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Phytochemical Analysis and Antifungal Activity of Brunei Propolis Against *Candida* sp. and *Cryptococcus* sp.

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Abstract. The propolis used in this study is propolis from Brunei Darussalam, including *G. thoracica, H. itama, and T. binghami*. This study focuses on evaluating phytochemicals, including the total content of polyphenols and flavonoids, marker compounds, and the anti-fungal activity of propolis Brunei. Until now, research on the compounds contained in propolis is still being carried out. However, the literature on the chemical compound of Brunei propolis is still limited. The results of the research on the content of Brunei propolis using LC-MS/MS found as many as 21 chemical compounds and three marker compounds, namely maslinic acid, D-(-) Mannitol, and 18-β-Glycyrrhetinic acid. The total content of flavonoid and polyphenolic propolis in Brunei was obtained using quercetin as a flavonoid standard and gallic acid as a polyphenol standard. In Brunei propolis, the total flavonoid content was higher than the total polyphenol content. Where the total polyphenol content of propolis *G. thoracica, H. itama,* and *T. binghami* were 78.79±17.06; 70.51±12.93 and 16.37±0.53 (mgGAE/g). while the total flavonoid content was 19.30±1.99; 101.10±6.26 and 61.63±4.53 (mg QE/g). The antifungal activity was carried out by agar diffusion and microdilution methods. Brunei propolis extract showed antifungal activity against *Cryptococcus* and *C. Albicans*, whereas propolis Brunei extract showed anti-fungal activity with intermediate resistance to both fungi.

Keywords: Anti-fungal; Brunei propolis; LC-MS/MS; Total flavonoid content; Total polyphenol content

1. Introduction

Stingless bees consist of more than 500 species, and it is possible that more than 100 unidentified species, usually stingless bees, can be found in dry and humid tropical forests and some subtropical areas (Michener, 2012). They are ecologically active and play an important role in the forest ecosystem. Stingless bees are also attractive because of their

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honey, wax, and propolis (Ajibola et al., 2012). Propolis is a natural resin produced from bees, derived from a mixture of bee saliva containing enzymes with tree sap or exudates (Pratami et al., 2020).

The chemical compound content of propolis itself depends on the plant source, geographical location, environmental conditions, and bee species (Pratami et al., 2020). The most common chemical compounds contained in propolis are polyphenols and flavonoids (Król et al., 2013; Kumar et al., 2008; Bankova et al., 2000; Hegazi et al., 2000). Propolis is widely used in traditional medicine, especially in people who have inadequate health and sanitation conditions (Sung et al., 2017; Veiga et al., 2017; Boukraâ et al., 2009; Trusheva et al., 2007; Uzel et al., 2005).

Fungal infections are still a health problem in Indonesia, with 5.3 million people suffering from fungal infections every year. In fact, the National Nosocomial Infections Surveillance System (NNISS) reports that Candida species are the fourth most common nosocomial bloodstream pathogen (Wisplinghoff et al., 2004). Mortality is estimated at 45% (Cheng et al., 2005), probably due to ineffective diagnostic methods and inadequate initial antifungal therapies (Morrell et al., 2005). Many medical fungi in circulation have undesirable or highly toxic side effects (amphotericin B), produce relapse, and indicate drug-drug interactions (azoles). Leading to the development of resistance (fluconazole, 5-flucytosine); some even show ineffectiveness (White et al., 1998).

Therefore, it is necessary to find and find new antifungal agents that are more effective and less toxic in overcoming these problems. Propolis has attracted the attention of scientists searching for an alternative therapeutic drug against infectious diseases and multidrug-resistant bacteria since the 1970s. Researchers interest in this complex substance has increased in recent decades based on further investigation of the chemical composition of propolis (Toreti et al., 2013). The use of propolis is very influential on human health and is used for various purposes. Currently, it is used as an antibacterial, antifungal, anti-inflammatory, antiviral, anesthetic, and antioxidant (Omar et al., 2017; Boukraâ et al., 2009).

Propolis is found in various regions of the world, one of which is Brunei Darussalam. However, there are still very few studies on phytochemicals, compounds contained in Brunei propolis, and antifungal biological activity against *Candida albicans* and Cryptococcus neoformans. Because of the background that underlies this research, it is shown to analyze the potential of phytochemicals and biological activity contained in propolis Brunei. This analysis is expected to broaden the spectrum of herbal medicines that can be used in antifungal treatment.

The Research was conducted with three different species of propolis because each stingless bee has unique and specific characteristics. Depending on different countries and regions that may have unique and specific species of stingless bees, local ecosystemadapts to this. According to Abdullah et al. (2020) there are at least 50 species in Kalimantan, including Brunei, Sabak, and Sarawak. Among them, the species *Geniotrigona thoracica, Heterotrigona itama,* and *Tetrigona binghami* are widely cultivated because their tree trunks are found in natural forests, are collected, and are easy to cultivate and care for in suburban areas.

2. Methods

2.1. Materials

The sample of propolis extract used came from Brunei Darussalam with three different species, namely *Geniotrigona thoracica, Heterortigona itama,* and *Terigona binghami*.

Three species are different in their colour and size. The average body size of *Heterotrigona itama* was 4.7 ± 1.55 mm whilst *Geniotrigona thoracica* was 7.44 ± 2.05 mm, *Tetrigona binghami* has the permanent space covering 2/3 of the size of the head.

Heterotrigona itama are black in color with grey wings, while on the contrary, *Geniotrigona thoracica* are brown in color with dark brown wings and white tips at the apex of the wings; Tetrigona binghami the wing color is black except for having a white patch at the tip of the wing (Azmi et al., 2019).

2.2. Identification of Propolis Brunei Content

2.2.1. Total Polyphenol Content (TPC)

Quantitative testing of polyphenol content was carried out using Feline Ciocalteu and Na_2CO_3 reagents. 50 mg of gallic acid was used as a standard, dissolved in methanol to a concentration of 1000 ppm, and then diluted with water to concentrations of 0, 12.5, 25, 37.5, 50, 62.5, 75, 87.5, 100, and 112.5 ppm. Every 0.5 mL of gallic acid with a different concentration of 0.5 mL of propolis sample was mixed into 5 ml of Folin reagent, then vortexed and allowed to stand for 5 minutes. Next, 4 mL of 1M Na_2CO_3 was added to the mixture and allowed to stand at room temperature for 15 minutes. Using a UV-VIS spectrometer, the resulting mixture was then measured at a wavelength of 752 nm. Measurements were carried out 3 times (Sahlan et al., 2020).

2.2.2. Total Flavonoid Content (TPC)

Quantitative testing of flavonoid content was carried out using standard quercetin as much as 10 mg dissolved in methanol to reach a concentration of 1000 ppm which was then diluted with water to concentrations of 0, 12.5, 25, 37.5, 50, and 62.5 ppm. 1 mL of propolis sample was put into a test tube, and 0.2 mL of 10% $AlCL_3$, 0.2 ml of KCH_3COO, 5.6 mL of water and 3 mL of methanol were added. Then the solution was vortexed and allowed to stand at room temperature for 30 minutes. The resulting mixture was then measured at a wavelength of 428 nm using a UV-VIS spectrometer. Measurements were carried out three times (Sahlan et al., 2020).

2.2.3. LC-MS/MS Test

In this study, the propolis compound was identified using LC-MS/MS UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS ThermoScientific in Advanced Research Laboratory IPB University Bogor, West Java. The eluent phase is formic acid and acetonitrile with a flowrate 0.2 mL/min for 30 minutes. Mass spectrum data was analyzed using Compound Discover 3.2 (Pratami et al., 2018).

2.3. Antifungal Activity

2.3.1 Propolis Preparation

Preparation of propolis samples for use as a test material includes dilution of propolis in various concentrations. The concentrations to be tested are 50%, 70% and 100%. This propolis preparation was carried out with the addition of DMSO which would later act as a positive control in the antifungal test.

2.3.2 Agar Diffusion Method

The principle of the agar diffusion method is to make several holes in the Mueller Hinton agar which has been planted with fungi, namely *Cryptococcus neoformans* and *Candida albicans*. First for three concentrations of Brunei propolis (50%, 70% and 100%), two for positive controls (K+) including amphotericin B and fluconazole, and the last for DMSO as a negative control (K-). Then the inhibition zone will be seen. showing the sensitivity response of *Cryptococcus and C. Albicans* to Brunei propolis extract and for positive-negative controls.

The first procedure was to test the antifungal activity, sterilize distilled water, and then pour into a test tube. In the second stage, remove the fungal culture (*Candida albicans* and/*cryptococcus*) with Ose and put it in a test tube containing sterile distilled water until

it reaches 0.5 McFarland. In the third stage, spread the suspension of fungi (*Candida albicans* and/*cryptococcus*) on the Müeller Hinton agar evenly using a spreader. The fourth stage is wetting the blank disc with three concentrations of Brunei propolis. The fifth step is to prepare a 5mg/ml Amphotericin B and 5mg/ml Fluconazole solution. The sixth step is to take a plain disc that has been soaked in sterile Brunei Propolis and Aquadest and a 5mg/ml solution of Amphotericin B disc with tweezers and place it on Mueller Hinton agar which has been smeared with *Candida albicans* suspension. The seventh stage, incubating at 37°C for 24 hours in an incubator (Apriyanto, 2002). The eighth stage measures the inhibition's diameter using a caliper from the back of the petri dish. in the ninth stages, the petri dish must be placed on a dark base with a flat surface. Then the diameter to be measured includes the diameter of the disc and is measured by the point closest to the emergence of microbes (Marston, 2011).

3. Mathematical Model

3.1. Propolis Brunei Content

This result will show quantitative analysis of the total content of polyphenols and flavonoids along with a qualitative analysis of LC-MS/MS to identify the compounds of Brunei Propolis.

3.1.1. Total Content of Polyphenols and Flavonoids

The samples used in the polyphenol and flavonoid content test came from Brunei with three different species, namely *Geniotrigona thoracica* (Thor code), *Heterotrigona itama* (IT code), and *Tetrigona binghami* (Bh code). In measuring the gallic acid calibration curve, the as you can see in Figure 1 results of the calibration curve equation obtained are y = 0.0093x + 0.0392 with a value of R^2 = 0.994. With the sample absorbance data, the calculation is carried out using the calibration curve equation (Table 2). Furthermore, the conversion was carried out so that the total polyphenol content.

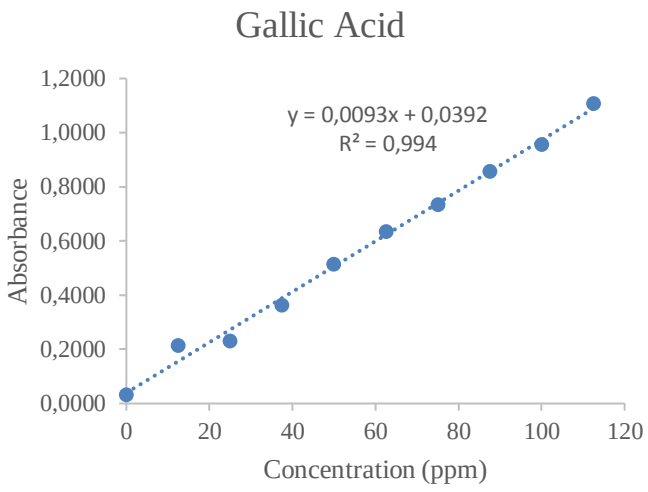


Figure 1 Standard Curve of Total Polyphenol Content

Table 1 Calculation of Total Polyphenol Content

m sample (g)	Sample Code	Absorbance	Concentration (ppm)	Concentration (mgGAE/g)	Everage Concentra-tion	Standard Deviation	TPC (mgGAE/g)
		0,7720	78,7957	91,1987			
0,432	Tor	0,7290	74,1720	85,8473	78,79	17,06	78,79±17,06
		0,5160	51,2688	59,3389			
		0,4910	48,5806	55,5843			
0,437	IT	0,6730	68,1505	77,9754	70,51	12,93	70,51±12,93
		0,6730	68,1505	77,9754			
		0,1780	14,9247	16,8832			
0,442	BH	0,1770	14,8172	16,7615	16,52	0,53	16,37±0,53
		0,1700	14,0645	15,9101			

(TPC) of EEP was 100% of each propolis species. Propolis from *G. thoracica* species had the highest polyphenol content, namely 78.79±17.06 mgGAE/g propolis. At the same time, the lowest polyphenol content value of 16.37±0.53 mgGAE/g propolis was owned by propolis from *T. binghami* species.

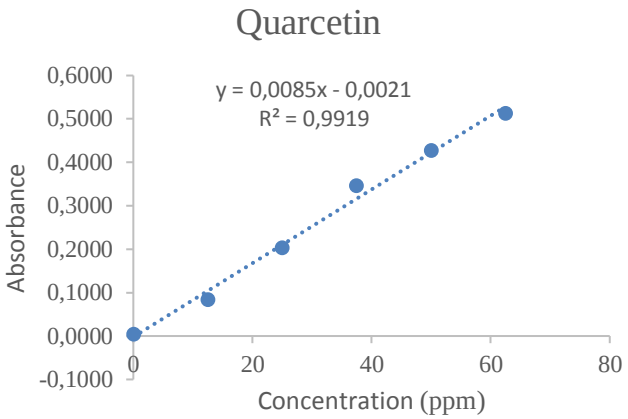


Figure 2 Standard Curve of Total Flavonoid Content

Meanwhile, in the test for the Total Flavonoid Content, the quercetin calibration curve was the measure (Figure 2) with the results of the calibration curve equation being y = 0.0085x - 0.0021 and the value of R^2 = 0.9919. Propolis from *H. itama* species had the highest flavonoid content value, 101.10±6.26 mg QE/g propolis. Meanwhile, the species with the lowest total flavonoid content was *G. thoracica* at 19.30±1.99 mg QE/g propolis (Table 2).

Table 2 Calculation of Total Flavonoid Content

m sample (g)	Sample Code	Absorbance	Concentration (ppm)	Concentration (mgGAE/g)	Everage Concentra-tion	Standard Deviation	TFC (mgGAE/g)
		0,142	16,9529	19,6215			
0,432	Tor	0,124	14,8353	17,1705	19,30	1,99	19,30±1,99
		0,153	18,2471	21,1193			
		0,76	89,6588	102,5845			
0,437	IT	0,698	82,3647	94,2388	101,10	6,26	101,10±6,26
		0,789	93,0706	106,4881			
		0,498	58,8353	66,5558			
0,442	BH	0,454	53,6588	60,7000	61,63	4,53	61,63±4,53
		0,431	50,9529	57,6391			

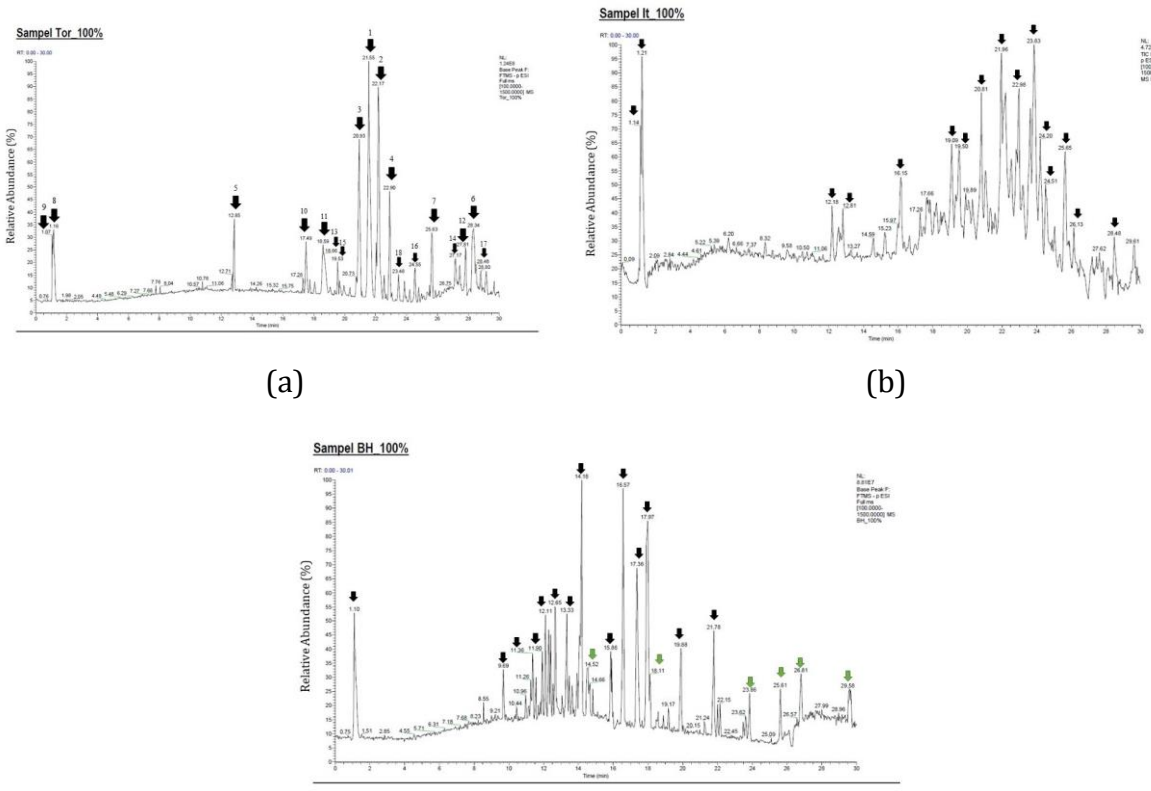
3.1.2. Analysis of LC-MS/MS Test Results

In this study, LC-MS/MS was used to identify the compounds present in 3 species of Brunei propolis. Identification of the compound was carried out in two parts. First, identify the highest peak of each propolis sample and identify the presence of the same compound in propolis. Meanwhile, the presence of the same compound in propolis was identified to search for marker compounds from Brunei propolis.

Spectra with peaks at different retention times were obtained by negative ionization mode. The peaks in the spectra can be formed due to the presence of an identified compound. In this study, the signal read on the LC-MS/MS tool was analyzed using the Thermo Scientific Xcalibur 4.2 software so that the chromatogram. Types of chromatograms are generally divided into Total Ion Chromatogram (TIC) and Base Peak Intensity (BPI).

The chromatogram on the TIC is made based on the sum of all ion currents in the mass spectra series as a function of retention time. Meanwhile, the BPI chromatogram is obtained by representing the base peak signal, which shows the highest ion intensity detected from each mass spectrum as a function of retention time (Murray et al., 2013).

Based on the results of compound readings (Fig 3.) using Compound Discover 3.2 software in Brunei propolis samples with Thor code (*G. thoracica*), 76 compounds were identified from the database. The identified compounds were obtained as many as 18 highest peaks or 18 compounds with the highest content in propolis Brunei *G. thoracica.* Meanwhile, in the *H. itama* sample, 133 compounds were identified from the database. List of compounds identified from the 17 highest peaks or 17 compounds with the most content in *H. itama* propolis. Then the sample of *T. Binghami,* obtained 91 compounds identified from the database. List of compounds identified from the 20 highest peaks or 20 compounds with the most content in *T. Binghami* propolis.



(a) (b)

(c)

Figure 3 LC-MS/MS Spectra of Brunei Propolis (a) *G. thoracica*, (b) *H. itama*, (c) *T. binghami*

In the second part, namely the identification of the presence of the same compound in propolis, the priority or criteria that can be used to determine the identification of propolis compounds are carried out. The compound identification process at this stage is carried out by searching the database owned by PubChem and ChemSpider. The results obtained in the first section were then processed into the second section, which obtained active compounds in each species of Brunei propolis, among others, from 18 compounds with the highest composition in Brunei propolis *G. thoracica,* there were four compounds identified. Then, from 17 compounds with the highest composition in Brunei *H. itama* propolis, there 10 compounds were identified. In Brunei *T. Binghami* propolis from 20 compounds with the most composition, there were seven compounds identified.

Table 3 Identification of propolis compounds using LCMS/MS

No	Compound Name	Molecular Formula	Propolis Code	Group
1	Mangostin	$C_{24}H_{26}O_6$	Thor	Fenol
2	Maslinic Acid	$C_{30}H_{48}O_4$		Triterpenoid
3	Luteolin	$C_{15}H_{10}O_6$		Flavonoid
4	D-(-)Mannitol	$C_6H_{14}O_6$		Alcohol and sugar
5	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$		Glycyrrhizic acid
6	Maslinic Acid	$C_{30}H_{48}O_4$		Triterpenoid
7	Asiatic acid	$C_{30}H_{48}O_5$		Pentacyclic triterpenoid
8	D-(-)Mannitol	$C_6H_{14}O_6$	It	Alcohol and sugar
9	Glyceryl 2-linolenate	$C_{21}H_{36}O_4$		Fatty Acid
10	(R)-Naproxen	$C_{14}H_{14}O_3$		Naphthalene
11	Flavesone	$C14_4H_{20}O_4$		Fenol
12	Apigenin	$C_{15}H_{10}O_5$		Flavonoid
13	Ursolic acid	$C_{30}H_{48}O_3$		Fenol
14	10,16-Dihydroxyhexadecanoic acid	$C_{16}H_{32}O_4$		Hydroxy fatty acid
15	(3aR,4R,5R,6aS)-5-Hydroxy-4-[(1E,3S)-3-hydroxy-1-octen-1-yl]hexahydro-2H-cyclopenta[b]furan-2-one	$C_{15}H_{24}O_4$		Aliphatic alcohol
16	Amiloxate	$C_{15}H_{20}O_3$	Bh	Cinnamic acid
17	Azelaic acid	$C_9H_{16}O_4$		Fenol
18	Oryzarol	$C_{26}H_{42}O_3$		Benzoate ester
19	18-β-Glycyrrhetinic acid	$C_{30}H_{46}O_4$		Glycyrrhizic acid
20	D-(-)Mannitol	$C_6H_{14}O_6$		Alcohol and sugar
21	Maslinic Acid	$C_{30}H_{48}O_4$		Triterpenoid

After identifying the compounds from the highest peak, three compounds were found in the majority of Brunei propolis. This shows that the three compounds can be marker compounds for Brunei propolis. The list of possible marker compounds includes Maslinic acid, D-(-)-Mannitol, and 18-β-Glycyrrhetinic acid.

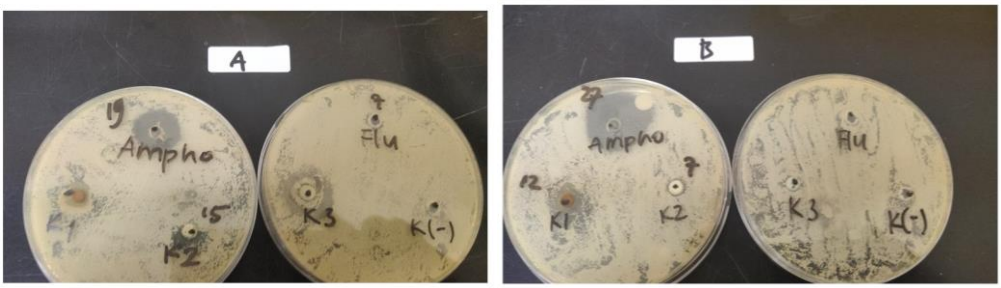
From the results of the LC-MS/MS test on the three Brunei propolis species, several active chemical compounds were obtained, each of which has various benefits. It can be seen from the explanation above therefore, the three species of Brunei propolis have the potential to have benefits as antimicrobial, antioxidant, anti-inflammatory, and antifungal agents (Rasul et al., 2013).

3.2. Antifungal Activity

The antifungal test with propolis was carried out using the agar diffusion method, where agar diffusion is a diffusion method used to determine the activity of antifungal agents. Fungal resistance to a radius of 3 mm below the control diameter, intermediate

when the inhibition zone has a radius ≥ 2 mm and is > 3 mm under control, while resistance is when the diameter of the inhibition has a radius of < 2 mm. Tables 4 and 5 show the results of the inhibition diameter measurements for both types of fungi with Brunei propolis.

Table 4 and Figure 4 shows the diameter of the antifungal test inhibition against *Cryptococcus neoformans*. The data was carried out twice to see a better level of accuracy. These data indicate that the diameter of inhibition resulting from the antifungal test using Brunei propolis indicates that Brunei propolis at concentrations of 50% and 70% has the moderate inhibitory ability as antifungal against *Cryptococcus neoformans* and this fungus has intermediate resistance to Brunei propolis.



(a) (b)

Figure 4 Inhibition Zone of Brunei Propolis against *Cryptococcus neoformans* (a) first experiment (b) second experiment

Table 4 Inhibition Zone of Brunei Propolis against *Cryptococcus neoformans*

	A (mm)		B (mm)		Average
Propolis 50%	K1	0	K1	12	6
Propolis 70%	K2	15	K2	7	11
Propolis 100%	K3	0	K3	0	0
DMSO 10%	K (-)	0	K (-)	0	0
Amphotericin B	K (+)	19	K (+)	27	23
Fluconazole	K (+)	7	K (+)	0	0.5

Table 5 and Figure 5 shows the diameter data of the antifungal test inhibitors against *Candida albicans*. The resulting inhibition diameter values indicate that *Candida albicans* have intermediate resistance to Brunei propolis, and Brunei propolis has moderate antifungal ability against *candida albicans*. And the Brunei propolis inhibition zone with a concentration of 100% greater than fluconazole indicates that Brunei propolis has the potential to be a better antifungal agent.

Table 5 Inhibition Zone of Brunei Propolis against *Candida albicans*

	A (mm)		B (mm)		Average
Propolis 50%	K1	9	K1	11	10
Propolis 70%	K2	7	K2	11	9.5
Propolis 100%	K3	10	K3	13	11.5
DMSO 10%	K (-)	0	K (-)	0	0
Fluconazole	K (+)	5	K (+)	16	10.5

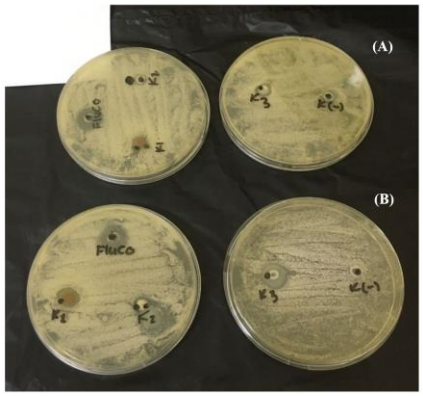


Figure 5 Inhibition Zone of Brunei Propolis against Candida albicans (a) first experiment (b) second experiment

4. Conclusions

The total flavonoid and polyphenol content test result in each Brunei propolis species showed that *H. itama* had the highest total flavolonid content of 61.63±4.53 mg QE/g propolis. Meanwhile, *G. thoracica* had the highest total polyphenol content of 78.79±17.06 mgGAE/g propolis. For the analysis of the identification of propolis compounds using LC-MS/MS, a total of 21 active chemical compounds and three marker compounds were found. The antifungal test results using the agar diffusion method showed that Brunei propolis had a larger inhibition zone for *candida albicans* than *cryptococcus neoformans*, and the resistance of both fungi was in the intermediate category.

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