Comparison of Antifungal Effects of Propolis Extract Apis mellifera carnica and Fluconazole against Candida glabrata in Vitro

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Abstract

C. glabrata has emerged as a major cause of adaptive resistance to azoles, echinocandins, and multidrugresistant agents. Therefore, an effort is needed to explore natural materials for antifungal activity, one of these natural ingredients is propolis. Propolis extract has chemical compounds such as flavonoids and phenolic acids which have antifungal activity which can inhibit the growth of the Candida glabrata. In this study, propolis was used from Apis mellifera carnica bees. This study aims to determine the antifungal effect of propolis Apis mellifera carnica against clinical isolates of Candida glabrata compared to fluconazole. This study used an in vitro experimental study conducted using the diffusion method. This study was divided into 5 groups, namely negative control (KN), positive control (KP) using fluconazole and treatment (P) with three variants of propolis Apis mellifera carnica extract concentrations, namely 50%w/v (P1), 75%w/v (P2), and 100%w/v (P3). Data analysis used the Kruskal-Wallis test and continued with the Mann-Whiteney test with significance ($p<0.05$). The average result of the diameter of the inhibitory zone sequentially starting from the smallest is negative control (KN) of 0 mm, P3 of 7.71 mm, P1 of 8.85 mm, P2 of 9.60 mm and the largest is positive control (KP) of 19.08 mm. The concentration of Apis mellifera carnica propolis extract had the greatest antifungal effect at a concentration of 75%w/v and the least at a concentration of 100%w/v. Based on the research above, it can be concluded that the propolis extract of Apis mellifera carnica has an antifungal effect against Candida glabrata but is not better when compared to fluconazole.

Keywords: antifungal, C. glabrata, propolis, Apis mellifera carnica, fluconazole

1. Introduction

Candidiasis is a broad term that refers to infections of the skin, mucosa and internal organs caused by fungi of the genus Candida, which can occur at any age [1]. From 1970 to 2000, C. albicans predominated as a pathogen causing candidemia and all forms of systemic candidiasis. However, in the last decade, there has been a significant increase of Candida non albicans where among a total of 79 proven cases of Candida non albicans infection, C. parapsilosis (36.8%) was the most common species, followed by C. glabrata (32.9%), C. orthopsillosis (11.4%), C.tropicalis (8.9%), C. krusei (5.0%) and C. guilliermondii (5.0%) [2]. Shifts are more pronounced in South Asian countries where Candida non albicans species are reported in 70–90% of cases [3].

Along with the increasing cases of Candidiasis by Candida non albicans, antifungal resistance has also increased rapidly, one of which is C. glabrata. Of all the Candida species, C. glabrata has emerged as the main cause of adaptive azole, echinocandin, and multidrug (MDR: azole + echinocandin) resistance [4]. Invasive candidiasis due to C. glabrata causes substantial morbidity and mortality of approximately 40-60%, this is due to the low susceptibility of C. glabrata to azoles [5]. Although the incidence of echinocandin-resistant and multidrug-resistant (MDR) C. glabrata is low, fluconazole-resistant C. glabrata isolates are increasingly being reported worldwide, at rates of 2.6%-10.6%, and these rates can reach 17 % [6][7][8].

This triggers an effort to explore existing and easy-to-obtain natural materials that aim to determine the potential of these materials for antifungal activity. Therefore, it is necessary to do research to find natural medicines as a solution to the problems above. The use of medicines from natural ingredients has begun to be developed in this modern era. One of them is propolis. Propolis is a plant-derived substance collected by honey

bees from various sources and contains many polyphenolic constituents, especially flavonoids and phenolic acids [9]. The content of flavonoids in propolis inhibits fungal growth with various underlying mechanisms, one of which is by damaging the plasma membrane, inducing mitochondrial dysfunction, and inhibiting the formation of cell walls, cell division, synthesis of RNA and protein, as well as outflow-mediated pumping systems [10]. In particular, phenolic acids have shown promising in vitro and in vivo activity against growth inhibition processes of Candida species [11].

This research is also aimed at exploring bees which are often cultivated in Indonesia. Many local beekeepers are starting to produce propolis, one of which comes from Apis mellifera carnica bees. This bee is a subspecies of the western honey bee, first described by Pollmann in 1879 [12]. Due to their relatively docile nature, good adaptation to extreme low temperatures, and abundant honey production in spring and summer (mainly produced on conifers), this honey bee is well received in many countries and is one of the popular bee varieties [13]. The content of flavonoids and phenols in Apis mellifera carnica propolis is very high, especially when compared to other cultivated bees such as Trigona sp.. Apis mellifera carnica propolis from Mojokerto has a high total flavonoid and phenol content compared to Trigona sp propolis from Mojokerto which has a low value of antioxidant activity, total flavonoids and total phenolics [14]. Therefore, in this study researchers used propolis derived from Apis mellifera carnica bees.

2. Materials and Methods

2.1 Study Design

This study used an in vitro experimental study carried out by the diffusion method. Observations made in this method were to measure the diameter of the inhibition zone of Candida glabrata which was cultured in 3 groups, namely negative control (KN), positive control (KP) and treatment (P). The negative control group (KN) was an isolate of C. glabrata culture on Mueller-Hinton agar plate + methylene blue + glucose media which was given a blank disk. Meanwhile, the positive control group (KP) consisted of C. glabrata culture isolates on Mueller-Hinton agar plate + methylene blue + glucose media that weas given a fluconazole disk. The treatment group (P) are C. glabrata culture isolates on Mueller-Hinton agar plate + methylene blue + glucose media were given a disc dripping with propolis extract Apis mellifera carnica with a concentration of 50% w/v (P1), 75%b/v (P2), and 100%b/v (P3). All groups were then incubated for 1 day at a temperature of 37^{o}C.Then the diameter of the inhibition zone was measured using a ruler or caliper and compared the results of the 3 groups. Stored isolates of C. glabrata used in this study were obtained from the Department of Microbiology, Faculty of Medicine, Airlangga University. Meanwhile, Apis mellifera carnica raw propolis was obtained from Wonorejo Pasuruan Village, East Java, Indonesia.

2.2 Statistical Analysis

The data analysis used was to look at the comparison of the inhibition diameter of Candida glabrata on Meller-Hinton agar + methylene blue + glucose media which was given a disk dripped with Apis mellifera carnica extract and as a comparison control was Candida glabrata on Mueller-Hinton Agar Plate media which was given a disk. fluconazole. Data analysis will be assessed statistically using descriptive analysis method. The descriptive analysis method was then analyzed using the SPSS 16.0 statistical software program. The data obtained were tested for normality and homogeneity first. Then statistical tests were carried out using the Kruskal-Wallis test method and continued with the Mann-Whitney test to determine the comparison between treatments. Results are said to be meaningful if $p<0,05$.

2.3 Ethical Acceptance

Approval for this research has been obtained from the Committee of Health Research, Faculty of Medicine, Universitas Airlangga (No. 211/EC/KEPK/FKUA/2021)

3. Results

The test results for the propolis extract content of Apis mellifera carnica can be seen in table 1 below.

Table 1 Test Results of propolis extract content of Apis mellifera carnica

Compound Identification	Parameter	Results
Flavonoids	Orange, Brick Red, Pink, Dark Red	(+) Positive
Tannins / Phenols	Dark Brown, Dark Blue	(+) Positive
terpenoids		
Steroids	Bluish Green	(-) Negative
Triterpenoids	Orange, Orange Brown	(+) Positive

Comparison of the average diameter of the Candida glabrata inhibition zone between groups can be seen in table 2 and figure 1 below.

Table 2 Diameter of antifungal inhibition zone of Apis mellifera carnica propolis extract against Candida glabrata (mm)

Diameter of Antifungal Inhibition Zone					
Sample	**Propolis extract concentration**			**Control**	**Control**
	P1 (50%w/v)	**P2 (75%w/v)**	**P3 (100%b/v)**	**(+)**	**(-)**
1	9,22	8,11	7,32	23.88	0.00
2	8.02	9.94	7,32	21.04	0.00
3	8.37	10.55	8.50	16.30	0.00
4	8,83	11.30	7,14	18.00	0.00
5	9,82	8,10	8,26	16,20	0.00
Average	8.85	9.60	7,71	19.08	0.00

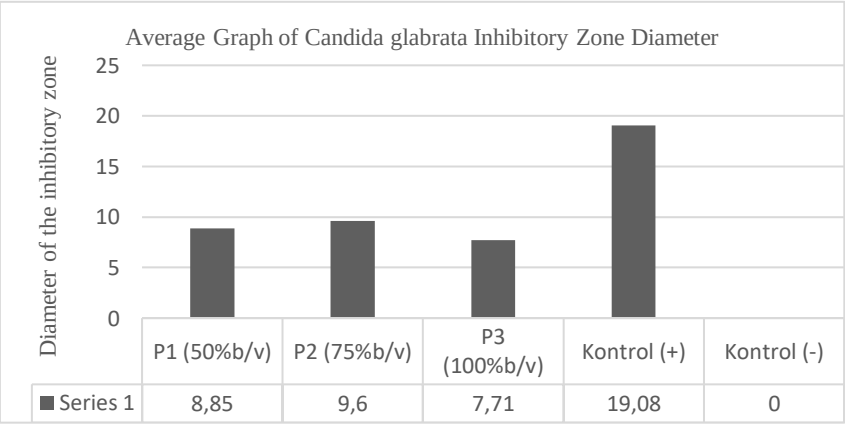

Figure 1 Graph of the results of measuring the diameter of the inhibition zone of Apis mellifera carnica propolis extract

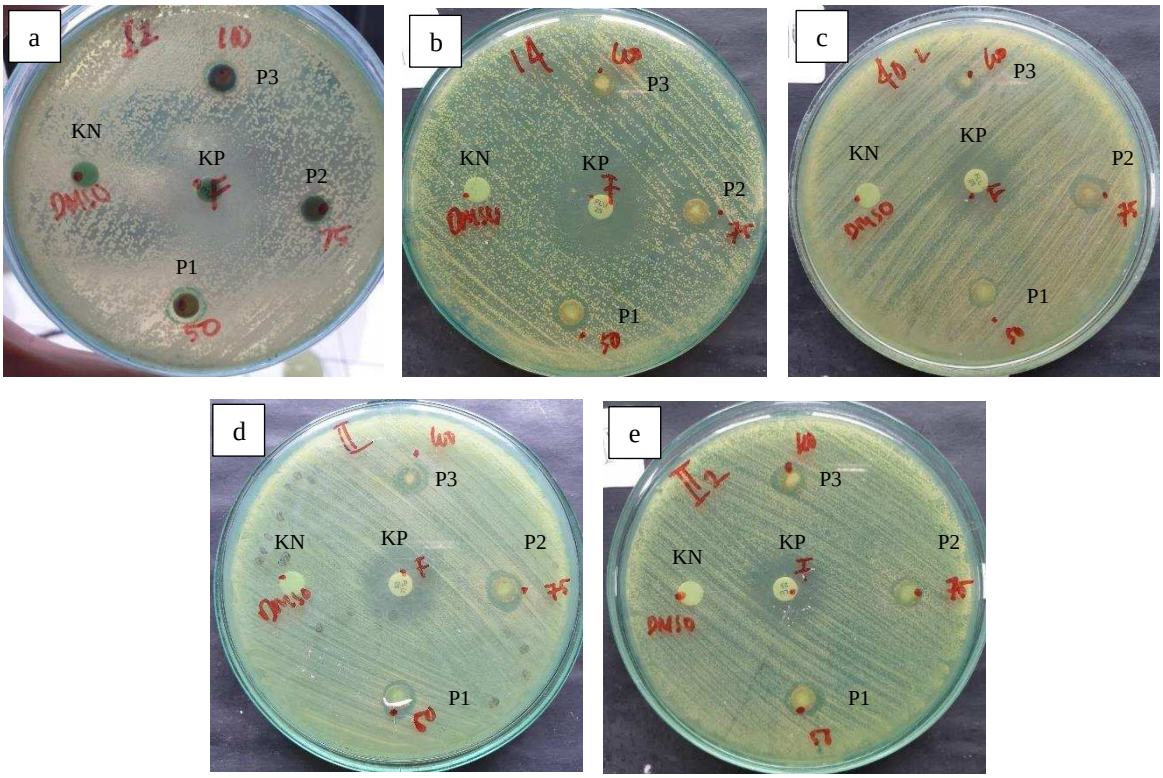

Figure 2 Comparative Picture of Inhibitory Zone Diameter of negative Control (KN), propolis extract with 50%w/v concentration as treatment 1 (P1), propolis extract with 75%w/v concentration as treatment 2 (P2), propolis extract with 100%w/v concentration as treatment 3 (P3) and Fluconazole as positive Control (KP).
(a) Sample 1; (b) Sample 2; (c) Sample 3; (d) Sample 4; (e) Sample 5

4. Discussion

In this study, Candida glabrata cultures were divided into 5 groups, each of which was given a different treatment. Each treatment group (P1, P2, P3) was given propolis extract with a concentration of 50%w/v, 75%w/v, 100%w/v. The negative control group used DMSO and the positive control used Fluconazole. The treatment groups starting from concentrations of 50%w/v, 75%w/v, and 100%w/v respectively produced an average inhibition zone of 8.85 mm, 9.60 mm and 7.71 mm. Antifungal activity is categorized as having low sensitivity if the diameter is 6-9 mm, then categorized as moderate if the diameter is between 9-12 mm and categorized as high sensitivity if the inhibition zone reaches >12 mm [15].

There was an increase in inhibition in the administration of propolis with a concentration of 75% w/v where the average inhibition zone was 9.60 mm. In contrast, there was a decrease in inhibition of propolis with a concentration of 100% w/v where the average inhibition zone was 7.71 mm. This is in accordance with previous research which examined the antifungal activity of propolis on C. albicans which showed inhibition and increased inhibition in the administration of 50% w/v propolis, which was an average of 11.7 mm, reaching a peak at a concentration of 80% w/v then decreased at a concentration of 100% w/v to 10.3 mm and 9.3 mm [16].

The decrease in the diameter of the inhibition zone in propolis with a concentration of 100% w/v was caused by the extract being too concentrated, which limited the ability of the extract to diffuse into the media [17]. When the concentration is higher, the bonds between molecules will be stronger, causing the size of the active compound to become larger [17]. This causes the molecules in Apis mellifera canrnica propolis extract to be unable to penetrate the pores of the agar medium so that the process of destruction of the fungal cell membrane by the active compounds contained in propolis is not maximized [17]. In this study it was found that the addition of the concentration of propolis extract did not always result in a larger diameter of the inhibition zone which means that the increase in the concentration of the extract was not always able to inhibit the growth of Candida glabrata.

Propolis extract has chemical compounds that have antifungal activity which can inhibit the growth of the fungus Candida glabrata. The main components of propolis are phenolic acids, flavonoids, terpenoids and tannins [18]. The flavonoid content in propolis inhibits fungal growth through different basic mechanisms including plasma membrane disruption, induction of mitochondrial dysfunction, and inhibition of cell wall formation, cell division, RNA and protein synthesis, and flow-mediated pumping systems [10]. Caffeic Acid Phenethyl Ester (CAPE) is one of the largest compounds in propolis and is a type of phenolic acid [19]. Phenolic acid is a very potent inhibitor of 12-lipoxygenase, where

Candida sprequires lipoxygenase for the enzymatic pathway to enter human endothelial cells [19]. Terpenoid antifungal activity induces linalool and LA in the G1 phase, citral and citronellal in the S phase, and benzyl benzoate in the G2-M phase, causing fungal cell death [20]. Ellagitanin and corilagin which are tannin compounds have the same activity as amphotericin B and ketoconazole against Candida glabrata [21]. The propolis extract used in this study came from Apis mellifera carnica bees where the compounds contained in it were not much different compared to propolis extracts in general, including phenolic acids, flavooids, terpenoids, and tannins which these compounds have the ability to inhibit fungal growth.

In this study fluconazole was used as a positive control. The use of fluconazole as a positive control is due to the similar mechanism of action of the chemical compounds contained in propolis extract where propolis content such as flavonoids, terpenoids, tannins and phenolic acids can also damage and disrupt the cell membrane structure of the Candida glabrata fungus [22] [23] [24] .

Fluconazole is a chemical synthetic drug that has been shown to have an antifungal effect, one of which is Candida glabrata. In this study, the effect of fluconazole was seen by inhibiting the growth diameter of Candida glabrata on agar media. This can happen because fluconazole works by inhibiting ergosterol synthesis to increase cellular permeability. Fluconazole interacts with 14-demethylase, a cytochrome P-450 enzyme responsible for catalyzing the conversion of lanosterol to ergosterol [25]. Ergosterol forms an important part of the fungal cell membrane, a decrease in ergosterol which is the main sterol to maintain membrane integrity causes the fungal cell wall to become permeable and the destruction of the fungus occurs [26].

Based on the research results, it was found that the mean diameter of inhibition of fluconazole against Candida glabrata was 19.08 mm which according to interpretation standards of the diameter of the inhibition zone and MIC Breakpoint were equivalent for Candida spp classified as susceptible or sensitive. Fluconazole is considered susceptible or sensitive if its diameter of inhibition is > 19 mm. However, these results can be said to be closer to SSD (Susceptible Dose Dependent). This is in accordance with previous studies where C. glabrata has emerged as the main cause of azole, echinocandin, and multidrug adaptive resistance (MDR: azole + echinocandin) [4].

In this study, Apis mellifera carnica propolis extract when compared to fluconazole had a significantly different effect where as Apis mellifera carnica propolis extract had no better effect when compared to fluconazole. The average diameter of the inhibition zone of propolis extract in this study was the largest at a concentration of 75% w/v, which was 9.60 mm, which was lower than the average inhibition zone for fluconazole, which was 19.08 mm. The reason for these results is possible because of the many active compounds contained in the propolis extract where each active compound has a different mechanism of action which can synergize or contradict each other in inhibiting the growth of Candida glabrata. In research with the diffusion method like this study,

5. Conclusion

In this study it can be concluded that the propolis extract of Apis mellifera carnica has an antifungal effect against Candida glabrata. The concentration of propolis extract of Apis mellifera carnica has the greatest antifungal effect at a concentration of 75%w/v and the smallest at a concentration of 100%w/v. The antifungal effect of propolis extract Apis mellifera carnica against Candida glabrata is not better when compared to fluconazole.

Acknowledgments

The researchers would like to thank the Laboratory of the Microbiology Unit, Department of Microbiology, Faculty of Medicine, Airlangga University for technical assistance during the research.

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