

# Phytochemical analysis and antifungal activity propolis Lombok against *Candida sp* and *Cryptococcus sp*

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**ABSTRACT:** Fungal infections become a serious problem, especially in developing countries. Antifungal drug classes that have developed shows some limitation due to poor selectivity, toxicity, and high resistance. Propolis is a natural resinous substance from flower buds, trees, and resinous exudates collected by bees. Propolis has several therapeutic properties such as antibacterial, anti-inflammatory, antifungal, and antiviral. This study explores antifungal activity of Ethanolic Extract of Propolis (EEP) Lombok against *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Cryptococcus neoformans*. Antifungal activity test were carried out with 2 different methods, agar diffusion and microdilution to see EEP Lombok's ability to inhibit fungal growth. In addition, this study analyzes phytochemical contents of Propolis Lombok by characterizing its substance and calculating the total phenolic content. The result shows the total polyphenol content of Lombok's EEP is around 222-278 mgGAE/g meanwhile total polyphenol content is 512-1100 mg QE/g. Based on the antifungal tests, propolis samples showed antifungal activity against *C. albicans*, *C. glabrata*, *C. krusei*, and *Cryptococcus neoformans* species. Propolis Lombok agar diffusion test showed an inhibition growth against *C. albicans*, *C. glabrata*, and *C. krusei*. While for microdilution test, propolis extract only affects *C. albicans*, *C. glabrata*, and *Cryptococcus neoformans* growth.

**KEYWORDS:** Propolis; Antifungal; Phytochemical Analysis; *Candida sp*; *Cryptococcus sp*.

## 1. INTRODUCTION

Propolis is a natural resin substance with various color and consistency depending on the resin. Propolis is produced by bees as a way to protect them from other insects and microorganisms[1]. Propolis has a lot of functions such as antibacterial, anti-inflammatory, antioxidant, and anticancer. Propolis is used as a traditional medicine in a number of nations, including Indonesia [2]. Beekeeping is starting to develop promptly in Indonesia, notably Lombok [3]. Based on a study conducted by Riendrisari and Krisnawati in 2017, Propolis Lombok production shows an increase from 2012 to 2014 [4]. Additionally, a prior study discovered a new compound in Propolis Lombok that possesses antibacterial activity against *S. aureus* and *E.coli* [5].

Fungal infections have grown in number and severity over the past decades. It becomes a major public health problem in the world, especially for developing countries [6,7]. Several species found in humans are *Candida sp*, *Cryptococcus sp*, *Aspergillus*, *Pneumocystis* [7]. Those fungi can be treated with several antifungal drugs such as polyenes, axoles, echinocandins, allyamines, and antifungal antimetabolites [8]. However, their use is limited due to poor selectivity, toxicity, and high resistance [6].

Fluconazole is one of the most prescribed drugs for candida infections [9]. This drug work as a fungistatic and has a higher risk of developing resistance than fungicidal medications [10]. Besides fluconazole, amphotericin B is also known for invasive fungal infections treatment caused by *Candida sp* and

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*Cryptococcus* sp [11, 12]. In this study, fluconazole and amphotericin B are used as a comparison with propolis as an antifungal agent.

On the other hand, antifungal agents that are derived from natural products, especially traditional medicines need to be studied for health matters in general [13]. In this regard, natural phenolics can be found in edible plants and are considered safe for humans [14]. Having said that, the significance to study natural antifungal is to prevent fungal growth in general.

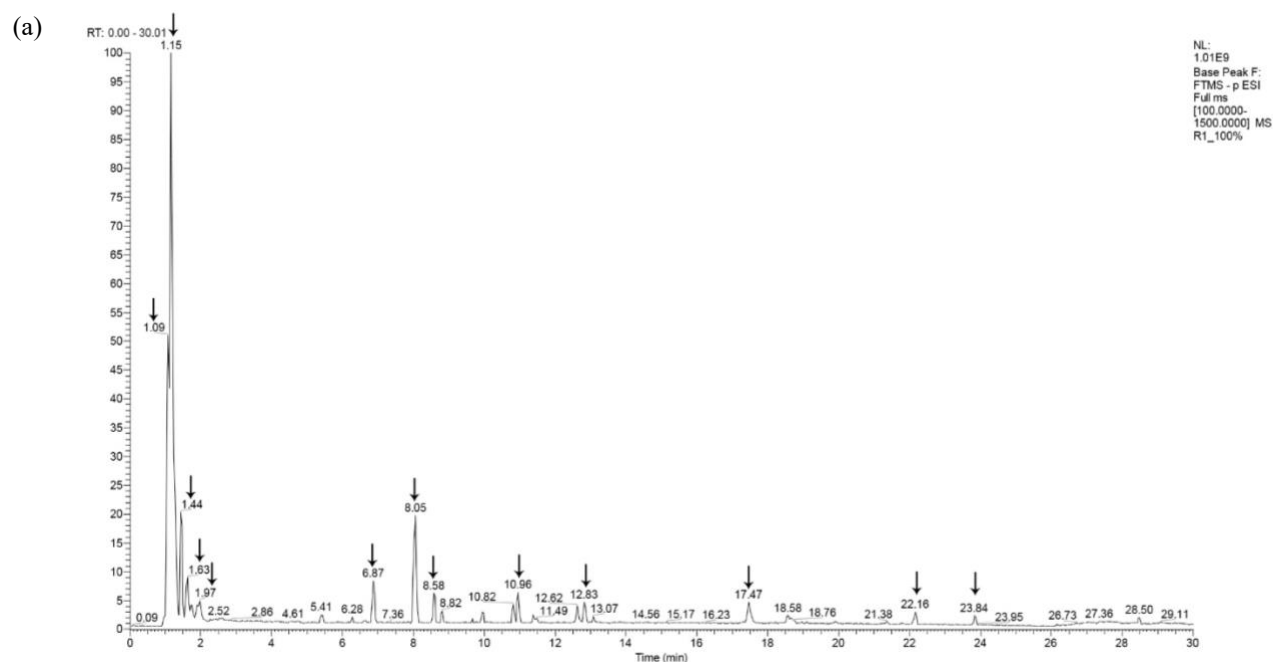
In order to reveal its significance, this study was conducted to show propolis Lombok antifungal potential to inhibit the growth of *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Cryptococcus neoformans*. The method is in vitro and studied the total content of polyphenols, flavonoids, and the identification of compounds using LC-MS/MS analysis of three samples of Propolis Lombok.

## 2. RESULTS

### 2.1. Phytochemical Analysis

Mass Spectrum detector as an LCMS-MS analysis method can detect compounds in a sample based on the ratio between mass per charge ( $m/z$ ) [15]. LCMS-MS spectrum modification and polyphenols site are shown in Figure 1. Spectrum data are analyzed using Compound Discover 3.2 software to identify polyphenol and flavonoid compound name, formula, molecular weight, retention time, and area. In this study, further analysis based on their classification, source, and function through online databases such as *Chemspider* and *PubChem* are performed on several highest peaks from each region.

In Propolis Rempek 110 molecules are detected and 65 compounds are identified. Based on further analysis 11 compounds were identified namely D-(-)-Mannitol, Gallic Acid Monohydrate, Methylmalonic acid, 3-Hydroxy-3-(methoxycarbonyl) pentanedioic acid, Mangiferin, Ellagic Acid, Salicylic acid, Apigenin, Asiatic Acid, Maslinic Acid, and 18- $\beta$ -Glycyrrhetic acid. Meanwhile in Propolis Sekotong 142 molecules detected and 81 compounds are identified. Twelve substances D-(-)-Mannitol, Citric acid, Gallic acid, Caffeic acid, Mangiferin, Paenol, Ellagic Acid, Luteolin, Asiatic Acid, 9,10-dihydroxystearic acid, Maslinic Acid, 18- $\beta$ -Glycyrrhetic acid are discovered after additional research. Lastly in Propolis Bayan 143 molecules detected and 83 compounds are identified. D-(+)-Galactose, Gallic acid, Aspirin, Rutin, Ellagic acid, Luteolin, Quercetin, 9,10-dihydroxystearic acid, -mangostin, 18- $\beta$ -Glycyrrhetic acid, and Maslinic Acid are the 11 compounds that analyzed. A total of 21 compounds in all Propolis Lombok sample are shown in Table 1.



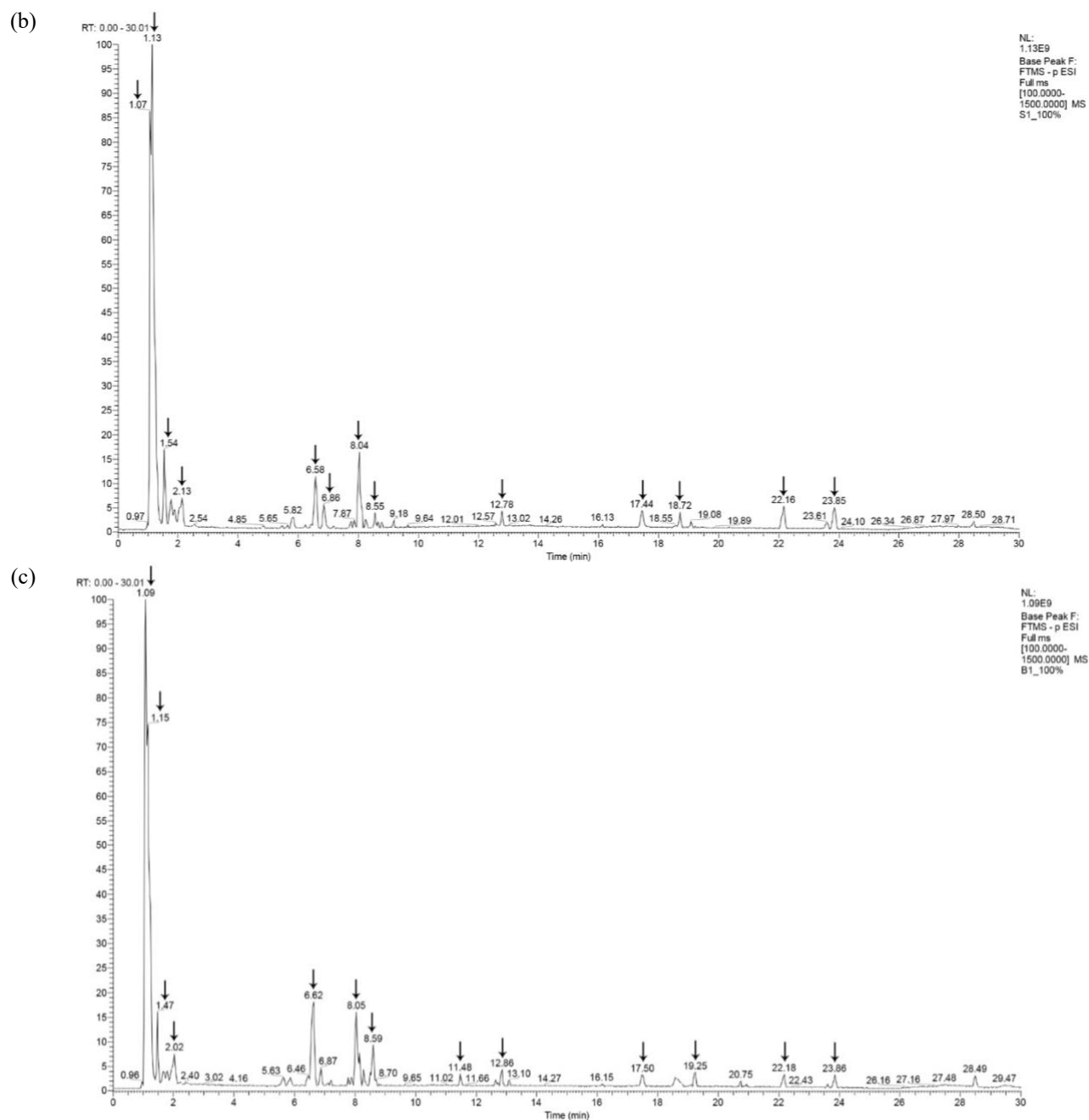


Figure 1. LCMS-MS Spectrum Propolis (a) Rempek, (b) Sekotong, (c) Bayan, Lombo

**Table 1.** Identified Propolis Lombok Compounds

Compounds	Chemical Formula	Groups	Sample Origin		
			Rempek	Sekotong	Bayan
D-(+)-Galactose	C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>	Alcohol and sugar			○
Gallic acid	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>	Polyphenol	○	○	○
3-Hydroxy-3-(methoxycarbonyl)pentanedioic acid	C <sub>7</sub> H <sub>10</sub> O <sub>7</sub>	Carbonyl acid	○		
Aspirin	C <sub>7</sub> H <sub>8</sub> O <sub>6</sub>	Polyphenol			○
Rutin	C <sub>27</sub> H <sub>30</sub> O <sub>16</sub>	Flavonoid			○
Ellagic acid	C <sub>14</sub> H <sub>6</sub> O <sub>8</sub>	Polyphenol	○	○	○
Luteolin	C <sub>15</sub> H <sub>10</sub> O <sub>6</sub>	Flavonoid		○	○
Quercetin	C <sub>15</sub> H <sub>10</sub> O <sub>7</sub>	Flavonoid			○
9,10-dihydroxystearic acid	C <sub>18</sub> H <sub>36</sub> O <sub>4</sub>	Hydroxy fatty acid		○	○
β-mangostin	C <sub>25</sub> H <sub>28</sub> O <sub>6</sub>	Xanthenes			○
18-β-Glycyrrhetic acid	C <sub>30</sub> H <sub>46</sub> O <sub>4</sub>	Triterpenoid	○	○	○
Maslinic Acid	C <sub>30</sub> H <sub>48</sub> O <sub>4</sub>	Triterpenoid	○	○	○
D-(-)-Mannitol	C <sub>6</sub> H <sub>14</sub> O <sub>6</sub>	Alcohol and sugar	○	○	
Citric acid	C <sub>6</sub> H <sub>8</sub> O <sub>7</sub>	Carboxylic acid		○	
Caffeic acid	C <sub>9</sub> H <sub>8</sub> O <sub>4</sub>	Polyphenol		○	
Paenol	C <sub>9</sub> H <sub>10</sub> O <sub>3</sub>	Polyphenol		○	
Mangiferin	C <sub>19</sub> H <sub>18</sub> O <sub>11</sub>	Xanthenes	○	○	
Salicylic acid	C <sub>7</sub> H <sub>6</sub> O <sub>3</sub>	Carboxylic acid	○		
Methylmalonic acid	C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	Carboxylic acid	○		
Apigenin	C <sub>15</sub> H <sub>10</sub> O <sub>5</sub>	Flavonoid	○		
Asiatic Acid	C <sub>30</sub> H <sub>48</sub> O <sub>5</sub>	Triterpenoid	○	○	

Total polyphenol content of Propolis Lombok is determined using a linear regression equation from gallic acid standard curve. The standard curve shows a relation between phenolic concentration (x-axis) with its absorbance (y-axis). The linear regression equation obtained is  $y = 0,0093x + 0,0392$  with  $R^2$  equals to 0.994. Total polyphenol content is converted to obtained total content in units mgGAE/g EEP. Whereas total flavonoid content are determined using quercetin standard solution with the derived linear regression equation is  $y = 0,0085x - 0,0021$  with an  $R^2$  value of 0.992. Final total flavonoid content data are in mgQE/g EEP units. Table 2 shows the calculation results of total polyphenol and flavonoid from 3 propolis Lombok samples.

**Table 2.** Total Phenolic and Flavonoid Content of Propolis Lombok

Sample	TPC (mg GAE/g)	TFC (mg QE/g)
R1	248.05±39.42	1023.71±140.15
S1	222.96±31.24	1104.22±73.90
B1	278.15±120.64	512.01±42.79

Based on the results, propolis from Bayan have the highest polyphenol content compared to Rempek and Sekotong which was 278.15±120.64 mg GAE/g ethanolic extract of propolis. Rempek sample have the highest total flavonoid content by 1023.71±140.15 mg QE/g. Meanwhile, Bayan has the smallest flavonoid content of 512.01±42.79 mg QE/g.

## 2.2. Antifungal test

As previously mentioned, two antifungal test methods are performed in this research, agar diffusion and microdilution. Three propolis concentrations used in the test are 50, 70, and 100%. Fluconazole and Amphotericin B were used as positive control whereas DMSO 10% was used as a negative control. The agar diffusion method shows fungal growth inhibition potential from propolis shown in Figure 2 and Table 3.

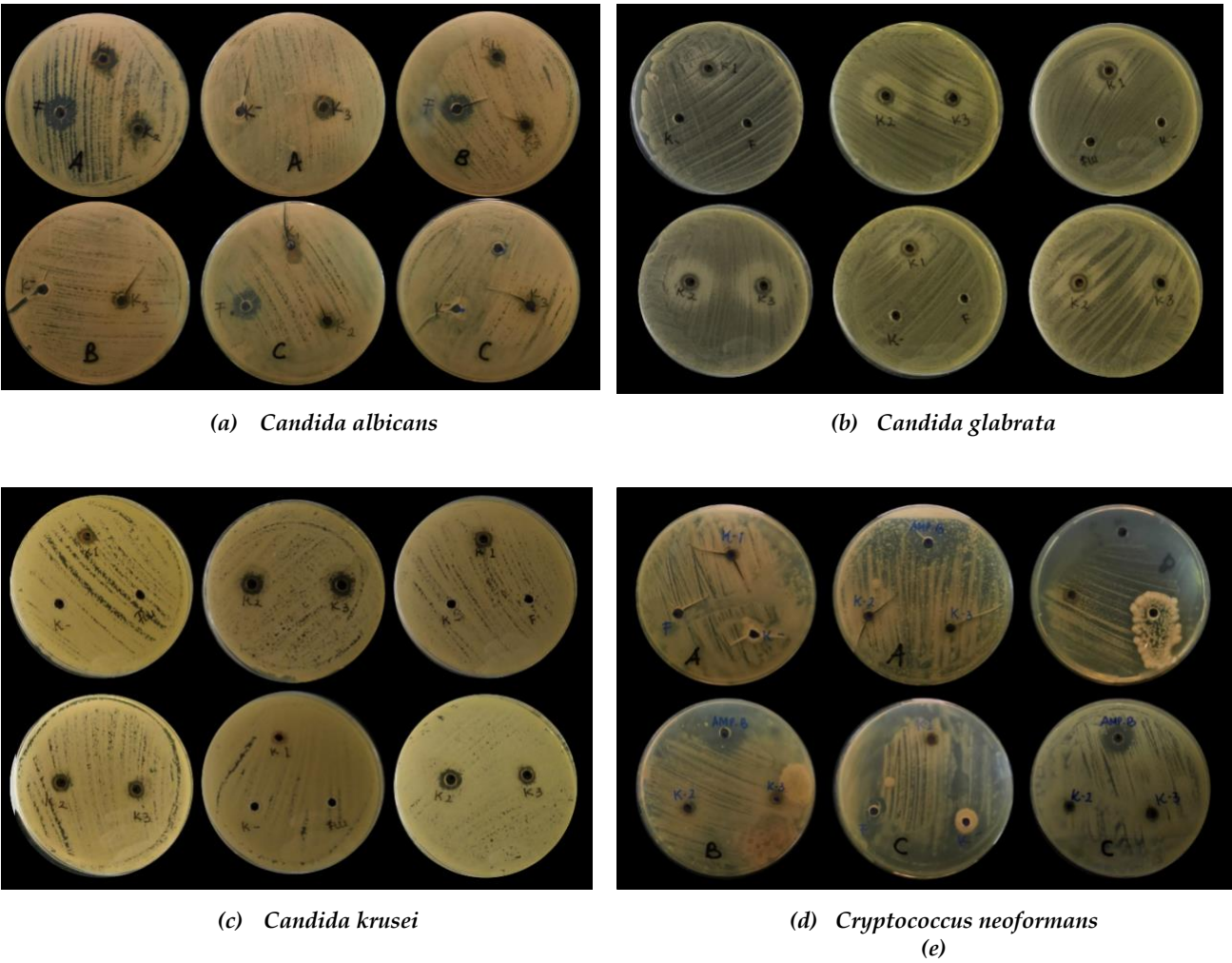


Figure 2. Agar Diffusion Fungal Culture Results

Table 3. Inhibition Zone (mm) Agar Diffusion Method on *C. albicans*, *C. glabrata*, *C.krusei*, and *C. neoformans*

Sample	Inhibition Zone (mm)			
	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C.krusei</i>	<i>C. neoformans</i>
Propolis 50%	7,93±6,99	10,0±1,0	5,67±4,93	0
Propolis 70%	9,33±2,25	10,0±0,0	9,67±0,58	0
Propolis 100%	10,4±1,97	9,67±1,15	9,33±1,53	0
DMSO 10%	0	0	0	0
Fluconazole	15,0±0,69	0	0	0
Amp B	-	-	-	20,0±0,0

Propolis with 100% concentration has the best inhibition zone on *Candida albicans* growth of 10.4±1.97 mm followed by propolis at 70% and 50%. However, compared to fluconazole, as a positive control, the inhibitory zone created by three doses of propolis was smaller. Propolis 50%, 70%, and 100% concentration had an effect on the growth of *Candida glabrata*. The best inhibition zone formed by propolis at 50% and 70% concentration, which was 10 mm. Antifungal potential of *C. krusei* was present in 50%, 70%, and 100% propolis samples, as shown by the existence of inhibitory zones respectively 5,67±4,93; 9,67±0,58; 9,33±1,53 mm. *Cryptococcus neoformans* agar diffusion tests show the presence of an inhibitory zone from amphotericin B, but there are no inhibition zones developed by all propolis samples.



**Table 4.** Fungal Growth Microdilution Data on *C. albicans*, *C. glabrata*, *C. krusei*, and *C. neoformans*

Fungal Species	Positive Control		Propolis 50%	Propolis 70%	Propolis 100%	Negative Control	Fungal Control	Media Control
	Flu	AmpB						
<i>Candida albicans</i>	-		-	-	-	+	+	-
<i>Candida glabrata</i>	+		+	-	+	+	+	-
<i>Candida krusei</i>	+		+	+	+	+	+	-
<i>Cryptococcus neoformans</i>	+	-	-	-	-	+	+	-

\*(+): fungal growth is detected after incubation; (-): fungal growth is not detected after incubation.

\*data is present as average data from three replications for each fungal species.

The data obtained in the microdilution test is the absorbance of the sample before and after incubation. Furthermore, this test also looks at fungal culture for each sample, to observe the possibility of any fungal growth shows in Table 4. Figure 3 shows example data from *Candida albicans* microdilution test, the right site shows the fluconazole effect, whereas left site is a propolis sample of 50%.



**Figure 3.** Microdilution test cultured fungi on SDA

The absorbance data for microdilution for *Candida albicans* decreased in each propolis sample. While using fluconazole as the positive control, the absorbance is increased. From the data culture, no fungal growth was detected in all propolis samples. Microdilution test on *Candida glabrata* shows that propolis Lombok decreased the absorbance after incubation process. Based on the culture results in several experimental samples, propolis can inhibit the growth of fungi when compared to the control. *Candida krusei* microdilution test shows a variety of data on absorbance before and after incubation. However, based on the culture results, fungal growth was found in the three concentrations of propolis samples. Lastly, *Cryptococcus neoformans* microdilution test shows in all propolis concentrations, there was a decrease in absorbance and no fungal growth was found. The culture findings obtained demonstrated no fungal growth in amphotericin B control, whereas growth of *Cryptococcus neoformans* continued in the fluconazole control.

### 3. DISCUSSION

Based on previous research, phytochemical compounds in propolis are in charge of antifungal activity on *Candida sp* [16]. Other research also says that flavonoid, saponin, and tannin compounds in propolis could inhibit fungal growth [17]. In this research, there are 21 compounds identified in Propolis Lombok that are classified as polyphenols, flavonoids, sugars, carboxylic acids, terpenoids, and others. The presence of those compounds shows the antifungal activity potential of Propolis Lombok.

Furthermore, biological antifungal activity propolis Lombok might be provided by gallic acid, ellagic acid, and caffeic acid compounds. Gallic acid was found in all 3 samples of propolis and acknowledged for its antifungal and antibacterial properties [18]. Besides gallic acid, ellagic acid is also found in all propolis samples are known for its potential as a natural antifungal agent [13]. Caffeic acid was found in propolis from Sekotong, and it is known for its fungistatic function and can interfere with biofilm structure [19].

Propolis Lombok shows the ability to inhibit *Candida albicans* growth in both methods, agar diffusion, and microdilution. Although Fluconazole gives a better inhibit zone,  $15,0 \pm 0,69$  mm compared to propolis Lombok  $10,4 \pm 1,97$  mm. Previous research done by Lutpiatina (2015) focused on the effectivity of *Trigona spp* propolis extract against *Salmonella typhi*, *Staphylococcus aureus*, and *Candida albicans*. The results show there are no inhibition zone formed by *Trigona spp* propolis extract against *Candida albicans* [20].

Positive results are given by Propolis Lombok against *Candida glabrata*. From agar diffusion data, all samples form an inhibit zone while positive control does not indicate an inhibition. For *Candida krusei*, propolis Lombok forms an inhibition zone, but from the microdilution test, fungal growth remains the same after propolis extraction treatment. This could happen because the inhibitory ability of the propolis sample is not proportional to the fungal growth that occurs. This finding shows a further potential of Propolis Lombok as a natural antifungal agent against *Candida krusei* especially due to the high level of resistance cases [21]. Lastly, in the agar diffusion method propolis extract does not produce an inhibitory zone, but in microdilution test, all propolis samples can inhibit fungal growth. Overall propolis Lombok shows a potential as a natural antifungal agent against *Candida albicans*, *Candida krusei*, *Candida glabrata*, and *Cryptococcus neoformans*. Additionally, further discussion regarding the appropriate formulation and lowest inhibitory concentration (MIC) of propolis Lombok as an antifungal agent is required.

#### 4. CONCLUSION

There are 21 compounds analyzed in Lombok Propolis, among these compounds, there are polyphenol, flavonoids, sugars, carboxylic acids, terpenoids, and others. There were 4 same compounds found in three samples of propolis Lombok namely gallic acid, ellagic acid, 18- $\beta$ -Glycyrrhetic acid, and maslinic acid. Lombok Bayan propolis has the best polyphenol content at  $278.15 \pm 120.64$  mg GAE/g, followed by Rempek at  $248.05 \pm 39.42$  mg GAE/g and Sekotong  $222.96 \pm 31.24$  mg GAE/g. The total flavonoid content in Lombok Rempek, Sekotong, and Bayan Propolis respectively was  $1023.71 \pm 140.15$ ;  $1104.22 \pm 73.90$ ;  $512.01 \pm 42.79$  mg QE/g. Based on the antifungal test results, propolis samples showed antifungal activity in *C. albicans*, *C. glabrata*, *C. krusei*, and *C. neoformans* species.

#### 5. MATERIALS AND METHODS

##### 5.1. Materials

Three samples of propolis was collected from different region in Lombok, Indonesia which was Bayan, Rempek (North Lombok), and Sekotong (West Lombok). Extract Ethanol Propolis (EEP) was prepared with maceration method according to Pratami (2017). 150 g of sample propolis mixed with ethanol in a ratio of 1:5 for 8 hours and left overnight. After that, the mixture is strained using Whatman filter paper. To obtain thick extract propolis, ethanol content in EEP is evaporated using rotary vacuum evaporator Buchi R-100.

##### 5.2. Phytochemical Analysis Propolis Lombok

In this study, two different analyses are conducted to characterize and quantify propolis Lombok compounds. The characterization of propolis Lombok was carried out using LCMS-MS (UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS ThermoScientif) analysis performed by Advanced Research Laboratory IPB University, Bogor, West Java. Formic acid and acetonitrile are use as eluent phase at flowrate 0,2 ml/min for 30 min. Mass spectrum data was analyzed using Compound Discover 3.2 then compare the data and with m/z ratio database.

Besides LCMS-MS, total phenolics and flavonoid content are also quantified in this study. Total phenolic content is tested using Folin Ciocalteu and  $\text{Na}_2\text{CO}_3$  reagents with gallic acid as the standard solution. First, 0.5 ml of EEP were pipetted to each test tube and then mixed with 5 ml of Folin Ciocalteu reagent using a vortex. Next step is to add 4 ml of 1M  $\text{Na}_2\text{CO}_3$  to the mixture and vortexed. Then the mixture absorbance was measured at a wavelength of 765 nm.

Total flavonoid content are quantified using quercetin standard solution. First, 0.5 ml of EEP are mixed with 1.5 mL of methanol, 0.1 mL of 10%  $\text{AlCl}_3$ , 0.1 mL of  $\text{KCH}_3\text{COO}$  1M, and 2.8 mL of water. The absorbance of the mixture was measured at 415 nm wavelength to quantify flavonoid content. This

measurement are done to quantify total phenolic compounds that carried out using UV-Vis Spectrophotometry.

### 5.3. Antifungal Activity

There are two methods of antifungal testing carried out in this study, namely agar diffusion and microdilution. Antifungal properties of propolis were tested at several concentrations, which are 100%, 70%, and 50%. This study employs fluconazole and amphotericin B as a positive control also DMSO 10% as a positive control.

The agar diffusion method uses mulle Hinton media by adding 266.5 mg mulle Hinton medium powder to 4000  $\mu$ L aquadest. Then the mixture is sterilized using an autoclave, put in a petri dish, and leave until it is set. Cultured fungi were transferred into a test tube filled with sterilized distilled water to reach standard turbidity, McFarland 0.5. After that, the fungi are inoculated into mulle Hinton agar using counter strike method. Next, the plain disk was immersed in each solution that had been prepared (positive control, negative control, medium, and propolis concentration) and placed on top agar. After the incubation process at 37°C for 24 hours, the inhibition zone that formed in the petri dish are measured using a ruler.

The microdilution test used in this research involved RPMI medium about 10.4 g along with 800 mL of aquadest and 2 g of NaHCO<sub>3</sub>. Second, fungal cultures must be prepared to its required standard turbidity, 0.5 McFarland. After that, in a 96-wells microplate, 50  $\mu$ L medium and 50  $\mu$ L fungi inoculum are added to each well. Next, various solutions (positive and negative controls, fungi, medium, and propolis sample) were applied to each well. The next step is to take 100  $\mu$ L of the mixture from the first well to the second well and so on to create a dilution. In this method, absorbance measurements are tested before and after incubation at a 405 nm wavelength. The incubation process lasted for 24 hours at 35°C. Observation process are continued by take 50  $\mu$ L solution from each well and cultured it on SDA medium. After 48 hours incubation at 35°C, record data of any fungal growth.

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