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Exploration of the Antifungal Potential of Indonesian Propolis from *Tetragonula biroi* Bee on *Candida sp.* and *Cryptococcus neoformans*

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Abstract: Fungal infection is one of the existing diseases in Indonesia. The most common fungal infections are the candidiasis and cryptococcosis disease which is caused by *Candida sp*. and *Cryptococcus sp*. fungi respectively. Propolis is known to have antifungal properties to *Candida sp.* and *Cryptococcus sp*. However, the compounds contained in propolis differs according to its source; the bee that produces it, and its environment, differentiating also its antifungal potential. The aim of this study is to determine the potential of Indonesian propolis, specifically from *Tetragonula biroi* bee as an antifungal agent in hopes to discover its ability as candidiasis and cryptococcosis drugs. The propolis used in the study was Indonesian propolis produced by *Tetragonula biroi* bee. The antifungal potential of Indonesian propolis was discovered by observing its antifungal activity to a few species of Candida and Cryptococcus and determining its content. Two types of Indonesian propolis, smooth and rough propolis in the form of ethanol extract propolis (EEP) were tested on *Candida albicans, C. krusei, C. glabrata, C. parapsilosis, C. tropicalis,* and *Cryptococcus neoformans* with the disc diffusion method. Ethanolic extract of Indonesian propolis is further tested with LC-MS/MS analysis. The content of Indonesian propolis itself in the form of polyphenol and flavonoid is discovered with the help of UV-Vis Spectrometry where it was discovered that smooth propolis has higher phenolic and flavonoid level at 18.32% and 17.45% respectively. Ethanolic extract of Indonesian propolis is further tested with LC-MS/MS procedure which results in the founding of three antifungal compounds. Adhyperforin was found in both rough and smooth propolis, and deoxypodophyllotoxin, as well as kurarinone, was found only in smooth propolis. Based on our study, Indonesian propolis is proven to have antifungal potential though its effectivity differs from one species of fungi to another where on *Candida albicans, C. krusei, C.tropicalis, and Candida glabrata*, rough propolis shows the higher diameter of inhibition.

Keywords: *Candida sp.*; *Cryptococcus sp.*; disc diffusion; fungal infection, propolis

1. Introduction

Fungal infection based on the site is divided into a superficial, cutaneous, subcutaneous, and systemic (deep) infection. Candidiasis is a fungal infection caused by *Candida albicans* and other *Candida spp.* which infects immunocompromised hosts. Candidiasis infection range from superficial that involves oral cavity, esophagus, intestine, vagina, and other epidermal and mucosal surface, to deep infection involving kidney, liver, brain, eye, heart, and other major organ tissues [1,2)]. Cryptococcosis mostly

caused by *Cryptococcus neoformans*, like candidiasis, range from cutaneous to systemic infection and infects immunosuppressed patients, especially those with HIV/AIDS infection [3].

Favorable sites for penetration of Candida species usually revolves around the oral mucosa and gastrointestinal tract through gastric mucosal layers and subsequently disseminated candidiasis in immunocompromised patients also in case of neonatal care units, Candida-related bloodstream infections are very frequent [4,5]. Studies on immunocompromised patients demonstrated culture-proven disseminated candidiasis due to *Candida albicans* or *C. tropicalis* infection with the involvement of the gastrointestinal tract [6]. In comparison to other Candida species, *Candida parapsilosis* has an extensive distribution in nature. Unlike *C. albicans* and *C. tropicalis*, *C. parapsilosis* is not an obligate human pathogen, having been isolated from nonhuman sources. This pathogen has a high affinity for parenteral nutrition, frequently colonizes the hands of health care workers, and forms biofilm on prosthetic surfaces and central venous catheters [7]. *Candida krusei* and *C. glabarata*, as one of the Candida species, are also found in systemic infections although its prevalence isn't as high as *C. albicans* [8]. The *C. krusei* infection is notable in ocular manifestation, endophthalmitis [9].

Cryptococcus neoformans is a basidiomycetous yeast which is haploid in nature and frequently comes into contact with a human, therefore becoming one of the prominent fungal pathogens. The immunocompetent individuals are able to control and contain the infection and do not develop cryptococcosis. However, in immunocompromised patients, *C. neoformans* can cross the blood-brain barrier and infect the brain, which leads to the development of meningitis [9].

In Indonesia, over 5.3 million people are infected with fungi in any given year [10]. In Jakarta alone, 21.9% of AIDS patients suffer from Cryptococcosis [11]. Meanwhile, in a RSCM hospital Jakarta, Invasive Candidiasis prevalence was 12,3% and caused mortality due to *C. albicans*, the most common etiologic pathogen [12]. Walangare (2014) found that *C. albicans* as a causative agent of oral candidiasis in HIV&AIDS patient in RSUD Dr. Saiful Anwar Malang, Indonesia [13].

Fungal infections have been treated with drugs by topical, oral, or intravenous preparation. However, fungal has its own resistance and increase in its resistance to reduce the effectiveness of existing drugs [14,15]. Propolis is a product from a beehive that is known to have antifungal and antimicrobial properties with its phenolic and flavonoid content [16,17]. However, propolis from different origins have a different composition and therefore the potential of one type of propolis is different from the other type [18]. Indonesia itself is a tropical country with a great potential for propolis and therefore propolis is available in large quantity. However, there are still very few studies focusing on the potential of Indonesian propolis.

Our study is done to determine the potential of Indonesian propolis, specifically from *Tetragonula biroi* bee as an antifungal agent in hopes to discover its ability as candidiasis and cryptococcosis drugs [19,20]. The quantitative polyphenol and flavonoid content are observed as a base for its antifungal potential and its own potential is further studied by testing the sample onto the cause of cryptococcosis, *Cryptococcus neoformans*, and the fungi causing candidiasis; *Candida albicans, C. krusei, C. parapsilosis, C. tropicalis*, and *C. glabrata*.

2. Materials and Method

2.1. Propolis Sample

The study uses Indonesian propolis from the *Tetragonula biroi* bee in the form of ethanol extract propolis (EEP) where the raw propolis itself is obtained from RIN Biotek Indonesia. The propolis used is differentiated into two categories based on the origin itself, texture, and also color. The first propolis is the regular propolis also known as smooth propolis, originating from inside the beehive with a softer texture and darker color. The second type of propolis which originates from the outer part of the beehive is known as rough propolis with a harder, rock-like texture, and lighter color. Propolis from raw propolis itself was extracted with the Muhamad Sahlan method where 1 kg of the raw propolis was macerated with 96% ethanol (5 L) [21,22]. The mixture was then left to age for 16 hours and went through filtration. Water was then added to dilute the mixture until it reaches 70% ethanol-water v/v which was then incubated in 50℃ water for 30 minutes (min). Left to age overnight in the freezer, the wax (bottom product) of propolis and the propolis itself was then separated by filtration. The filtrate was then evaporated using rotary vacuum evaporator (Rotavapor R-205, Büchi, Switzerland) which results in the propolis used as the sample, a highly viscous residue.

2.2. Microorganism

The fungal sample used in the study was obtained from the stock culture of the Department of Parasitology, Faculty of Medicine, Universitas Indonesia. The study uses six types of fungi. *Candida albicans, C. tropicalis, C. krusei, C. parapsilosis, C. glabrata*, and *Cryptococcus neoformans*. Each stock culture used has a minimum age of 48 hours (h).

2.3. Antifungal Test

The test was done with the disc diffusion method described by Silici (2006) and Pereira et al (2013) with slight modifications on the six types of fungi [23,24]. Each type of fungi was prepared for later inoculation to the Muller Hinton medium in the petri dish. The preparation was done to obtain standard turbidity, McFarland 0.5 which is equal to a concentration of 1.5 x 108 / ml cell

density. The fungi were then transferred to its medium using the counter strike method. As the fungi not only consist of the five different species of Candida but also *Cryptococcus neoformans*, for positive control, fluconazole is used. Blank discs were later soaked in extract ethanol propolis with 3 different concentration, 1 5 and 7% (%w/v) before being used. After 48 h of incubation at room temperature, the diameter of inhibition was measured using vernier caliper.

2.4. Polyphenol and Flavonoid Content with Spectrometry

Propolis sample of both types was prepared by dissolving 50 mg of propolis with ethanol until it reaches a concentration of 1000 ppm. The phenolic content of propolis was determined using the Folin and Ciocalteau reagent method with slight modifications [25]. The aluminium chloride colorimetric method was used to determine the flavonoid content [26,27].

2.4.1. Polyphenol Content

Quantitative test on polyphenol content was done with the help of Foline Ciocalteau reagent and Na_2CO_3. Gallic acid (50 mg) is used as the standard where it was dissolved with methanol until it reaches a concentration of 1000 ppm which was then further diluted with water until it reaches concentrations of 50, 100, 150, 200, 250 and 300 ppm. Propolis sample (0.5 ml) and 0.5 ml of each gallic acid with different concentration were taken and then added with 5 ml of the foline reagent with the help of vortex and let to mix thoroughly for five minutes. Na_2CO_3 1M (4 ml) was then added to the mixture and left to incubate for fifteen minutes. The resulting mixture was then measured with UV-VIS spectrometer at the wavelength of 765 nm. The measurement was done in triplicate.

2.4.2. Flavonoid Content

Quercetin (10 mg), a type of flavonoid is used as the standard where it was dissolved with methanol until it reaches a concentration of 1000 ppm which was then further diluted with water until it reaches concentrations of 12.5, 25, 50, 75, 100, and 200 ppm. Propolis sample (0.5 ml) and 0.5 ml of each quercetin with different concentration were taken and then added with 1.5 ml of methanol, 0.1 ml of aluminum chloride 10%, 0.1 ml of KCH_3COO 1M, and 2.8 ml of water. The solution was then left to incubate for 30 min. The resulting mixture was afterward measured with UV-VIS spectrometer at the wavelength of 415 nm. The measurement was done 3 times.

2.5. Chemical Antifungal

Compounds in Propolis with LC-MS/MS Spectrometry. The antifungal compounds in propolis were found with the help of LC-MS/MS spectrometry with slight modifications [21]. The type of LC-MS used was UPLC-TOF-MS. The instrument used was ACQUITY UPLC H-class system (Waters, US) with a 2.1 x 50 mm ACQUITY UPLC BEH C18 (Waters, US) column and Xevo G2-ST Qtof (Waters, US) mass spectrometer. With a flow rate of 0.2mL/min at 50°C column temperature, the solvents added to the column were 0.05% (w/v) of formic acid in water (A) as well as 0.05% of formic acid in acetonitrile (B) with different compositions over time. Ionization in the mass spectrometer was performed by electrospray ionization (ESI) in the positive mode. Data was yielded and processed using the software MassLynx (Waters). Composition of LC-MS/MS solvents over time was consisted of A: B as follows: t= 0 min 95% A; t= 3 min 75% A; t= 14 min 0% A; t= 19 min 95% A.

3. Result and Discussion

3.1. Quantitative Polyphenol and Flavonoid Content

The test was done using the UV-Vis spectroscopy where quercetin is used as the standard for flavonoid testing and gallic acid is used as the standard for polyphenol testing. From the standard concentrations, a linear equation for each standard was made from the standard plotted curve where concentrations provide as the x-axis and absorbance is the y-axis. The linear equation for polyphenol using the gallic acid standard is $y = 0.0052x + 0.1861$ with $R^2 = 0.9911$; the linear equation for flavonoid using the quercetin standard is $y = 0.0068x – 0.0081$ with $R^2 = 0.9986$.

Both smooth and rough propolis in a concentration of 200 ppm were measured three times, resulting in absorbance data which were later averaged and vulnerable to error as visualized in Figure 1. From the data, it is known that smooth propolis has an average of 18.32% polyphenol and rough propolis has an average of 14.72% polyphenol. On the other hand, the flavonoid content of smooth propolis has an average of 17.45% and rough propolis has an average of 7.83%. This data, as well as Figure 1, shows that smooth propolis contains higher flavonoid and polyphenol content than rough propolis, testifying that even from the same beehive, propolis can have different characteristic, content, and properties. In this case, propolis originating from inside the hive, smooth propolis, is higher in polyphenol and flavonoid content than the propolis originating from outside the hive.

Indonesian propolis from *Tetragonula biroi* bee has polyphenol content of 14.72-18.32% which also translates as having 29.44 - 36.71 µg/ml phenols in 200 µg/ml propolis. This is in range with Malaysian propolis from *Heterotrigona itama* bee having phenolic content of 29.1 µg/ml, and lower than the Malaysian propolis from *Geniotrigona thoracica* bee with phenolic content of 56.9 µg/ml [28]. Meanwhile, the flavonoid content of Indonesian propolis is more superior compared propolis from other countries as Indonesian propolis has 7.83-17.45% of flavonoid when compared to Taiwanese propolis with

2.82-3.07% flavonoid, Brazil with 3.26% flavonoid, and China with 5.37-7.73% flavonoid [27].

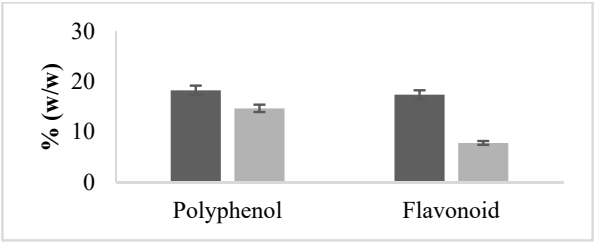

Fig. 1. Polyphenol and flavonoid content in smooth and rough propolis. Dark-grey indicates smooth propolis and light-grey indicates rough propolis.

3.2. Antifungal Test

The result of antifungal test shown in Figure 2 and 3 is available after 48 h of incubation in room temperature.

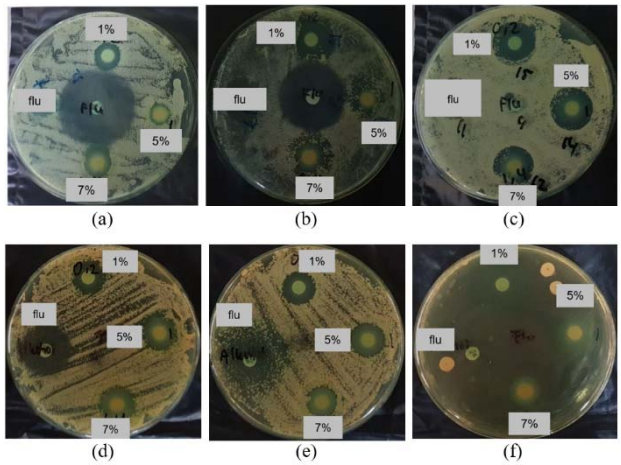

Fig. 2. Diameter of inhibition zone using rough propolis on (a) *Candida albicans* (b) *C. tropicalis* (c) *C.krusei,* (d) *C. parapsilosis* (e) *C. glabrata* (f) *Cryptococcus neoformans*

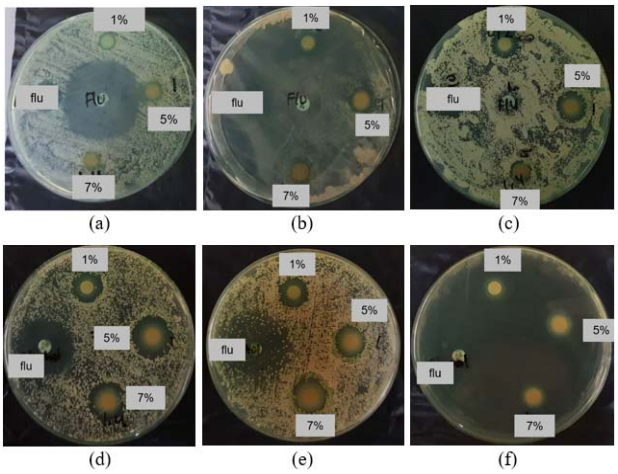

Fig. 3. The diameter of inhibition zone using smooth propolis on (a) *Candida albicans* (b) *C. tropicalis* (c) *C. krusei,* (d) *C. parapsilosis* (e) *C. glabrata* (f) *Cryptococcus neoformans*

The data obtained on the diameter of inhibition by fluconazole was divided into three categories based on its susceptibility: susceptible for diameter ≥ 19 mm; susceptible dose-dependent (SDD) for 15-18 mm; and resistant for ≤ 14 mm [29]. On the other hand, the interpretation of the disc diffusion result using propolis was done using the Stokes Disc Diffusion Technique to categorize the resulting data into sensitive, intermediate, and resistant. Sensitive is when the zone radius is ≤ 3 mm below the control. Intermediate is when the zone radius is ≥ 2 mm and is > 3 mm than control. While resistant has a zone radius < 2 mm [30].

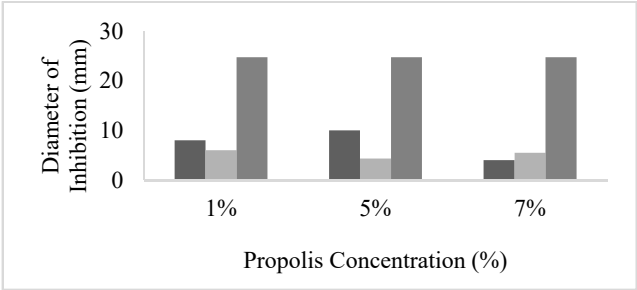

Fig.4. The diameter of inhibition on *Candida albicans*. Dark-grey indicates smooth propolis, light-grey indicates rough propolis, middle-grey indicates fluconazole as a control.

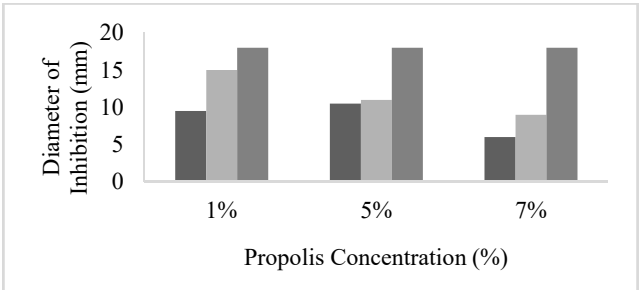

Fig. 5. The diameter of inhibition on Candida tropicalis. Dark-grey indicates smooth propolis, light-grey indicates rough propolis, middle-grey indicates fluconazole as a control.

The data of the diameter of inhibition from the antifungal test on Candida albicans is shown in Figure 4. The data shows that the diameter of inhibition of fluconazole on *Candida albicans* is 24.75 mm, therefore proving that fluconazole is susceptible to *Candida albicans*. The diameter of inhibition for smooth propolis reaches 8 mm for the concentration of 1%, 10 mm at 5% and decreases to 4 mm at 7%. For rough propolis, the diameter of inhibition is highest at 1% reaching 6 mm, then 4.3 mm at 5%, and increases back to 5.5 mm at 7%. For *Candida albicans*, smooth propolis has a higher inhibition diameter than rough propolis at the concentration of 1% and 5%, while at 7% rough propolis has a better result. As such, propolis although the resulting diameter of inhibition is relatively low, both smooth propolis and rough propolis are categorized to have intermediate susceptibility on *Candida albicans*.

Figure 5 shows the antifungal test results on *Candida tropicalis*. Fluconazole for the *Candida tropicalis* tested

has a diameter of inhibition in a value of 18 mm, therefore it signifies that fluconazole is susceptible dose dependent for *Candida tropicalis*. On *Candida tropicalis*, smooth propolis with a 1% concentration can inhibit its growth to 9.5 mm, smooth propolis with a 5% concentration inhibits to 10.5 mm, and at 7% smooth propolis inhibits to 6 mm. Rough propolis on *Candida tropicalis* has a diameter of inhibition 15 mm at 1% concentration, 11 mm at 5% concentration, and 9 mm at 7% concentration. From Figure 4 itself, it can be seen that rough propolis has a higher diameter of inhibition at all concentration compared to the smooth propolis. The diameter of inhibition shows that both type of propolis compared to the control has an intermediate susceptibility.

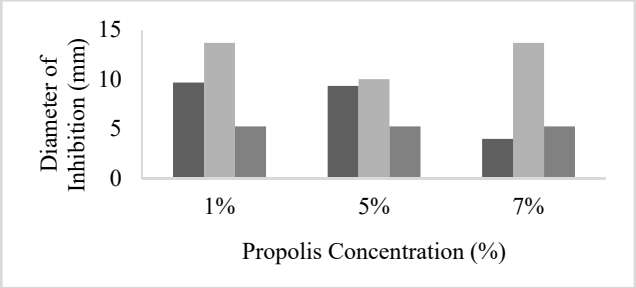

Fig. 6. The diameter of inhibition on *Candida krusei*. Dark-grey indicates smooth propolis, light-grey indicates rough propolis, middle-grey indicates fluconazole as a control.

C. krusei like other fungi is tested with smooth propolis, rough propolis, and fluconazole as shown in figure 6. From the data, it is shown that *C. krusei* is resistant to fluconazole as its diameter of inhibition only 5,25 mm on the disk tested. In this part of the test result, rough propolis also shows that its inhibition value on *C. krusei* is better than smooth propolis at all concentration. Smooth propolis has a diameter of inhibition of 9.67 mm at 1% concentration, 9.33 mm at 5% concentration, and 4 mm inhibition diameter at 7% concentration. Rough propolis which has a higher inhibition diameter than smooth propolis has it's a value of 13.67 mm at 1% concentration, 10 mm at 5% concentration, and back to 13.67 mm at 7% concentration. From the data, propolis is known to have better antifungal properties than fluconazole on *C. krusei*, except for the smooth propolis at 7% concentration.

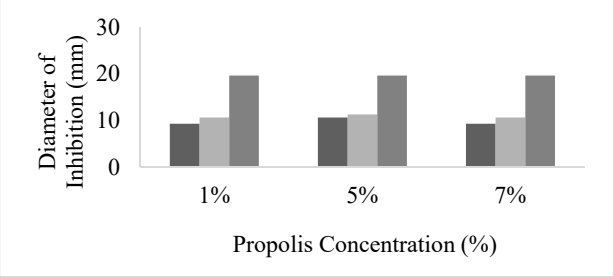

Fig.7. The diameter of inhibition on *Candida parapsilosis*. Dark-grey indicates smooth propolis, light-grey indicates rough propolis, middle-grey indicates fluconazole as a control.

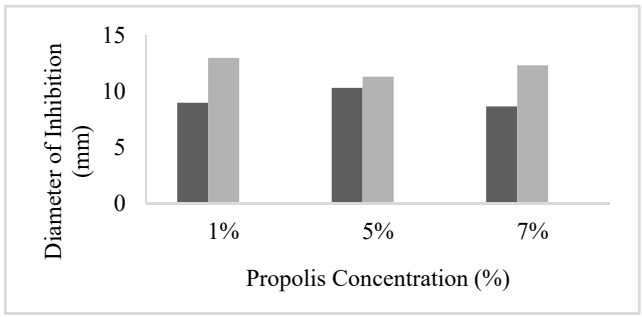

Fig. 8. The diameter of inhibition on *Candida glabrata*. Dark-grey indicates smooth propolis and light-grey indicates rough propolis.

Data on Figure 7 shows the inhibition diameter of propolis and fluconazole on *Candida parapsilosis*. Like on *Candida krusei* and *Candida tropicalis*, the data from the test done on *Candida parapsilosis* shows that rough propolis has better antifungal properties than smooth propolis. The fluconazole tested on *Candida parapsilosis* shows that it has a diameter of inhibition at 19.67 mm which indicates that *Candida parapsilosis* is susceptible to fluconazole. The smooth propolis on *Candida parapsilosis* at the concentration of 1%, produces a 9.33 mm diameter of inhibition; at the concentration of 5%, produces a 10.67 mm diameter; and at the concentration of 7%, produces a 9.33 mm diameter. Rough propolis on this test is able to inhibit *Candida parasilosis* to 10.67 mm at 1% concentration, 11.33 mm at 5% concentration, and 10.67 mm at 7% concentration. Compared to fluconazole, both smooth and rough propolis has an intermediate susceptibility on *Candida parapsilosis*.

As seen in Figure 8 which shows the result of the antifungal test on *Candida glabrata*, there is no bar showing the diameter of inhibition for fluconazole. This is because *Candida glabrata* is resistant to fluconazole that the control was not able to produce a diameter of inhibition as is referenced. In contrast to other *Candida* species, *C. glabrata* is not dimorphic; consequently, it is found as blastoconidia both as a commensal and as a pathogen. *C. glabrata* infections are difficult to treat and are often resistant to many azole antifungal agents [30]. However, propolis from this test has shown that it is able to inhibit the growth of *Candida glabrata*. Smooth propolis is able to inhibit the fungi to 9 mm at 1% concentration, 10.33 mm at 5% concentration, and 8.67% at 7% concentration. Rough propolis has a higher diameter of inhibition value where it reaches 13 mm at 1% concentration, 11.33 mm at 5% concentration, and 12.33 mm at 7% concentration. Therefore, it can be said that *Candida glabrata* has a higher susceptibility to rough propolis than smooth propolis.

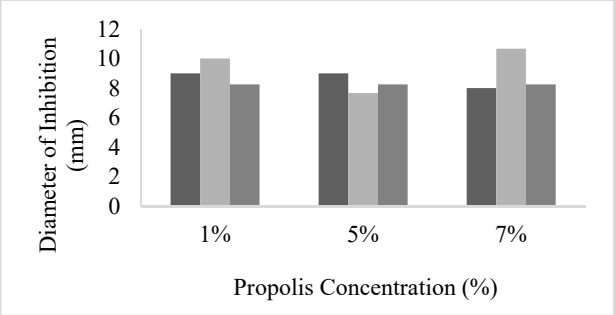

Fig 9. The diameter of inhibition on *Cryptococcus neoformans*. Dark-grey indicates smooth propolis, light-grey indicates rough propolis, middle-grey indicates fluconazole as a control.

Figure 9 shows the antifungal test result in *Cryptococcus neoformans*. On *Cryptococcus neoformans*, fluconazole has an inhibitory diameter of 8.25 mm which indicates that although there is still an inhibition zone, *Cryptococcus neoformans* is still considered to be resistant to fluconazole. Smooth propolis has a diameter of inhibition of 9 mm at the concentration of 1% and 5%, and 8 mm at the concentration of 7%. Rough propolis has a diameter of inhibition of 10 mm at 1% concentration, 7.67 mm at 5% concentration, and 10.67 mm at 7% concentration. From the test, rough propolis has a higher diameter at 1% and 7% concentration while at 5% concentration, smooth propolis has a higher concentration. As at 1% and 5% concentration smooth propolis has a higher diameter of inhibition than fluconazole, and at 7% concentration the difference between fluconazole and smooth propolis is only 0.25 mm, *Cryptococcus neoformans* can be categorized to have a sensitive susceptibility to smooth propolis when compared to fluconazole. This is the same on rough propolis as a concentration of 7% and 1%, rough propolis has a higher diameter of inhibition than fluconazole and at 5% concentration, the difference in diameter between fluconazole and rough propolis is only 0.58 mm.

From all the data above, it can be said that the difference of concentration at 1%, 5%, and 7% of both smooth and rough propolis does not affect the inhibition ability of propolis in a form of trend. This is because the concentration tested itself doesn't differ much and it may be that none of the concentration is the most effective concentration, thus further research is needed to know the most effective concentration. When compared between propolis, on most fungi; *Candida tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. glabrata*, rough propolis has a higher inhibitory diameter than smooth propolis.

3.3. Chemical Antifungal Compounds in Propolis

The LC-MS/MS produced spectras from which the elemental composition of the samples was analyzed. From that analysis was discovered several elements including flavonoids and other elements, some of which with antifungal properties. Three such compounds were found. In the ethanolic extract of both smooth and rough propolis, adhyperforin was found at retention time 15.14 minutes [30-33]. The intensity of this compound, however, differ in the two propolis types. The intensity of adhyperforin in rough propolis is higher at 15-20% compared to the 5-20% in smooth propolis, in accordance to its volume, 3.86% in the former, and 3.76% in the latter. While in the rough propolis, the only adhyperforin was found, in the smooth propolis, two other compounds were found, deoxypodophyllotoxin [34,35] at retention time 11.20 minutes and kurarinone [36–40] at retention time 13.05 minutes. Deoxypodophyllotoxin in smooth propolis has 5-10% intensity and a volume of 2.27%. Kurarinone has 15-20% intensity and 1.29% volume. From these compounds, it can be proved that ethanolic extracts of Indonesian propolis do have antifungal properties. In this particular study, however, the antifungal properties of ethanolic extract of propolis were tested on six different kinds of fungi in which the potency of each compound may differ on each kind. Therefore, a further study involving each of the compounds on the fungi is needed. Then we need study the influence of temperature and atmospheric moisture (water content) conditions for various fungi reproduce to fungal proliferation and colony formation[41]. Further research needs to be done on the content of propolis in other regions in Indonesia by utilizing internet technology[42]. So that we can obtain a database of active compounds contained in propolis from various regions in Indonesia.

Table 1. Antifungal Compounds Found in Propolis

Compound	Molecular Formula	m/z	Group	Plant of *genus*
Adhyper-forin	$C_{36}H_{54}O_5$	551,5042	Phloro-glucinol	*Hypericum*
Deoxypodo-phyllotoxin	$C_{22}H_{22}O_7$	399,1436	Lignan	*Podophy-llum*
Kurarinone	$C_{26}H_{30}O_6$	439,2092	Flavo-noid	*Sophora*

4. Conclusion

Indonesian propolis is proven to have antifungal properties as it is able to inhibit the growth of all the fungi tested; *Candida albicans, C. tropicalis, C. krusei, C. parapsilosis, C. glabrata*, and *Cryptococcus neoformans*. When the fungi tested is resistant to the control, both smooth and rough propolis is able to produce a higher inhibitory diameter than the control. As the compounds in smooth and rough propolis differ, most fungi are more susceptible to rough propolis than smooth propolis. The phenolic and flavonoid content of smooth propolis is however higher than of rough propolis. To know the differences in each of the active compounds as well as to determine the antifungal mechanism, ongoing study was conducted to identify the cell signaling involved in the process.

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