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Research Article

Antifungal Activity Testing of Extract and Fractions from *Tectona grandis* Linn. F Leaves Using the Microdilution Method

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Abstract

Teak leaves (*Tectona grandis* Linn. F) are one of the plants that have empirically been used as antifungal, antioxidant, antibacterial, and antidiabetic agents, due to their content of secondary metabolite compounds including saponins, tannins, alkaloids, and sterols. This research aims to determine the secondary metabolite compounds present in the extract and fractions of teak leaves (*Tectona grandis* Linn. F) and the antifungal activity of the teak leaf extract and fractions against *Candida albicans* using the microdilution method. The ethanol extract was fractionated using a partition method with solvents n-hexane, chloroform, and ethyl acetate. Antifungal testing was carried out against *Candida albicans* using the Broth Microdilution method to measure the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) values. Based on the phytochemical screening results, teak leaves contain alkaloids, phenolics, flavonoids, and terpenoids. The MIC results for the antifungal activity of the ethanol extract, n-hexane fraction, chloroform fraction, and water fraction against Candida albicans were all >500 µg/mL. In the meantime, the MIC and MFC values for ketoconazole 200 mg against *Candida albicans* were 64 µg/mL. These results indicate that there is a difference in MIC and MFC values between the samples and the positive control, with the antifungal activity of the samples being weaker compared to the positive control.

Keywords: *Candida albicans*, Microdilution, Teak leaf, *Tectona grandis* L.

Uji Aktivitas Antifungi dari Ekstrak dan Fraksi Daun *Tectona grandis* Linn. F dengan Metode Mikrodilusi

Abstrak

Daun jati (*Tectona grandis* Linn. F) merupakan salah satu tanaman yang secara empiris digunakan sebagai antifungi, antioksidan, antibakteri dan antidiabetes, karena memiliki kandungan senyawa metabolit sekunder diantaranya saponin, tanin, alkaloid, dan sterol. Penelitian ini bertujuan untuk mengetahui senyawa metabolit sekunder yang terkandung dalam ekstrak dan fraksi daun jati (*Tectona grandiss* Linn. F) serta aktivitasnya antifungi terhadap *Candida albicans* dengan metode mikrodilusi. Ekstrak etanol difraksinasi dengan metode partisi menggunakan pelarut n-heksana, kloroform dan etil asetat. Uji antifungi dilakukan terhadap fungi *Candida albicans* dengan metode *Broth Microdilution* dengan mengukur nilai *Minimum Inhibitory Concentration* (MIC) dan *Minimum Fungicidal Concentration* (MFC). Berdasarkan hasil skrining fitokimia ekstrak dan fraksi daun jati positif mengandung senyawa alkaloid, fenolik, flavonoid, dan terpenoid. Hasil uji MIC aktivitas antifungi ekstrak etanol, fraksi n-heksana, fraksi kloroform, dan fraksi air terhadap Candida albicans diperoleh yaitu >500 µg/mL. Sementara itu, nilai MIC dan MFC pada ketokonazol 200 mg terhadap Candida albicans yaitu 64 µg/mL. Hasil ini menunjukkan bahwa terdapat perbedaan nilai MIC dan MFC antara sampel dan kontrol positif, dimana aktivitas antifungi sampel lebih lemah dibandingkan dengan kontrol positif.

Kata Kunci: *Candida albicans*, Daun jati, Mikrodilusi, *Tectona grandis* L.

1. Introduction

Infection disease is one of the major public health issues in both developed and developing countries. Infection diseases are caused by the entry and proliferation of microorganisms, which constitute a broad group of microscopic organisms consisting of one or many cells, such as bacteria, fungi, parasites, and viruses.[1]

Candida albicans is one of the opportunistic fungi that can cause infectious diseases. Several risk factors for infection include a weakened immune system and physiological changes in the body, one of which occurs in individuals with diabetes mellitus.[2] The administration of synthetic antifungals like fluconazole, nystatin, and amphotericin B is the therapy typically employed for oral candidiasis. However, it is currently reported that there are numerous cases of drug resistance in *Candida albicans* against these drugs. Resistance to antimicrobial agents in pathogenic fungi is a phenomenon that can have implications for human health.

The sensitivity pattern research indicates that *Candida albicans* is resistant to fluconazole and itraconazole at rates of 1.29% and 4.31%, respectively. The sensitivity pattern of *Candida* sp. to various antifungals also shows resistance to some antifungals, although their sensitivity remains high.[3] The continuous and irrational use of drugs that inhibit sterol synthesis can lead to drug resistance in *Candida albicans,* making future medical treatment more challenging. Therefore, it is necessary to seek alternative options, such as herbal remedies that have similar effects to antifungal drugs like ketoconazole. This has led to the discovery of herbal plants containing compounds with antifungal properties.[4]

One of the plants that can be used is the teak tree (*Tectona grandis* Linn. F). Teak is a tropical plant that grows in countries such as Indonesia, India, Thailand, Nigeria, and Myanmar. Teak cultivation in Indonesia covers a total area of 923.92 hectares.[5] Teak trees are commonly found in Java, Madura, Sulawesi Island, Munam Island in the Bima region of Sumbawa Island, and Buru Island.[6] *Tectona grandis* Linn. F. have metabolic compound are alkaloid, flavonoid, terpenoid and has anti fugal activity.[7]

It is known that teak trees have antifungal activity.[8] Teak trees contain secondary metabolite compounds such as saponins, tannins, alkaloids, sterols, and other substances that have been tested to possess antifungal, antibacterial, and antioxidant properties. Phenolics, which are secondary metabolites, serve the purpose of protecting the plant from pests and diseases, including yeast. Teak leaf extracts can be obtained through an extraction process.[9,10]

2. Method

2.1. Tools

The tools and equipment used in this research are glassware, graduated cylinder, separatory funnel, erlenmeyer flask, dropper pipet, spatula, rotary vacuum evaporator (Buchi Interface I-100® from China), Uv-Vis spectrophotometer (Genesys 10s UV-Vis from Germany), micropipette (Scilogex from North America), hot plate, petri dish, laminar air flow (Robust Multilab Solusindo®), and microplate reader (Thermo Fisher Scientific®). These tools and equipment are essential for conducting the research.

2.2. Materials

Teak leaves were obtained from Lapulu Village, Abeli District, Kendari City, Southeast Sulawesi and has been determined in the laboratory of the Biology Unit development at the Faculty of Teacher Training and Education, Universitas Halu Oleo Kendari with determination letter number 65/BIO/PB/IX/2021; 96% ethanol (Emsure® Merck, Germany); n-Hexane; chloroform; ethyl acetate; distilled water (Aquadest, OneLab®, Bandung); sodium bicarbonate; ketoconazole 200 mg (Tablet OGB HJ); *Candida albicans* ATCC 10231; and sterile NaCl 0,9 %. These resources serve a variety of functions in the research.

2.3. Procedure

2.3.1. Preparation of *Tectona Grandis* Linn.F.

Leaf Extract and Fractions

A total of 800 grams of powder simplisia plant material was macerated in 10 liters of 96% ethanol solvent until the plant material was completely covered. The maceration process was performed three times within 24 hours. The resulting macerate was evaporated at a temperature of 50°C, producing a concentrated extract. The extract was then separated from the plant material residue using Whatman filter paper.

The concentrated teak leaf extract was separated using the solvents n-hexane, chloroform, and ethyl acetate through the application of a partition method. A mixture of 30 grams of teak leaf extract and 1 liter of distilled water was combined to obtain a diluted solution. The resulting diluted mixture was poured into a separating funnel, to which 100 mL of n-hexane solvent was added. The contents were briskly shaken to achieve the separation of layers. The lower layer contained the remaining water, while the upper layer comprised the n-hexane fraction.

Allow the two layers to settle for 5-10 minutes. Collect the upper layer, which is the n-hexane fraction, and repeat the process for the chloroform and ethyl acetate fractions. After obtaining the three fractions (n-hexane, chloroform, and ethyl acetate), concentrate each fraction using a rotary evaporator at a temperature of 50°C. This process results in the isolation of different compounds from the teak leaf extract into these three fractions.[11,12]

2.3.2. Tectona grandis Linn.F. Extract and Fraction Phytochemical Screening

The testing of chemical concentration in the fractions involves the utilization of samples from the ethanol extract, n-hexane fraction, chloroform fraction, ethyl acetate fraction, and water fraction derived from teak leaves. Screening was carried out individually for each fraction to identify the presence of diverse phytochemical substances.

a. Alkaloid Test

Two drops of Mayer's reagent was added into fractions and the methanol extract. The appearance of a white to yellowish precipitate is a sign of success.

b. Flavonoid Test

Sodium hydroxide was added and the color of each fraction and methanol extract was changed. If the colors changed into Red, orange, or yellow its mean that fraction and methanol extract containing flavonoid.

c. Terpenoid Test

Methanol extract and each fraction added one drop of sulfuric acid and 3 drops of concentrated HCl (hydrochloric acid), then observe the color change. A positive result is indicated by a color change to red, reddish-brown, or purple.

d. Saponin Test

Methanol extract and each fraction added 10 mL of hot water and shake vigorously for a few moments. If permanent froth or foam was formed upon the addition of one or two drops of 2N hydrochloric acid (HCl), it indicates a positive result for saponins.

2.3.1. Antifungal Activity Test Using the Microdilution Method

Using a microplate and the microdilution technique, the antifungal activity is assessed. The Clinical and Laboratory Standards Institute (CLSI) advises using the microdilution technique. This technique, which is well-liked in the fields of clinical research and microbiology, enables the evaluation of the samples' antifungal abilities.

a. Establishing the Minimum Inhibitory Concentration (MIC)

The determination of MIC includes a control for fungal growth, a negative control (10% DMSO), a positive control (ketoconazole 200 mg), and the test extract and fractions. The testing is carried out in triplicate. A total of 100 µL of Sabouraud Dextrose Broth (SDB) medium is added to each well.

100 L of the sample solution are added to column well 12 containing the medium in the sample. After being blended, the solution is transported to well 11. To create a series of

serial dilutions with 5 distinct concentration values in each well (500, 250, 125, 62.5, and 31.25 g/L), serial dilution is carried out step by step toward the smallest column.

The MIC is established as the lowest concentration at which nothing is visible. After incubation, the development of colonies in the solution (cloudiness in the microplate) denotes fungal growth. The MIC is the lowest concentration at which no discernible turbidity or fungal growth is seen.

b. Establishing Minimum Fungicidal Concentration (MFC)

From each clear portion, a 5 L aliquot is cultivated on Sabouraud Dextrose Agar (SDA) medium and incubated at the proper temperature and for a predetermined amount of time. Three copies of each test are run. The MFC is established as the lowest concentration at which no fungal growth is seen. The MFC concentration is necessary to kill as opposed to only suppress fungus.

3. Result

The total yield of the ethanol extract from *Tectona grandis* Linn. F leaves obtained in this study is 178.6 grams. Subsequently, the extract is fractionated. Fractionation of the ethanol extract is carried out using different solvents. For n-hexane, the aqueous phase is in the lower layer, and n-hexane is in the upper layer due to the higher density of water, which is 1 g/mL, compared to the density of n-hexane, which is 0.6548 g/mL. For chloroform, the aqueous phase is in the upper layer, and chloroform is in the lower layer because chloroform has a higher density of 1.489 g/mL. For ethyl acetate, the aqueous phase is in the lower layer, and ethyl acetate is in the upper layer because ethyl acetate has a lower density of 0.894 g/mL compared to the density of water. This separation process is based on the differences in the densities of the solvents and the aqueous phase, which allows for the isolation of different compounds present in the extract.

Phytochemical screening results for the ethanol extract and fractions from *Tectona grandis* Linn. F leaves indicate the presence of alkaloids, flavonoids, terpenoids, and tannins. Saponins were only identified in the ethanol extract. This information reveals the phytochemical composition of the extract and fractions, providing insights into the types of compounds present.

The MIC test results for the antifungal activity of the ethanol extract, n-hexane fraction, and chloroform fraction against *Candida albicans* at 24, 48, and 72 hours were all >500 µg/mL. The water fraction had a MIC value of >500 µg/mL at 24 and 48 hours and a MIC value of 125 µg/mL at 72 hours. Meanwhile, the ethyl acetate fraction had a MIC value at 24, 48, and 72 hours each as follows 500, 250, and 125 µg/mL. The MFC value for all samples was stated to be >500 µg/mL.

In contrast, the MIC value for ketoconazole 200 mg against *Candida albicans* was 64 µg/mL with the same MFC value of 64 µg/mL. These results indicate that the tested samples have weaker antifungal activity compared to ketoconazole, as the MIC and MFC values for the samples were significantly higher.

4. Discussion

4.1. The yield results of the extract and fractionation of *Tectona grandis* Linn. F leaves

The total yield of the teak leaf extract in this study was 178.6 grams. The yield of the ethanol extract is related to the ability of ethanol solvent to extract compounds from the sample during the maceration process. The yield of the extract provides important information about the efficiency of the extraction process and the concentration of bioactive compounds in the extract. In this case, the yield indicates that 178.6 grams of extract were obtained from the given amount of plant material. When a solid material (sample) comes into contact with a solvent, the components that are soluble in the solid material move into the solvent. This results in the transfer of the mass of active components from the solid material to the solvent. The longer the extraction time, the greater the percentage of the yield obtained. This is

because the contact time between the plant material and the solvent is extended, allowing for more efficient extraction of the desired compound.[13]

Based on Table 1, the fraction with the highest yield is the water fraction, with a weight of 12.5 grams. The highest yield in the water fraction is because it contains a larger number of compounds compared to the n-hexane, chloroform, and ethyl acetate fractions. Differences in yield can be influenced by various factors, including extraction time, drying conditions, sample particle size, as well as the ratio of the amount of sample to the solvent used. These factors can affect the efficiency of compound extraction from the plant material.[14]

3.2. Compound Content of Extract and Fractions from Tectona grandis Linn. F Leaves

Teak leaf fractions and the ethanol extract were subjected to phytochemical screening in order to determine the secondary metabolite content. This allowed for the discovery of secondary metabolites that may have antifungal activity. The results of the phytochemical screening for the ethanol extract, n-hexane fraction, chloroform fraction, ethyl acetate fraction, and water fraction can be found in Table 2.

When using the Dragendorff reagent, an orange to reddish-brown precipitate forms, indicating the presence of alkaloids. Alkaloid potassium precipitates like this one. Due to ligand exchange, the precipitation reaction takes place.[15] Alkaloids can be detected in both the sample's extract and its fractions.

The presence of flavonoids is confirmed through a noticeable shift in color, transitioning to red, yellow, or orange upon the introduction of magnesium powder and concentrated HCl. The outcomes acquired suggest that flavonoids are present in both the sample's extract and its fractions. This is because flavonoid compounds can be divided into several types, each type of flavonoid has different polarities depending on the number and position of hydroxyl groups in each type of flavonoid, which affects the solubility of flavonoids in the solvent.[16]

Heating and shaking are used to identify saponins by looking at the foam that forms. Since substances with both polar and nonpolar groups have surface activity, a sample containing saponins will form micelles, according to Robinson (1995). The results show that the extract is positive for saponins and that the fractions do not, as shown by the lack of foam formation, contain saponins.

The terpenoid test is carried out by mixing H_2SO_4 with anhydrous acetic acid. Colors such as red, orange, yellow, and purple are produced by terpenoids. Both the extract and the fractions tested positive for terpenoids, according to the outcome. This is because terpenoid compounds belong to a group that is soluble in non-polar to semi-polar solvents.[17]

3.3. Antifungal Activity of Extract and Fractions from Tectona grandis Linn. F Leaves

In order to establish the MIC, antifungal activity testing is carried out using the broth microdilution method. The broth microdilution method was chosen because it requires a small amount of sample, is cost-effective, and is not affected by the thickness of the medium.[18] This method is suitable for assessing the antifungal activity of the samples.

The positive control used is 200 mg ketoconazole, while the negative control is 10% DMSO. The use of 200 mg ketoconazole

Table 1. Results of Fractions from *Tectona grandis* Linn. F Leaves

Extract Weight (g)	Fraction	Fraction weight (gram)	Yield (%)
40	n-hexane	1.72	5.73
	Chloroform	3.96	13.2
	Ethyl acetate	12.2	40.66
	Water	12.5	41.6

Table 2. Phytochemical Screening Results of Extract and Fractions from Tectona grandis Linn. F Leaves

Compound	Sample	Result	Reagen	Observation result
Alkaloid	Extract	+	Dragendorff	Brown precipitate
	n-hexane	+		
	Chloroform	+		
	Ethyl acetate	+		
	Water	+		
Flavonoid	Extract	+	Magnesium	Coloured Red
	n-hexane	+		
	Chloroform	+		
	Ethyl acetate	+		
	Water	+		
Saponin	Extract	+	-	Foam is formed
	n-hexane	-	Water	No foam is formed
	Chloroform	-		
	Ethyl acetate	-		
	Water	-		
Terpenoid	Extract	+	Liebermann burchard	Blackish red
	n-hexane	+		
	Chloroform	+		
	Ethyl acetate	+		
	Water	+		

as the positive control is because its inhibition mechanism disrupts the function of the membrane and increases permeability, which inhibits fungal growth.[19]

The results of the antifungal testing using the test samples can be seen in Table 3 and Figure 1. The MIC values for the ethanol extract, chloroform fraction, and n-hexane fraction at 24, 48, and 72 hours are >500 µg/mL, indicating very weak activity in inhibiting *Candida albicans*. The ethyl acetate fraction shows MIC values indicating weak antifungal activity. In contrast, ketoconazole has an MIC value of 64 µg/mL at 24, 48, and 72 hours. This indicates that ketoconazole has a stronger antifungal activity compared to the samples, which have weaker inhibitory effects on *Candida albicans* can be seen in figure 1.

Finding the MFC was the next step in the test, and the absence of fungal colonies proved that it was successful. After visual inspection of the wells that remained clean after incubation, the MFC values were evaluated using SDA media. Table 4 and Figure 2, displays the MFC outcomes that were attained.

The MFC values for the extract and fractions of teak leaves are >500 µg/mL according to Table 5. This shows that the teak leaf extract and fractions only have fungistatic activity at these doses and do not have fungicidal activity. Ketoconazole, in contrast, exhibits fungicidal action against Candida albicans with an MFC value of 64 µg/mL.

The effectiveness of an antifungal substance in inhibiting growth depends on the nature of the fungus, concentration, and the duration of contact. The results of the extract, chloroform fraction, n-hexane fraction, and water fraction are lower than the ethyl acetate fraction and ketoconazole. This may be due to the lower concentration of secondary metabolite compounds in these samples, which are not potent enough to inhibit the growth of *Candida albicans*. It is also possible that the secondary metabolite content in these samples is not specific for inhibiting Candida albicans.[8,20]

5. Conclusion

In comparison to the positive control

Table 3. Antifungal MIC Measurement Results with Strength Categories Following the CLSI Guidelines (2012)

Sample	MIC (µg/ml)		
	24 hours	48 hours	72 hours
Ethanol extract	>500	>500	>500
Chloroform fraction	>500	>500	>500
n-hexane fraction	500	>500	>500
ethyl acetate fraction	500	250	125
Water fraction	>500	>500	125
Ketoconazole	64	64	64

ketoconazole 200 mg, the test findings of the extract and fractions of Tectona grandis Linn. F leaves demonstrate poor antifungal activity against Candida albicans.

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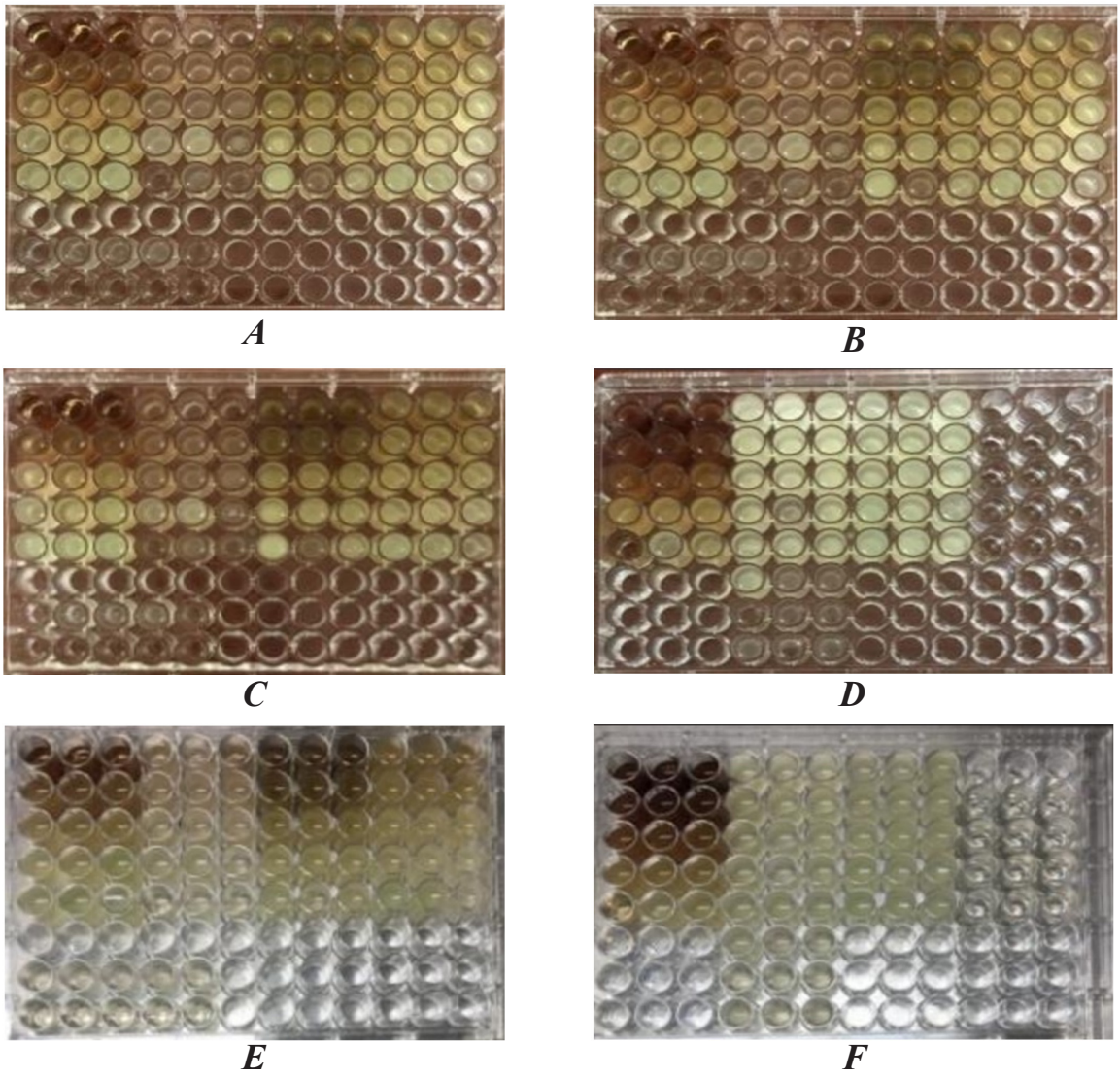

Figure 1. Minimum Inhibitory Concentration (MIC) Candida albicans in samples; (a) Ethanol extract, chloroform fraction, n-hexane fraction, and ethyl acetate fraction incubated for 24 hours; (b) Water fraction and ketoconazole incubated for 24 hours; (c) Ethanol extract, chloroform fraction, n-hexane fraction, and ethyl acetate fraction incubated for 48 hours; (d) Water fraction and ketoconazole incubated for 48 hours; (e) Ethanol extract, chloroform fraction, n-hexane fraction, and ethyl acetate fraction incubated for 72 hours; (f) Water fraction and ketoconazole incubated for 72 hours.

Table 4. Antifungal MFC Measurement Results

Sample	MIC (µg/ml)		
	24 hours	48 hours	72 hours
Ethanol extract	>500	>500	>500
Chloroform fraction	>500	>500	>500
n-hexane fraction	>500	>500	>500
ethyl acetate fraction	>500	>500	>500
Water fraction	>500	>500	>500
Ketoconazole	64	64	64

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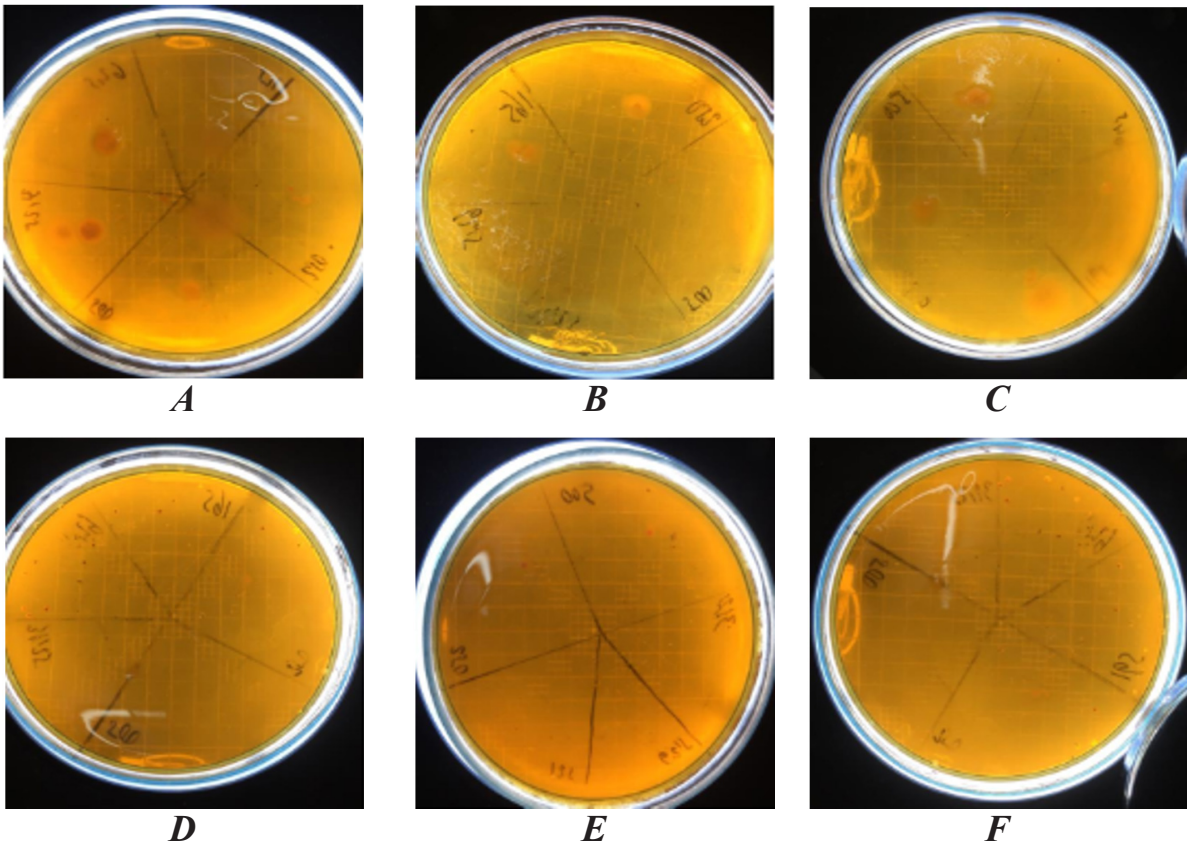

Figure 2. Minimum Fungicidal Concentration (MFC) Candida albicans in samples incubated for 72 hours; (a) Ethanol extract; (b) Chloroform fraction; (c) n-hexane fraction; (d) ethyl acetate fraction; (e) Water fraction; (f) Ketoconazole

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