drugs, and they easy to obtain.⁵

Coffee beans are divided into two types, arabica coffee beans (Coffea arabica) and robusta coffee beans (Coffea canephora).⁶ Arabica beans are the most popular coffee beans in the world. Arabica coffee beans are many and easy to find in the wider community, so the price is cheaper than robusta coffee.⁷

Recently, studies have shown that coffee beans have therapeutic effects such as anti-inflammatory, antifungal, and antibacterial.⁸ Prior research with boiling technique, with aquadest
solvent, and using dilution method found that arabica coffee beans have an inhibitory effect to \textit{C. albicans} stronger than robusta coffee beans with 1 ml of arabica coffee beans giving 20,3\times10^{4} \text{ CFU/mL} inhibition.\textsuperscript{9}

In this study, we collected \textit{C. albicans} isolates that were found in Candidiasis patient because they had higher pathogenicity and virulence compared with pure strains such as ATCC.\textsuperscript{10} Here, we examine the antifungal concentration effectiveness test from ethanol seed arabica coffee (\textit{Coffea arabica}) extract against the growth of \textit{C. albicans} isolate from patient with in vitro method.

2. Material And Method

2.1 Design, Place and Time

The type of research used in this study is an experimental study with the research design used is posttest only control group design. This research was conducted at Microbiology Laboratory Faculty of Medicine, Universitas Pembangunan Nasional "Veteran" Jakarta. Preparation of ethanol extract of arabica coffee beans (\textit{Coffea arabica}) was done at Balai Penelitian Tanaman Rempah dan Obat (BALITTRO), Bogor. The study was conducted in July 2017.

2.2 Number and Sampling Method

\textit{C. albicans} isolate samples were taken from patient from Dermatology Venereology and Obstetric Ginecologic Clinic at RSPAD Gatot Subbroto Jakarta, which was diagnosed clinically or suspected Vulvovaginalis Candidiasis. Sampling was conducted during July 2017 and was conducted for one month.

The sample of each group is calculated using Federer's formula with \( n \) is the number of samples, and \( t \) is the number of groups. The formula used is as follows: \( (n-1)(t-1) \geq 15 \)

Based on the formula, each sample is repeated as many as four times.

2.3 Materials and Tools

The tools used include digital screw term, hand scoon, test tube, bunsen, disc paper size 6 mm, 100 pieces, Bekker glass, petri dish, syringe, centril oce, measuring cup, autoclave, aluminum foil. The ingredients used were ethanol extract of arabica coffee beans, Saboraud Dextrose Agar (SDA), CHROMAgar Candida, \textit{C. albicans} suspension from patient isolate and \textit{C. albicans} ATCC 90028 culture, 100 ml sterile distilled water, 10\% sterile NaCl 0.9\%, ketoconazole tablet 200 mg, BaCl2 1\% 0.5 ml, H2SO4 1\% 99.5 ml.

2.4 Research procedure

Sampling of \textit{C. albicans} isolates was performed after the patient read the informed consent and filled the respondent sheet. Sampling is carried out by the medical officer at the Presidential Hospital RSPAD Gatot Soebroto Jakarta. After the specimen was taken and inserted into a tube containing NaCl, the researcher gave the tube identity and brought it to the Microbiology Laboratory of the Medical Faculty of the National Development University "Veteran" Jakarta.

The isolate sample was grown into CHROMAgar Candida medium, then incubated with 37\(^{\circ}\)C for 48 hours to determine colonization. The Candida species can be determined using CHROMAgar Candida according to the manufacturer's instructions that produce the media. After that, the green-colored isolates on CHROMAgar media are performed differentiation test with an incubation on SDA medium with temperature of 45\(^{\circ}\)C for 48 hours. This process aims to distinguish between \textit{C. albicans} and \textit{C. dubliniensis} because they have a color similarity in the CHROMAgar Candida media.

Once confirmed the growing colonies were \textit{C. albicans}, the colony was taken and suspended \textit{C. albicans} isolates patients with a standard of 0.5 McFarland. The suspension is applied to the SDA media, then give the disc paper that has been immersed in each test group. Then the media was incubated in 37\(^{\circ}\)C for 1x24 hours. After incubation is complete, the Kirby-bauer method were performed by observed the inhibitory zone around the disc paper.

2.5 Data analisis

Inhibitory zone data were analyzed by using Kruskal-Wallis test. If the results of the analysis show significant differences between the control group and the treatment group, then the Post Hoc
Mann-Whitney test is followed. Data processing and data analysis is done using SPSS for Windows version 17.0.

3. Results And Discussion

In this study, seven patients who have been diagnosed with vulvovaginalis Candidiasis diagnosed and are willing to take samples by health workers at Presidential Hospital RSPAD Gatot Soebroto Jakarta. In the differentiation test results using CHROMAgar Candida media, we obtained six samples from patients that contain colonies of *C.albicans* and one sample of patients there are no colonies of *C.albicans*.

The measurement results of this experiment have different sizes, the smallest inhibitory starts at a concentration of 10% and the greatest inhibitory at 80% concentration (Table 1.) The inhibitory zone formed showed the effectiveness of inhibition of ethanol extract of Arabica coffee beans (*Coffea arabica*) on the growth of *C.albicans* isolates. The result showed that the negative control group has no inhibitory zone. The negative control group had no effect in inhibiting the growth of *C.albicans*. While the ethanol extract group of Arabica coffee bean (*Coffea arabica*) and the positive control group showed an inhibitory zone, where the barrier started to form on ethanol extract of Arabica coffee bean (*Coffea arabica*) with concentration of 10% and the diameter increased according to addition of concentration of extract tested (Table 1.). The experimental results show that there is a zone of resistance to the growth of *C.albicans* patient isolates. Zone is formed clear but still visible presence of faint patches around the paper disc which is a fungus metabolite.

| Repetition | Inhibitory zone on Candida patient isolate (mm) | Inhibitory zone on Candida ATCC 90028 (mm) |
|------------|-----------------------------------------------|------------------------------------------|
|            | 10%  | 20%  | 40%  | 80%  | Contro l (+)  | Contro l (-) | 10%  | 20%  | 40%  | 80%  | Contro l (+)  | Contro l (-) |
| 1          | 0.61 | 1.46 | 2.74 | 4.55 | 12.74       | 0             | 1.56 | 2.41 | 3.69 | 5.5  | 14.04       | 0             |
| 2          | 0.70 | 1.49 | 2.44 | 4.70 | 13.42       | 0             | 1.65 | 2.44 | 3.39 | 5.65 | 14.37       | 0             |
| 3          | 0.63 | 1.62 | 2.20 | 4.43 | 13.54       | 0             | 1.58 | 2.57 | 3.15 | 6.02 | 14.49       | 0             |
| 4          | 0.69 | 1.33 | 2.58 | 4.46 | 13.34       | 0             | 1.64 | 2.43 | 3.53 | 5.41 | 14.29       | 0             |
| Σ          | 0.65 | 1.47 | 2.49 | 4.53 | 13.26       | 0             | 1.56 | 2.41 | 3.69 | 5.5  | 14.04       | 0             |

The average inhibitory zone produced from ethanol extract of Arabica coffee bean (*Coffea arabica*) 80% concentration is 4.53 mm (Table 1). This concentration provides the greatest inhibitory effect of the antifungal effectiveness of ethanol extract of Arabica coffee beans (*Coffea arabica*) against the *C.albicans* isolates tested. At concentrations of 40%, 20% and 10% respectively give an inhibit zone average of 2.49 mm, 1.47 mm and 0.65 mm.

The result with *C.albicans* ATCC 90028 showed broader inhibition compare with than the *C.albicans* patient isolates. This occurs due to the virulence from patient isolate which is more higher than *C.albicans* ATCC 90028.

Ethanol extract of Arabica coffee bean (*Coffea arabica*) 80% concentration has the greatest inhibition power compared with other concentrations, while extract with the smallest inhibitory power is the concentration group of 10%. In dilution of ethanol extract of Arabica coffee beans (*Coffea arabica*) from concentration of 100% to 80%, 40%, 20% and 10%, there

is reduction of dissolved active substances in each of these concentrations, therefore the inhibitory effectiveness is lower with the smaller concentration of the extract tested. This is evident from the large difference in inhibitory diameter of growth of *C.albicans* isolate, while with the lower concentration of extract tested, the inhibitory power diameter is smaller.

According to Davis and Stout (1971) the power of antifungal inhibition is divided into 4 categories, namely weak, medium, strong and very strong. Based on these criteria, the result of diameter zone inhibition of ethanol extract of Arabica coffee bean (*Coffea arabica*) on *C.albicans* isolate at concentrations of 10%, 20%, 40%, and 80% was classified into the weak inhibitory category (≤ 5mm). This is in contrast to the results of ethanol extract test of Arabica coffee bean (*Coffea arabica*) with *C.albicans* ATCC 90028 which has moderate inhibition category (5-10 mm) at 80% concentration.

Table 1. Average Measurement Results On *C.albicans* Patient Isolate and ATCC 90028

| Repetition | Inhibitory zone on Candida patient isolate (mm) | Inhibitory zone on Candida ATCC 90028 (mm) |
|------------|-----------------------------------------------|------------------------------------------|
|            | 10%  | 20%  | 40%  | 80%  | Contro l (+)  | Contro l (-) | 10%  | 20%  | 40%  | 80%  | Contro l (+)  | Contro l (-) |
| 1          | 0.61 | 1.46 | 2.74 | 4.55 | 12.74       | 0             | 1.56 | 2.41 | 3.69 | 5.5  | 14.04       | 0             |
| 2          | 0.70 | 1.49 | 2.44 | 4.70 | 13.42       | 0             | 1.65 | 2.44 | 3.39 | 5.65 | 14.37       | 0             |
| 3          | 0.63 | 1.62 | 2.20 | 4.43 | 13.54       | 0             | 1.58 | 2.57 | 3.15 | 6.02 | 14.49       | 0             |
| 4          | 0.69 | 1.33 | 2.58 | 4.46 | 13.34       | 0             | 1.64 | 2.43 | 3.53 | 5.41 | 14.29       | 0             |
| Σ          | 0.65 | 1.47 | 2.49 | 4.53 | 13.26       | 0             | 1.56 | 2.41 | 3.69 | 5.5  | 14.04       | 0             |
This may be due to the \textit{C.albicans} isolate having higher virulence than the ATCC 90028 strain, so the inhibitory power generated at the isolate growth is lower than that of the pure strain.

The higher the concentration of extract tested, the more active substances contained. Conversely, the lower the concentration of the extract, the less active substance, so that the inhibitory power effectiveness will be reduced.\textsuperscript{12}

The concentration of the extract affects the speed of the diffusion of a nutritious substance. The greater the concentration of extract, the faster the diffusion occurs, so the greater the inhibitory power and the greater the diameter of the inhibitory zone formed.

Kruskal-Wallis test results have a significance value of 0.000. Because of the significance value <0.05, from the results of this test it can be concluded that there are significant differences in the results between the concentration groups tested against the growth of \textit{C.albicans} patient isolates.

The data analysis was followed by a Mann-Whitney post-hoc test to identify significant differences on different treatment groups. Mann-Whitney test statistics showed that each group of ethanol extract of Arabica coffee bean (\textit{Coffea arabica}) had a significant difference. It can be seen from the significance value less than 0,05 (Table 2.)

| Table 2. Mann-Whitney Post-hoc Test |
|------------------------------------|
| Treatment Group                  | Sig. |
|-----------------------------------|------|
| Positive Control                  |      |
| 10%                               | 0.000|
| 20%                               | 0.000|
| 40%                               | 0.000|
| 80%                               | 0.000|
| 10% Concentration                 |      |
| 20%                               | 0.000|
| 40%                               | 0.000|
| 80%                               | 0.000|
| 20% Concentration                 |      |
| 40%                               | 0.000|
| 80%                               | 0.000|
| 40% Concentration                 |      |
| 80%                               | 0.000|

The anti-fungal effect of Arabica coffee bean extract (\textit{Coffea arabica}) is caused by chemical compounds in Arabica beans (\textit{Coffea arabica}). The chemical compounds include classes of compounds such as caffeine, phenol, alkaloids, flavonoids, and saponins (Table 3.).

Alkaloid compounds affect the isolates of \textit{C.albicans} by inhibiting the biosynthesis of fungal nucleic acids, prevented the fungus to develop and eventually die. In addition to alkaloids, phenols also play a role in inhibiting the growth of \textit{C.albicans} isolates by denaturing the protein bonds in the cell membrane and making the cell membrane lysate and allowing the phenol to penetrate into the cell nucleus and inhibit the \textit{C.albicans} development.\textsuperscript{13} This is because \textit{C.albicans} is a very sensitive on phenol compounds.\textsuperscript{14}

Flavonoids works by disrupting the formation of pseudohifa during the course of pathogenesis and saponins work by affecting the permeability of molds and yeasts of \textit{C.albicans}. In addition, flavonoids are also able to inhibit the action of enzymes that play a role in the synthesis of \textit{C.albicans} cell walls such as manan synthase, chitin synthase and glucan synthase. If the enzyme is inhibited activity then the formation of fungal cell wall will be disrupted and the formation of pseudohifa will be inhibited.\textsuperscript{15}

| Table 3. Phytochemical Test Results Arabica Beans |
|-----------------------------------------------|
| Phytochemical Test                             | Arabica coffee bean extract |
| Alkaloids                                      | +                             |
| Saponins                                       | +                             |
| Tannin                                         | +                             |
| Phenolic                                       | +                             |
| Flavonoids                                     | +                             |
| Triterpenoids                                  | +                             |
| Steroids                                       | -                             |
| Glycoside                                      | +                             |
Saponins are antifungal by binding to the ergosterol present in the cell wall of the fungus, so the permeability of fungal cells will be disrupted and may inhibit the growth and even death of the fungal cells. Then, there is a tannin that works by interfering with the permeability and causing the shrinking of the cell wall of the fungus that can lead to the death of the fungal cell.

The solvent used in this study is ethanol solvent because ethanol is a safe and good universal solvent for the extraction process of all secondary metabolites, so it can dissolve all active ingredients contained in a natural material such as coffee beans.

4. Conclusion

The ethanol extract of Arabica coffee beans (Coffea arabica) has an inhibitory effectiveness on the growth of isolates of Candida albicans by disc diffusion method. Then, there was a significant difference in each concentration of ethanol extract of Arabica coffee bean (Coffea arabica) in inhibiting the growth of Candida albicans isolate. Then, the largest concentration (80%) of ethanol extract Arabica beans (Coffea arabica) is the most effective concentration in inhibiting the growth of C.albicans isolates.

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