

# Comparison of In Vitro Testing Antifungal Activity between Rosemary Essentials Oil and Fluconazol on *Candida species* Isolate from HIV/AIDS Patients with Candidiasis Oral

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## ABSTRACT

**Background:** The most common opportunistic infection obtained in patients with Human Immunodeficiency Virus (HIV)/Acquired Immune Deficiency Syndrome (AIDS) is Oral Candidiasis (OC). Currently widely reported about increased fluconazole resistance, as a therapy that is often used in patients with HIV/AIDS with OC. For this reason, effective antifungal agents are needed as alternative therapies. Several studies have reported some antifungal activity from *rosemary* essential oils.

**Purpose:** The aim of this study is to evaluate the antifungal activity between *rosemary* essential oils and fluconazole against the isolates used by *Candida sp.* taken from HIV / AIDS patients with OC. **Methods:** This research was an experimental laboratory study with a post-test only design of 40 *Candida sp.* isolates from HIV / AIDS patients with OC in the Outpatient Unit and Inpatient Installation of the Infectious Disease Intermediate Care Unit (UPIPI) Dr. Soetomo General Academic Teaching Hospital, Surabaya. Examination of antifungal activity using conventional test methods with disk diffusion. **Result:** Disk diffusion test result revealed average inhibition zone of *rosemary* essential oil in *Candida albicans* 1,75±3,64 mm and *Candida non-albicans* 1,5±3,08 mm, while the average inhibition zone of fluconazole in *Candida albicans* 17,9±8,62 mm and *Candida non-albicans* 4,85±7,9 mm. There was significant difference ( $p < 0.001$ ) between the inhibition zone formed in *Candida albicans* by fluconazole and *rosemary* essential oil, but no significant difference of inhibition zone formed in *Candida non-albicans*. **Conclusion:** Antifungal activity fluconazole has greater than *Rosemary* essential oils in *Candida albicans*.

**Keywords:** Antifungal activity, *Candida albicans*, *Candida non-albicans*, essential oils, flukonazole, *Rosmarinus officinalis*.

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## BACKGROUND

Oral candidiasis (OC) is an opportunistic infection by the fungus *Candida sp.* which attacks the oral cavity. *Candida sp.* is a normal flora in the body and is most abundant in the oral cavity.<sup>1</sup> OC is the most common opportunistic infection found in patients infected with Human Immunodeficiency Virus (HIV).<sup>2</sup> Acquired Immunodeficiency Syndrome (AIDS) is a disease with high mortality and morbidity rates. In some countries it is known as a leading cause of death. The development of HIV / AIDS cases in Indonesia based on data from the Directorate General of Disease Control and Environmental Health Ministry of Health (Ditjen PP & PL Ministry of Health) in 2017 shows the number of new cases of patients with HIV / AIDS as many as 14,640 people and the number of cumulative cases from 1987 to 2017 as many as 280,623 people. East Java Province ranks second highest after DKI Jakarta for the number of HIV infections, amounting to

39,633.<sup>3</sup>

*Candida albicans* is the most reported etiology of fungal-causing fungi, but at this time an etiological change has begun to change to *Candida non-albicans*. Other studies report a significant correlation between the number of low cluster of differentiation-4 (CD4) 200 cells /  $\mu\text{L}$  on the incidence of OC.<sup>4</sup> Retrospective research in 2013 at RSUD Dr. Soetomo Surabaya showed 81.1% of patients suffering from HIV / AIDS with OC in the Infectious Disease Intermediate Care Unit of the total patients with skin manifestations.<sup>5</sup>

The World Health Organization (WHO) recommends fluconazole and miconazole as first-line therapy in cases of HIV & AIDS with OC.<sup>6</sup> Fluconazole is a safe, inexpensive, effective, and available antifungal option in Indonesia. In the clinical practice guide for Indonesian dermatologists in 2017 and practice guide for Dermatology Functional Medical Unit Dr. Soetomo Surabaya in 2014 from the

two guidelines, fluconazole was a drug that was used quite often.<sup>7,8</sup>

The widespread use of fluconazole in cases of OC was reported to be associated with a decrease in sensitivity of in vitro fluconazole to *Candida* sp. Several other factors that influence the decrease in sensitivity to OC therapy with azole groups against *Candida* sp. among others, changes in causative species from *albicans* to non-*albicans* that have intrinsic resistance such as *C. glabrata* and *C. krusei*. Since it was first discovered in 1990, the incidence of antifungal agent resistance to *Candida* species from HIV / AIDS patients with OC has been reported to increase.<sup>9</sup> Fluconazole resistance data at RSUD Dr. Soetomo by using the disk diffusion method as a resistance test method, found fluconazole resistant to 18 of 37 (48.6%) *Candida* resistance isolates and most species were *Candida non-albicans*.<sup>10</sup>

Lately, research on herbal essential oils has turned out to have antifungal effects. The results are expected to be obtained as an alternative therapy to replace antifungal drugs that have been resistant. The pharmacological properties of these medicinal plants have been extensively studied in phytotherapy. In several studies reported the presence of antifungal effects of rosemary essential oils.<sup>11,12</sup> Antifungal activity has been reported in research by Delich et al investigated rosemary essential oil compared with fluconazol against *Candida* species isolates, showing means of diameter zone inhibition of 5% rosemary essential oil was  $11.17 \pm 0.19$  and better when compared to 2mg / ml fluconazole.<sup>12</sup>

## METHODS

This research was an experimental laboratory study with a posttest only design conducted in Dr. Soetomo General Hospital, Surabaya. Forty isolates divided in two groups 20 isolates of *C. albicans* and 20 isolates *Candida non-albicans* used in this study, were obtained from forty patients Candidiasis oral with HIV/AIDS infection. *Candida non-albicans* species included in this study consisted of 9 species of *Candida krusei*, 4 species of *C. glabrata*, 2 species of *C. dubliniensis*, 2 species of *C. parapsilosis*, 2 species of *C. tropicalis* and 1 species of *C. lypoitica*. Analyzed essential of Rosemary (*Rosmarinus officinalis* L.), were commercial product produced by Youngliving, Ltd. (Canada, United State), compare of flukonazol 2mg/dl. This essential oil were analyzed by gas chromatograph (GC) containing 45.12% *1.8-cineol*, 12,78% *alpha-pinene*, 10,79% *camphor*, 3,50% *borneol*. Examination of antifungal activity using the

disc diffusion method. *Candida* suspension are removed to Muller Hinton agar supplemented with 2% glucose and 0.5 µg / mL methylene blue (GMB). Paper disks / disks containing rosemary and fluconazole with different potential are placed on Muller Hinton agar which has been planted with yeast isolates as much as  $1 \times 10^6$  CFU / ml and to be dried at room temperature for 15 minutes. After 15 minutes, 5 mm of wells were made, and 100 µL of rosemary and fluconazole essential oil were deposited into each well. Yeast susceptibility testing by radial diffusion compares the two active ingredients incubated at 37 ° / C for 24 hours, and halo inhibition is measured in millimeters. The zone sizes were interpreted as per Clinical Laboratory Standards Institute (CLSI) guidelines. The obtained results were analyzed using SPSS (mean, standard deviation). The data for diffusion methods were analyzed by *MannWhitney* in Statistic. Statistical significance was determined at  $p \leq 0.05$ . This research has been reviewed and approved by the Ethics Committee at Dr. Soetomo General Academic Hospital (1647/KEPK/XI/2019).

## RESULT

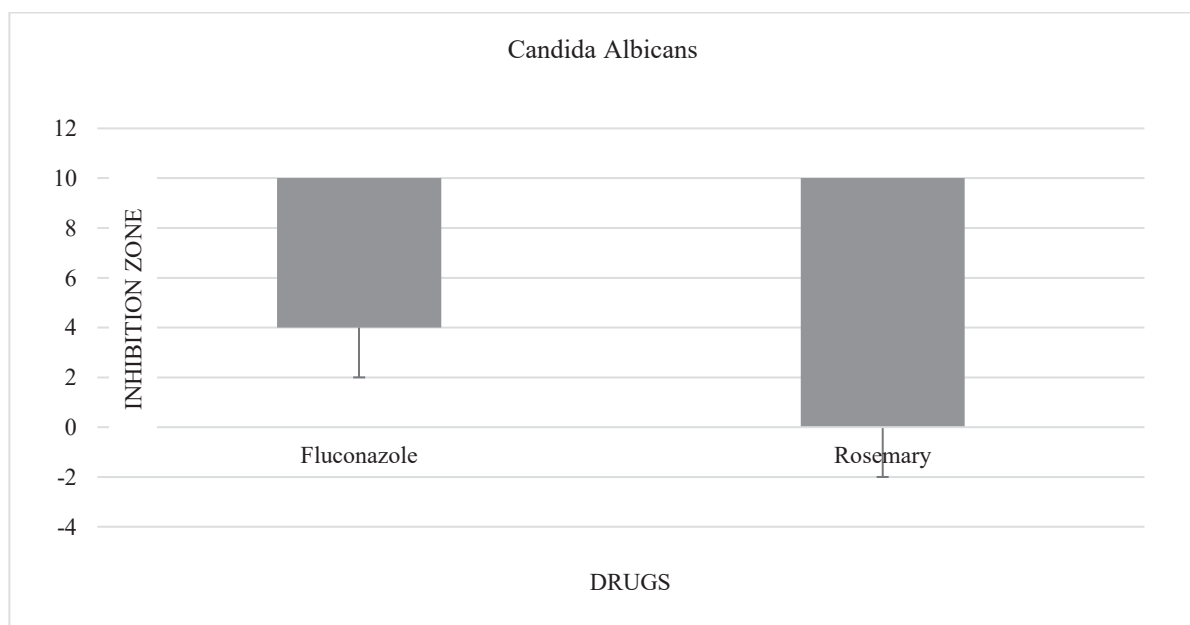
The demographic distribution of isolates from HIV / AIDS patients with OC in this study is presented in Table 1. The male patients were 31 patients (77.5%) and female patients were 9 patients (22.5%). The age of patients from isolates in this study varied between the age group 15-24 years to 55-64 years. The most age range is 19 subjects (47.5%) in the 25-34 years group the least number in the 55-64 years group is 3 patients (7.5%). The most common major complaint was white patches in the oral cavity of 36 patients (90%), and 23 patients (57.5%) accompanied by swallowing pain. The most common type of OC this study were pseudomembranous type, found in 39 patients (88%), followed by acute type of cheilitis and atrophic each of 4 (9%) and 1 (3%).

Figure 1 shows the inhibition zone formed by fluconazole is greater when compared with rosemary essential oil. The mean value of inhibition zone formed by fluconazole was  $17.9 \pm 8.62$  mm while rosemary essential oil was  $1.75 \pm 3.64$  mm. Shapiro Wilk test confirmed that the data were not normally distributed ( $p < 0.05$ ); therefore, nonparametric statistical tests, the Mann Whitney test, was performed. The Mann Whitney test results showed a statistically significant difference ( $p < 0.001$ ) between the inhibition zone formed by fluconazole and the inhibition zone formed by rosemary essential oil.

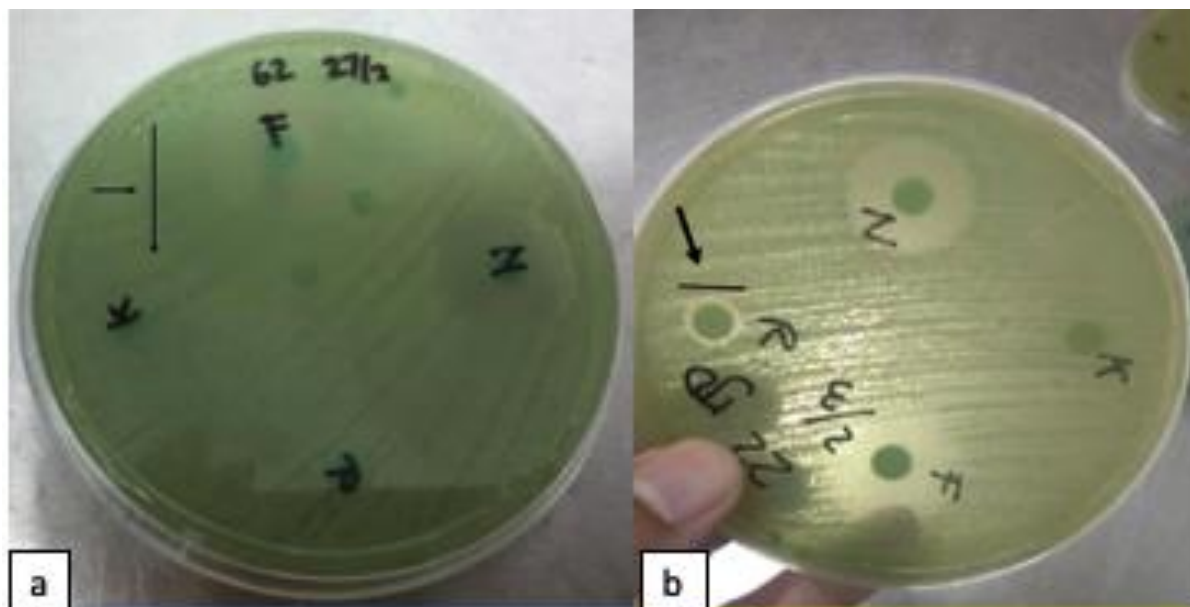
**Table 1.** Demographic data of research

No	Basic Data		N	%
	Categories	Group		
1	Gender	Male	31	77.5
		Woman	9	22.5
2	Age	15 – 24 years	6	15
		25 – 34 years	19	47.5
		35 – 44 years	4	10
		45 –54 years	8	20
		55-64 years	3	7.5
		> 65 years	0	0
3	*Major complain	Red patches in the oral cavity, patches and sores on the lip corners	3	7.5
		Red and red patches on the oral cavity	0	0
		White patches on the oral cavity	36	90
		White patches in the oral cavity, patches and sores on the lip corners	1	2.5
4	*Diagnosis	Pseudomembranous candidiasis	39	88
		Plaque hyperplasia candidiasis	0	0
		Atrophic candidiasis	1	3
		Cheilitis candidiasis	4	9
5	Pain	Yes	23	57.5
		No	17	42.5
6	Total CD4 absolute	< 200 sel/ $\mu$ L	28	70
		> 200 sel/ $\mu$ L	12	30

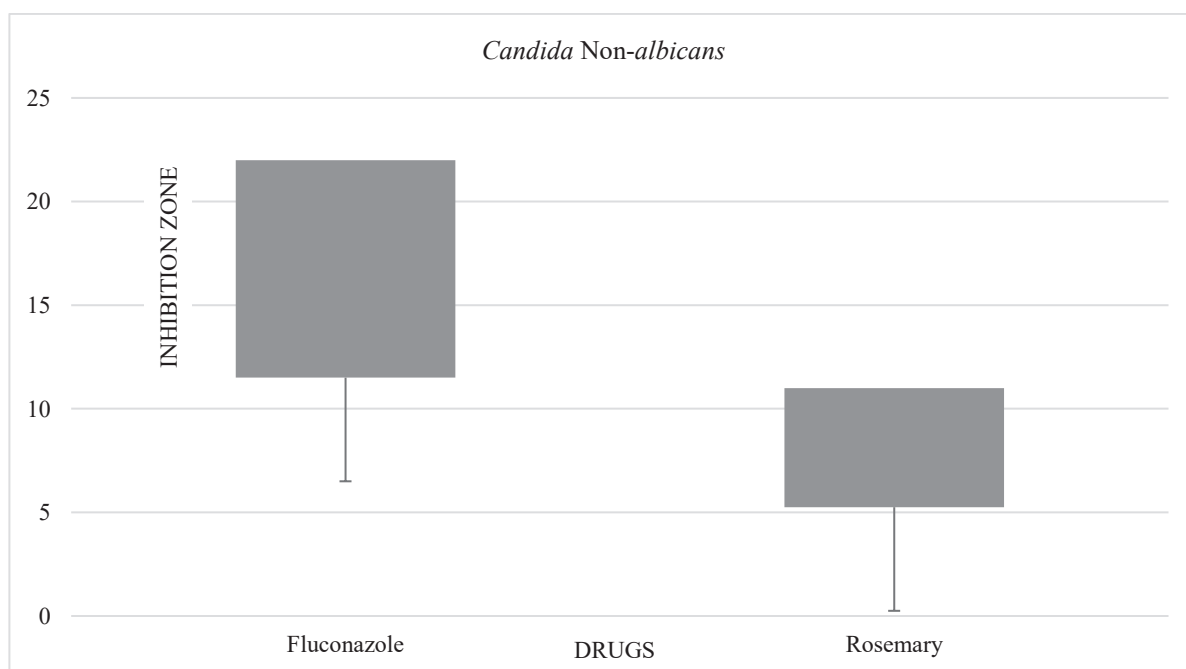
\* Patients can have > 1 major complaint, diagnosis.



**Figure 1.** The diameter inhibition zone of *Candida albicans* growth between fluconazole and rosemary



**Figure 2.** Inhibition zones against *C. albicans* a. fluconazole with diameters 30mm b. rosemary with diameters 7mm

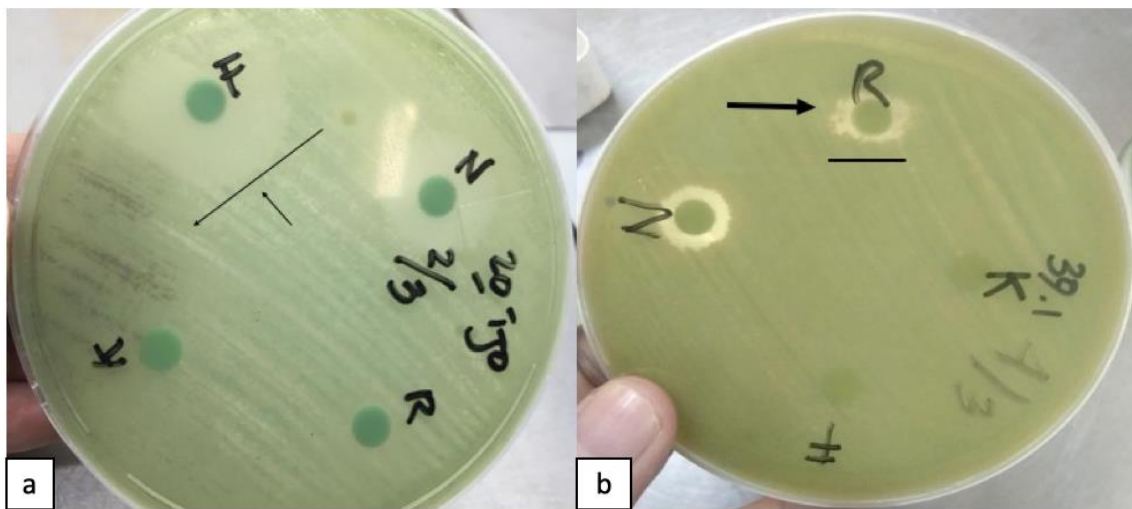


**Figure 3.** The diameter inhibition zone of *Candida non-albicans* growth between fluconazole and rosemary essential oil in disc-diffusion assay

Figure 3 shows the inhibition zone formed by fluconazole is greater when compared with rosemary essential oil. The mean value of inhibition zone formed by fluconazole was  $4.85 \pm 7.9$  mm while rosemary essential oil was  $1.5 \pm 3.08$  mm. Shapiro Wilk test confirmed that the data were not normally distributed ( $p < 0.05$ ); therefore, nonparametric statistical tests, the Mann Whitney test, was performed. The Mann Whitney test results showed a no statistically

significant difference ( $p > 0.05$ ) between the inhibition zone formed by fluconazole and the inhibition zone formed by rosemary essential oil.

Based on data from Table 2 and Table 3 shows all of research subject had history of treatment before with fluconazole nystatin and the combination of both. Kruskal Wallis test results showed no significant differences in the inhibition zone formed with a history treatment, on the use of fluconazole or rosemary ( $p > 0.05$ )



**Figure 4.** Inhibition zones against *C. non-albicans* a. fluconazole with diameters 22mm b. rosemary with diameters 8mm

**Table 2.** Correlation between inhibitory zones of *C. albicans* with a history treatment

Drug	History treatment	N	Mean±SD	Median (min – max)	Values
Fluconazole	Fluconazole	5	23,4 ± 3,97	22 (20 – 30)	0,096
	Nystatin	9	18,3 ± 7,94	20 (0 – 26)	
	Fluconazol & nystatin	6	12,7 ± 10,27	16 (0 – 24)	
Rosemary	Fluconazole	5	0,0 ± 0,0	0 (0 – 0)	0,057
	Nystatin	9	3,9 ± 4,70	0 (0 – 10)	
	Fluconazole & nystatin	6	0,0 ± 0,0	0 (0 – 0)	

\* SD: Standard deviation

**Table 3.** Correlation between inhibitory zones of *Candida non- albicans* with a treatment history

Drug	History treatment	n	Mean±SD	Median (min – max)	Values
Fluconazole	Fluconazole	5	5,5 ± 11,00	0,0 (0 – 22)	0,159
	Nystatin	9	0,0 ± 0,0	0 (0 – 0)	
	Fluconazole & Nystatin	6	7,5 ± 8,29	5,5 (0 – 21)	
Rosemary	Fluconazole	5	2,0 ± 4,0	0 (0 – 8)	0,480
	Nystatin	9	2,5 ± 3,89	0 (0 – 8)	
	Fluconazole & Nystatin	6	0,7 ± 2,21	0 (0 – 7)	

\* SD: Standard deviation

**DISCUSSIONS**

The basic characteristics of the research subjects are presented in Table 1, showing that there were more male subjects than female (77.5% and 22.5%). This research supports a report from the Ministry of Health of the Republic of Indonesia in 2017 that the proportion of male patients is twice as frequent as women. This is related to the number of HIV / AIDS patients more in men.<sup>3</sup> The most age group is in the age range of 25-34 years with 19 subjects (47.5%), this finding is similar to the statement in research by Putranti et al in 2018, that HIV infection is more common in adulthood which makes it more likely to engage in unsafe sexual

behavior that is at risk of HIV transmission.<sup>13</sup>

Most of these patients complained of the presence of white patches in the oral cavity of 36 patients (90%), and 23 patients (57.5%) accompanied by swallowing pain. This condition can be caused by *Candida* sp. attached to the mucosal surface of the oral cavity or the tongue through its hyphae can be detached and leave a red mucosa and sometimes accompanied by mild bleeding. This can cause pain and burning in the oral cavity as a sign of inflammation.<sup>2</sup> Complaints white patches in the mouth found in knockout type of pseudomembranous and hyperplasia of plaque is the most common type of OC. Fourty of *Candida* species



as research subjects, most were taken from HIV / AIDS patients with OC who had an absolute CD4 count <200 cells /  $\mu$ L of 28 patients (70%). This finding is similar to the statement in research by Nugraha et al in 2018, OC patients were more likely to be found in patients with an absolute CD4 cell count <200 cells /  $\mu$ L of 61 patients (89.71%). This is related to the pathogenesis of HIV / AIDS in OC. Interaction between HIV Trans-Activating Transduction or Transcriptional Activator (TAT) protein with *C. albicans* cell wall enables the occurrence of specific gene transduction in HIV / AIDS with CD4 T lymphocyte counts <200 cells /  $\mu$ L.<sup>14</sup>

The results obtained in the study of inhibition zone measurements showed the average diameter of inhibition zone of *C. albicans* formed by fluconazole was  $17.9 \pm 8.62$  mm and rosemary essential oil formed was  $1.75 \pm 3.64$  mm. The Mann Whitney test results showed a statistically significant difference ( $p < 0.001$ ) between the inhibition zone formed by fluconazole and the inhibition zone formed by rosemary essential oil. These results are adjusted to the standard criteria from the Clinical and Laboratory Standards Institute (CLSI) for the sensitive, intermediate, or resistant criteria of each antifungal drug and from the measurement results found that fluconazole is included in the criteria of intermediate and rosemary essential oil there is no standards measurement criteria until now. The results showed the inhibitory zone created by fluconazole growth of *Candida non-albicans* was greater when compared to rosemary essential oil. The mean value of inhibitory zones that form fluconazole is  $4.85 \pm 7.9$  mm while rosemary essential oil is  $1.5 \pm 3.08$  mm. The mean value of inhibitory zones that form fluconazole is included in the resistant category. *Candida non-albicans* species included in this study consisted of 9 species of *C. krusei*, 4 species of *C. glabrata*, 2 species of *C. dubliniensis*, 2 species of *C. parapsilosis*, 2 species of *C. tropicalis*, and 1 species of *C. lyophilic*. This study shows that the zone of inhibition of fluconazole determines the greatest of 2 species of *C. dubliniensis*, with diameters of 22 mm and 21 mm respectively, which are classified as sensitive categories. Fluconazole inhibition zone in *Candida non-albicans* species included in the intermediate category is 1 species of *C. parapsilosis*, where the diameter value is 15 mm. Fluconazole inhibition zone in resistant *Candida non-albicans* species are 9 *C. krusei*, 4 *C. glabrata* species, 1 *C. parapsilosis* species, 2 *C. tropicalis* species, and 1 *C. lyophilic* species.<sup>15</sup>

Result of fluconazole resistant in this study were 50% consist of (10% *C. albicans* and 40% *Candida non-albicans*). The results of this study indicate the fluconazole resistance of *Candida non-albicans* species, the data are in accordance with the results of a

study conducted by Murtiastutik et al in 2019 about the sensitivity of antifungal fluconazole to *C. species* in HIV / AIDS patients in Dr. Soetomo at 48% and the most species are *Candida non-albicans*.<sup>10</sup> The mechanism of fluconazole resistance in *Candida non-albicans* species varies depending on each species, and is grouped into two namely intrinsic resistance and extrinsic resistance. Intrinsic resistance associated with *Candida* species causing OC has occurred since the beginning of antifungal therapy without any prior drug exposure (primary resistance). Fluconazole is intrinsically resistant to several *Candida non-albicans* species, such as *C. krusei*, *C. glabrata*. Extrinsic resistance occurs due to the use of antifungal drugs that are widespread, not according to the dosage and standard, and not according to indications in HIV / AIDS patients causing changes and mutations in the genes in *Candida*. *C. krusei* in particular has a low affinity of the ERG11 gene naturally (intrinsically) so that the fluconazole drug cannot bind to the ERG11 protein, *C. glabrata* has the ability to absorb exogenous sterols so that changes in the composition of the fungal cell membrane are not recognized by the azole antifungal, *C. tropicalis* has the ability inactivating the ERG3 gene that causes changes in the composition of the fungal membrane sterols and both species are also able to increase the expression of the ERG11 gene which causes the azole agent to not bind.<sup>16</sup>

The results of research on the presence of antifungal activity of rosemary essential oil on the growth of *C. albicans* and *Candida non-albicans* by invitro are still different. The statistical test results obtained mean fluconazole inhibition zone is greater than rosemary essential oil, this is possible because of several factors from the tested drug or from the implementation of the research itself. Rosemary essential oil is more volatile than fluconazole, thus allowing the amount of active ingredients to evaporate before diffusing on paper discs that can reduce its effectiveness. Research on antifungal activity with essential oil often produces data that contradicts the theory, because there is no standard technique for evaluating antifungal using essential oil that have been standardized by CLSI.<sup>11</sup> Previous research conducted by Delich et al. in 2013 showed the inhibition zone diameter in  $11.17 \pm 0.19$  and fluconazole was resistant. The difference in the results of the inhibition zone diameter in this study with the research conducted by Delich et al is in the diffusion method carried out. This study uses the disk diffusion method while the research of Delich et al used agar well diffusion method for the antifungal activity of essential oil the results are often inconsistent, because of the factors of volatility, water solubility and viscosity.<sup>12</sup>

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