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ANTIFUNGAL ACTIVITY OF CHINESE BELT HERB (Peperomia pellucida L. Kunth) ETHANOL EXTRACT ON THE GROWTH OF Candida albicans

Ni Putu Widayanti*, Ayu Saka Laksmita W., Desak Putu Risky Vidika Apriyanthi, Ni Made Widnyani
Universitas Bali Internasional, Jl. Seroja, Gang Jeruk, Nomor 9A, Tonja, Denpasar Timur. Denpasar, Bali 80234, Indonesia
*wida.yantisp@gmail.com

ABSTRACT
When the host's phagocytic resistance diminishes, Candida albicans can enter the bloodstream and cause a systemic fungal infection known as candidiasis. One fungus that has become resistant to antifungals is Candida albicans. The herb sirih cina is one way to discover novel antifungals made of natural elements. This study aims to determine the antifungal activity of Chinese betel leaf extract against the growth of the Candida albicans fungus. The sirih cina plant (Peperomia pellucida L. Kunth) was used in this experimental work, which used 70% ethanol as a solvent for the extraction process using maceration. Alkaloids, flavonoids, tannins, saponins, and phenolics are among the secondary metabolites found in the ethanol extract of the Sirih cina plant. Using the well diffusion test method, the antifungal efficiency of the ethanol extract of the Sirih cina plant at concentrations of 25%, 50%, 75%, and 100% was evaluated. Candida albicans growth is inhibited by the ethanol extract of the Sirih Cincina plant, which is categorized as medium category and has the highest inhibition zone diameter at 100% concentration of 8.20 ± 0.14 mm.

Keywords: antifungal; *candida albicans*; *peperomia pellucida l. kunth;* secondary metabolites

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INTRODUCTION

Candida is a normal flora found mainly in the vagina, urethra, mucous membranes of the respiratory tract, and skin. However, too much Candida can suppress the immune system. Candida sp. is an organism that does not cause disease or infection in a normal immune system, but can infect people with a poor immune system. Candida sp. is a saprophyte that lives in the gastrointestinal area, vagina, skin, and under nails(Puspitasari et al., 2019). One of the most common causative agents of skin, genital and oral mucosal infections is Candida albicans, which causes candidiasis. In addition, non-albicans species, such as C. tropicals, C. parapsilosis, C. guillermondii, C. kruzei, and some rarer Candida species, also frequently cause infections. Candidiasis can occur in body folds, especially in moist and warm areas such as the groin and other skin folds (Puspitasari et al., 2019).

Candidiasis is a common disease in Indonesia, with a prevalence of 20–25%. Since 1980, fungal infections have increased in a wide variety of patients. Indonesia's tropical climate can cause skin to become sweaty and damp, as well as poor personal hygiene and a lack of health awareness, all of which are big risk factors for fungal growth. Apart from being diagnosed as dermatitis, many treatments for candidiasis are not carried out according to procedures (Puspitasari et al., 2019). Candidiasis treatment usually uses antifungals and antibiotics. The

most common antifungal treatment is azole, but this drug can also have positive and negative effects (Hofer, 2022). As a result, the use of antifungal agents for treatment has increased, which may lead to clinical consequences such as antifungal resistance (Pfaller, 2011). Antifungal resistance certainly requires the discovery of alternatives. Medicinal plants are an affordable alternative for the community because the cost of treatment cannot be afforded by everyone (Agustantina & Soekartono, 2021). Plants play many important roles for humans, such as traditional medicinal uses. The use of natural ingredients as traditional medicines is also increasing, and some have been made on a large scale.

Indonesia has rich biodiversity, with various types of useful medicinal plants. The Chinese betel plant is one that is traditionally used for health. The secondary metabolite content of Chinese betel (Peperomia pellucida L. Kunth) includes phenolics, phytosterols, tannins, alkaloids, terpenoids, saponins and flavonoids, according to several studies (Ahmad *et al*., 2022). Chinese betel plants contain flavonoids, tannins and saponins which can inhibit fungal growth. The way flavonoids work can change organic components and nutrient transport, causing toxic effects on fungi (Rai et al., 2022). Chinese betel leaf plants have been used for the treatment of various diseases, including antimicrobial, antiprotozoal, anti-inflammatory, analgesic, antipyretic, anticancer, antiulcer, and antioxidant, according to previous research (Saputri et al., 2021). Currently, the ethanol extract of Chinese betel herb is only used in the antifungal field. According to this description, researchers wanted to know how the ethanol extract of Chinese betel herb affects the growth of fungi, especially Candida albicans, which has not been widely studied. This study aims to determine the antifungal activity of Chinese betel leaf extract against the growth of the Candida albicans fungus.

METHOD

Preparation of ethanol extract from Chinese betel herb

Three kilograms of Chinese betel herbs were washed with running water to remove impurities attached to the plants. Cleaned and air-dried, the Chinese betel fruit is left in the air for two days until it wilts,then dried in the oven at 40 degrees Celsius for six hours. After the bark is dry, it is blended and sieved using an 80 mesh sieve to produce a fine powder.

Making Chinese betel herbal extract

Extraction is carried out through a maceration process. A total of 300 grams was put into a glass jar and poured with 1000 milliliters of 70% ethanol solution. Leave it for 24 hours. Then, filtering is carried out and the ethanol solution is macerated again. Then, the filtrate obtained was concentrated at a temperature of 40 degrees Celsius using a rotational vacuum evaporator. It produces a thick extract from the Chinese betel plant.

Phytochemical screening of ethanol extract of Chinese betel herb

Phytochemical tests include the addition of concentrated HCl and Mg powder to flavonoids; addition of water to saponin; addition of FeCl3 to tannin; and the addition of methanol and FeCl3 to polyphenols (Karimatulhajj, 2020; Dubale et al., 2023).

Making potato dextrose agar (PDA) media

After weighing 3.9 grams of dextrose agar, 100 milliliters of distilled water was suspended in an Erlenmeyer flask. Cotton wool and aluminum foil were placed on top of the Erlenmeyer flask, and an autoclave was used to clean it for 15 minutes at 1210C. PDA was added as much as 25 mL into the petri dish and then left to stand. To prevent the growth of microorganisms other than fungi, antibiotics should be added to the PDA medium with 100 mL of PDA with 1 mL of antibiotic (1% concentration). The media that has been created is left for some time

before use. The antibiotic Nystatin was used as a positive control, and DMSO was used as a negative control (Azzahra et al., 2020)**.**

Determination of minimum inhibitory concentration

To test MIC, the test materials used were negative control, positive control, and ethanol extract of Chinese betel herb with concentrations of 25%, 50%, 75%, and 100%. Each solution of Chinese betel herb ethanol extract was mixed into the medium with a ratio of 1:25, then the medium was allowed to solidify in a petri dish. A 25% transmissible fungal suspension is streaked on the surface of the media containing the test material with a sterile spreader (swab theory). After being left for a while until the fungal inoculum is absorbed by the medium, the petri dish is incubated for 24 to 48 hours at 370C. Each petri dish showed mold growth. The minimum inhibitory level is the amount of test material that is still able to inhibit or not grow by fungi.

Testing the antifungal effectiveness of Chinese betel herb extract

Testing the antifungal performance of the ethanol extract of Chinese betel herb using disk diffusion. Once liquid, the potato dextrose agar (PDA) medium was placed in a sterile petri dish and allowed to solidify. After the medium became solid, 50 µ of fungal suspension was applied evenly to the surface of the medium. A 6 mm sterile paper disc was dipped in each concentration of test extract, positive control, and negative control, and then placed on the surface of the media. For one day, the petri dish was incubated at 370C. Measurement of the inhibition zone formed is carried out with a caliper and the strength of the inhibition zone will be interpreted. In this study, samples of Chinese betel herb ethanol extract were used in various levels proportional to the number of treatment groups (25%, 50%, 75%, and 100%). Apart from that, it was also used as a positive control for nystatin and a negative control for DMSO.

RESULTS

Phytochemical screening of ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth)

The results of the phytochemical screening test show that the ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth) contains secondary metabolite compounds such as alkaloids, flavonoids, tannins, saponins and phenolics, as shown in Table 1.

Table 1.
Phytochemical screening of Ethanol Extract of Chinese Betel Herb (Peperomia pellucida L. Kunth)

Compound	Reactor	Observation result	Note
Alkaloids	Mayer	White precipitate	+
	Dragendroff	Brick red solution	+
Tannin	FeCl3 1%	Black precipitate	+
Flavonoids	Concentrated HCl + Mg Powder	Brick red precipitate	+
Saponins	Hot water + 1 N HCl	Stable foam is formed	+
Phenolic	FeCl3 10%	Black precipitate	+

Note: (+) = Contains the compound in question
(-) = Does not contain the compound in question

Evaluation of Candida albicans Fungus

Fungi are identified using culture and identification techniques. To identify the Candida albicans fungus, the fungus can be cultivated on slanted and flat potato dextrose agar (PDA) media. Then, using a microscope that had been dripped with KOH previously and observed at a microscope with 10 times and 40 times magnification, the results were positive. The results are presented in Table 2.

Table 2.
Identification Results of Candida albicans Fungi

Identify mushrooms	Specification	Results
Colony morphology test	Form	Round
	Edge	Flat
	Surface texture	Smooth and shiny
	Elevation	Convex
	Color	Yellowish white
Microscopic test	Form	Round and oval
	Edge	Thin walled
	Arrangement	*Chlamdospores, Pseudohypha, Blastopora*

To identify the Candida albicans fungus, potato dextrose agar (PDA) media was used to isolate the fungus and then incubated for 24 hours at 37°C. The fungus can be observed macroscopically with a round shape like yeast, flat edges, yellowish white color, small size, smooth surface texture, and emerging colonies. Microscopic observation (with 10x and 40x magnification) shows morphological characteristics, such as hyphae and pseudohyphae in intermediate, round and oval shapes. Blastopores and chlamdospores are located at the tip of the fungus.

Antifungal Activity Test Results of the ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth)

The aim of testing the antifungal activity of the ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth) is to determine how much the Candida albicans fungus inhibits at each concentration of ethanol extract. The well diffusion method was used. In this study, nystatin was used as a positive control to show inhibition, and the results were 9.85 ± 0.011 mm and negative control via DMSO, which showed that the test medium did not experience inhibition. Figure 5.1 shows the results of the inhibition zone test of the ethanol extract of Chinese betel herb against the Candida albicans fungus.

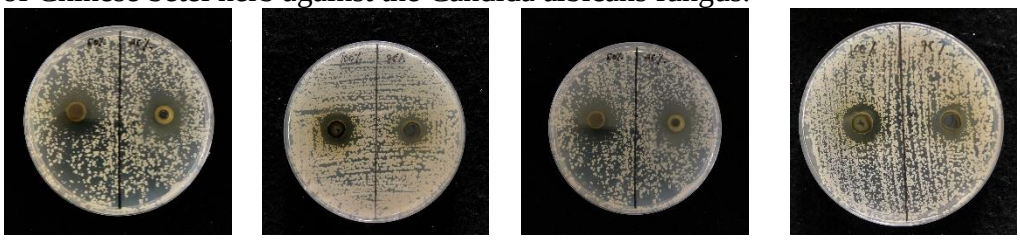

Figure 1. Antifungal test results of ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth) against Candida albicans fungus

Measurement of the diameter of the inhibitory zone of the ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth) against Candida albicans fungi with concentrations of 25%, 50%, 75% and 100% showed that there was an inhibitory zone around the well, which varied between weak and moderate. With a concentration of 25%, the ethanol extract of Chinese betel herb showed weak antifungal activity with an inhibition zone of 2.89 ± 0.06 mm, while with a concentration of 50% it showed moderate antifungal activity with an

inhibition zone of 4.46 ± 0.23 mm. The antifungal activity was moderate with an inhibition zone of 6.72 ± 0.16 mm at a concentration of 75%, and the largest inhibition zone was 8.20 ± 0.14 mm at a concentration of 100%. This information is presented in Table 3.

Table 3.
Antibacterial activity of Chinese betel leaf extract

Treatment	Inhibition Zone Diameter±SD (mm)	Information
Control (+)	9.86 ± 0.08	Currently
Control (-)	0.00 ± 0.00	There isn't any
Extract 25%	2.89 ± 0.06	Weak
Extract 50%	4.46 ± 0.23	Weak
Extract 75%	6.72 ± 0.16	Currently
Extract 100%	8.20 ± 0.14	Currently

DISCUSSION

Parts of the stems and leaves of Chinese betel (Peperomia pellucida (L.) Kunth) from Angseri village in Baturiti District, Tabanan Regency, Bali, were used for this research. The purpose of collecting Chinese betel is to ensure that the level of compound content in the plant does not change due to changes in environmental conditions (Pandey, 2022). This study aims to identify the classes of secondary metabolite compounds found in the ethanol extract of Chinese betel herb and how effective the ethanol extract of Chinese betel herb (Peperomia pellucida L. Kunth) is as an antimicrobial to stop the development of the Candida albicans fungus. The results of phytochemical screening showed that the ethanol extract of Chinese betel herb contains secondary metabolite compounds, including alkaloids, flavonoids, tannins, saponins and phenolics. The alkaloid compound test with Meyer's reagent showed a color change to white, and another test with Dragendroff's reagent showed a color change to a precipitate. Alkaloids are a group of colorless compounds that are crystalline and form heterocyclic rings with at least one nitrogen atom which is basic. The sample alkaloid test must be carried out by adding HCl solution because most alkaloids are insoluble in water but can react with acids to produce water-soluble salts. If the result is positive, it means that the alkaloid sample contains alkaloids (Slacek et al., 2023).

In the alkaloid test with Dragendroff's reagent, the brick red precipitate changes color. This is due to the fact that bismuth salts are easily hydrolyzed to form bismuth ions (BiO+). Next, the Bi3+ ion from bismuth nitrate reacts with potassium iodide to form a black precipitate of bismuth(III) iodide, which then dissolves in excess potassium iodide to prevent the hydrolysis reaction. According to research conducted by Komala et al. (2019), alkaloid metabolite compounds function as antifungals by inserting between the cell wall and DNA. Then, this stops the fungal DNA from replicating, halting the growth of the fungus. The tannin test uses adding 1% $FeCl_3$ to the test sample to differentiate condensed and hydrolyzed tannins. A bluish black or green color change appeared after adding FeCl3, indicating that the Chinese betel herb ethanol extract sample was positive for containing tannin metabolite compounds. A study conducted by Astuti & Respatie (2022) showed that the tannins contained in turi leaves have the ability to stop the growth of Staphylococcus aureus and Candida albicans. Tannins have the ability to stop the synthesis of chitin, which is responsible for building fungal cell walls, and damage cell membranes, stopping the development of bacteria and fungi (Rai et al., 2022). Tannins easily bind to fungal cell walls because they are lipophilic (Rai et al., 2022).

The flavonoid test includes the test sample in a test tube after adding magnesium powder and concentrated HCl solution. The purpose of adding both is to reduce glycoside bonds with flavonoids, which is needed to identify glycoside bonds with flavonoids in plants. HCl will interact with the carbonyl group of flavones and experience resonance, which results in the

formation of new bonds and the release of double bonds. Thus, HCl can dissolve flavones and flavonoids can be separated from other chemical groups to form salts. Red, yellow or orange colors indicate that flavonoids are present (Oktavia & Sutoyo, 2021). The test results on the ethanol extract sample of Chinese betel herb showed a brick red color, which indicates that there are flavonoids in the test sample.

Flavonoids have the ability to inhibit fungal growth through their mechanism of action which reduces the permeability of fungal cell membranes. Flavonoids also have the ability to form complexes with extracellular and soluble proteins as well as with fungal cell walls. Flavonoid hydroxyl groups can alter organic components and nutrient transport, which can cause toxic effects on fungi (Aboody & Mickymaray, 2020). It is possible that mitochondrial membrane potential is reduced as a result of the action of flavonoids that inhibit mitochondrial electron transport. Inhibition of protons in the respiratory chain can cause inhibition, resulting in decreased ATP production and fungal cell death. Gradually, this condition can stop the growth of Candida albicans by creating a defense system against it (Nurafia, 2023).

The saponin test uses foam that forms stably. The presence of glycosides, which have the ability to form foam in water that hydrolyzes to glucose and other substances, is demonstrated by the foam test. Glycones, or reducing sugars, and glycones, or nonsugars, form glycosides. The saponin test on samples of Chinese betel herb ethanol extract was carried out by adding hot water and 1 N HCl to a test tube, and then shaking vigorously for 10 seconds. The test results showed that the sample contained positive saponin (Oktavia & Sutoyo, 2021). According to research conducted by Tivani et al. (2020), saponins function as antifungals by changing the surface tension of the sterol membrane of Candida albicans cell walls, increasing cell permeability. Intracellular fluid can become more concentrated due to increased permeability.

Phenolic compounds are the largest group of compounds that have one or more hydroxyl groups attached to aromatic groups. Phytochemical tests of phenolic compounds using FeCl3 reagent showed good results. The color change to black occurs when FeCl3 reacts with the hydroxyl groups in phenol compounds (Astutoi & Respatie, 2022). Phenolics and their derivatives can cause protein denaturation in fungal cell walls and change the permeability mechanisms of lysosomes, microsomes and fungal cell walls (Oktavia & Sutoyo, 2021). Culture examination is useful for identifying fungal contaminants for pharmaceutical products. Potato Dextrose Agar (PDA) media used to culture the fungus Candida albicans is one of the most commonly used culture media because of its simple formulation and low pH, namely 4.5–5.6, which allows the growth of various fungi (Talapko et al., 2021). To make PDA, tubes or plates can be used and incubated for 24–48 hours at 37°C. After three days, convex-shaped, yellowish-white colonies of Candida albicans appeared on the surface of the medium, had a smooth and slippery start, and had a characteristic yeast odor.

By using a KOH solution, direct microscopic examination can be carried out easily and shows the relationship between the number and shape of the fungus and the tissue reaction. Because Candida albicans multiplies rapidly at room temperature, direct examination should be performed as soon as a clinical source is obtained. To support the diagnosis of superficial candidiasis, images of pseudohyphae on direct preparations can be confirmed through culture examination (Lopes & Leonakis, 2021). To test the antifungal power of the ethanol extract of Chinese betel herb, holes were made in solid media inoculated with the fungus Candida albicans. The well diffusion technique was used. Because the isolate is active down to the bottom, the well diffusion method makes it easier to measure the area of the inhibition zone

formed. To ensure that the growing microorganisms are evenly distributed on the surface of the agar media, test the effectiveness using the spreading plate method. The aim of the antifungal effectiveness test is to determine how inhibitory each concentration of ethanol extract is against Candida albicans ATCC 10231.

The antifungal effectiveness test used Potato Dextrose Agar (PDA) media, which is a media that is often used for fungal growth because it is rich in nutrients and can inhibit the growth of bacteria that can grow at a neutral pH, namely pH 7.0 and at an ideal growth temperature of between 25-300C . Before fungal inoculation on the test medium, a fungal suspension was prepared based on 0.5 McFarland I standard solution, which is equivalent to 1.5 times 108 CFU/mL. This is done to keep the number of growing fungal colonies under control. This antifungal test uses nystatin as a positive control. Nystatin is made from Streptomyces noursei and is a polyene antibiotic. Only sensitive fungi or yeast can bind nystatin. The presence of bonds with sterols in fungal or yeast cell membranes, especially ergosterol, determines antifungal activity. Changes in cell membrane permeability are caused by the binding between sterols and antibiotics, which results in the loss of a number of small molecules. To date, there is no evidence that nystatin confers resistance to Candida albicans (Siddaiah et al., 2024). The diameter of the inhibition zone in the positive control was 9.86 ± 0.08 mm, indicating moderate antifungal results.

In this research, DMSO was used as a negative control to determine whether the test media used was contaminated or not. DMSO is not bactericidal, the ethanol extract of Chinese betel herb has pure antifungal properties that fight Candida albicans without being affected by the solvent used (Empress, 2012). In the negative control, the diameter of the inhibition zone was 0.00 ± 0.00 mm, indicating that there was no antifungal activity. All types of bacteria and fungi are included in the inhibition zone diameter category, according to Bouz & Dolezal (2021). Inhibitory zones with a diameter of more than 20 mm are considered to have very strong inhibitory activity; sizes 10–20 mm are considered to have strong inhibitory power; sizes 5–10 millimeters are considered to have moderate resistance; and sizes below 5 mm are considered to have weak inhibitory power. The results of the antifungal effectiveness test showed that the ethanol extract of Chinese betel herb with a concentration of 100% had a higher antifungal level than other concentrations of 25%, 50% and 75%. This is indicated by the larger diameter of the clear zone.

Based on the antifungal effectiveness test of the ethanol extract of Chinese betel herb, the diameter of the inhibition zone formed from four treatment replications can be classified into the following categories: strength of antifungal activity. With Chinese betel herb ethanol extract at a concentration of 25%, the average diameter of the clear zone was 2.89 ± 0.06 mm, and this result was classified in the weak category. At a concentration of 50%, the diameter of the inhibition zone was 4.46 ± 0.23 mm, the results were moderate; at a concentration of 75%, the diameter of the inhibition zone was 6.72 ± 0.16 mm, the results were moderate; and at 100% concentration, the diameter of the inhibition zone was 8.20 ± 0.14 mm.

Post-Hoc LSD follow-up tests were performed to examine mean differences between groups. The results show that the significance value of the average difference between groups is 0.000 ($p < 0.005$), which indicates that there is a significant difference in the average antifungal inhibition zone between groups. This is caused by compounds containing antifungals having different concentrations from the addition of DMSO solvent. Based on a concentration of 100%, the inhibition zone formed has good effectiveness, namely 8.20 ± 0.14 mm. The LSD

value shows that the higher extract concentration is proportional to the value of the inhibition zone formed due to the amount of antifungal compounds absorbed in the test medium.

In the positive control test, the value obtained was $p = 0.000$ ($p < 0.005$) so it could be said that there was a significant difference. The positive control (nystatin) has the greatest inhibitory power compared to other concentration variants because nystatin has an antifungal effect which can bind ergosterol to fungal cell membranes. So, based on the test results obtained in this study, the ethanol extract of Chinese betel herb with different concentrations showed the formation of an inhibition zone which indicates that each concentration tested has antifungal effectiveness against the Candida albicans fungus.

The research results, as shown by Sinarsih et al. (2021), shows that the diameter of the inhibition zone increases along with the extract concentration. The diameter of the inhibitory zone tends to increase in proportion to the indication of the inhibitory effect indicated by the absence of a clear zone. Increasing the concentration can help the antifungal compound get closer to the fungal cell, causing damage to the cell membrane. Damage to cell membranes can also disrupt nutrient transport, causing fungal cells to lack nutrients necessary for their growth (Garcia-Salazar et al., 2022). This is in accordance with research findings which show that, as a result of large differences in the concentrations of the antifungal active substances used, different inhibition zones are formed with each concentration.

Oktavia & Sutoyo (2021) stated that the turbidity of the fungal suspension affects the diameter of the antifungal growth inhibition zone. If the suspension is too turbid, the diameter of the inhibition zone will be smaller, and if the suspension is too clear, the diameter of the inhibition zone will be larger, and the number of microorganisms present in it is unknown. To measure suspension turbidity, the ideal measurement is with a nephelometer, but in this study, due to equipment limitations, macroscopic measurements of turbidity were carried out visually.

Incubation temperature can influence the area of inhibition of fungal growth. The ideal incubation requirement is 350C. Higher incubation temperatures can make disinfectants or microbial agents more effective. This is caused by chemicals that can destroy microorganisms through chemical reactions (Oktavia, 2021). Incubation temperatures below 350C can cause a larger diameter zone of inhibition. In addition, the incubation time must be adjusted to the growth of the fungus because the area of the inhibition zone is determined within the first few hours after inoculation on the agar medium; then, after fungal growth, an inhibition zone can be observed (Tivani, 2020).

The thickness of the media can contribute to the extent of the area inhibiting fungal growth. Diffusion of the test substance into the agar is influenced by differences in the thickness of the agar medium, which is effectively around 4 mm. The thicker the media used, the smaller the diameter of the inhibition zone. Changes in media composition can also affect antifungal assays. Changes in media composition can change the properties of the media, causing changes in the diffusion distance. This may affect the activity of some fungi, as well as the rate of diffusion and growth of antifungals. The diameter of the inhibition zone can also be influenced by the influence of pH because there are differences in the pH of the media used, which can cause differences in the test substance that diffuses (Tivani, 2020). The two main components that influence the width of the inhibition zone are the inoculum density and its size. A smaller amount of inoculum can result in the extract diffusing further, so that the resulting zone of inhibition is larger (Tivani, 2020).

CONCLUSION

The metabolite compounds contained in the ethanol extract of Chinese betel herb consist of alkaloids, tannins, saponins, steroids and phenolics. The largest inhibition zone diameter is found at a concentration of 100%8.20 ± 0.14 mm so that it can inhibit the growth of the Candida albicans fungus.

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