Antimicosis Chloroform Fraction

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Antimycotic activity of chloroform fraction of ethanol extract soursop leaves (Annona muricata, L.)

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ABSTRACT

Fungal infections are often found in the community, some antifungal drugs have been less effective so it is necessary to look for new candidate drugs as antimycotic, one of the candidates is soursop leaf (*Annona muricata*, L.). Soursop leaves contain compounds alkaloids, polyphenols, flavonoids, triterpenoids that function as antifungals. The purpose of this study was to determine the antifungal activity of the n-hexane fraction of the Soursop ethanol extract of the fungus *Candida albicans* and determine the class of compounds that act as antimycotic. Soursop leaf was extracted by maceration method using ethanol 96% then fractionated by partition method using chloroform solvent. The chloroform fraction was tested for antifungal activity against the fungus *Candida albicans* by diffusion method with a concentration variation of 10%, 20%, and 30%. then identified by UV-VIS and FTIR spectrophotometry to determine active compounds as antifungals. The results showed that the Soursop leaf chloroform fraction had the highest antifungal activity at a concentration of 30% with an inhibitory diameter of 25.4 mm which was categorized strong. Compounds that are suspected to have antifungal activity from the soursop leaf chloroform fraction are terpenoid compounds. The results of this study are expected to provide scientific information to the public about the benefits of soursop leaves as an alternative treatment for fungal infections caused by the fungus *Candida albicans*.

INTRODUCTION

Fungal infections are often found in people in tropical countries including Indonesia because of the hot and humid climate. In general, organisms that cause fungal infections and are at high risk are *Candida* sp.[1] Candidiasis is a primary or secondary infection of the genus Candida especially *Candida albicans*. In general, candidiasis treatment uses azoles, polyenes, and echinocandins.[2] The use of local and systemic antifungal drugs has disadvantages such as amphotericin B has side effects of kidney damage (nephrotoxic) and nystatin cannot provide systemic effects.

Some antimycotic drugs are no longer effective because of resistance. Research in Ilam-Iran shows that 150 isolates of *Candida albicans* have been

resistant to fluconazole, itraconazole, ketoconazole, clotrimazole, voriconazole, posaconazole, and nystatin which illustrate that some antifungal agents are no longer effective.[3] Therefore, the discovery of new drugs is an alternative that must be done to replace synthetic drugs that are no longer effective or that have excessive side effects. One alternative medicine used for candidiasis is the soursop plant (*Annona muricata* L.).

Soursop (*Annona muricata* L.) has been used for generations by some Indonesian people to treat diseases. Soursop leaves are used as an alternative treatment for cancer treatment, namely by consuming soursop leaf boiled water. In addition to the treatment of cancer, soursop plants are also used for the treatment of fungal infections mainly caused by the fungus *Candida albicans*.[4] Soursop leaves contain flavonoid compounds, which compounds can function as antiseptic disinfectants. Soursop leaves contain secondary metabolites such as tannins, steroids, alkaloids, terpenes, acetogenin, flavonoids, and lectins.[5] One of the research showed that Soursop leaf ethanol extract can inhibit the growth of *Candida albicans* ATCC 10231 at the concentrations of 15%, 30%, and 60% indicated with the formation of inhibitory/ clear areas around the well that contains ethanol extract of soursop leaves.[6] Inhibitory zones formed in soursop leaf extract due to the presence of active substances contained in soursop leaves which influence in inhibiting fungal growth.[7]

Research that has been done is not yet known which compounds are most effective in dealing with fungal infections. For this reason, further research needs to be carried out to obtain the active compounds of soursop leaves fractionated with chloroform and to determine its activity as an antimycotic. The purpose of this study was to early identify the active compounds of the chloroform fraction of soursop leaf extract and to test antifungal activity by looking at the MIC and MBC concentration values.

MATERIALS AND METHODS

Materials

The materials that will be used in this study include simplistic leaf *Annona muricata* L (obtained from Materia Medica, Batu, Malang), the fungus *Candida albicans* culture (obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, ethanol (Merck), chloroform (Merck), alcohol, SDA (Saboraud Dextrose Agar) (Merck), SDB (Saboraud Dextrose Broth) (Merck), Aquadest, Nystatin, ketoconazole, DMSO (Dimethyl Sufoxide).

Sample Preparation

Soursop leaves are cleaned of dust and dirt by washing. After that, it is dried in the oven at 45 ^{o}C for 72 hours. Furthermore, it is blended with a blender and sieved to obtain a fine powder.

Soursop Leaf Extraction

Soursop leaf powder 500 grams soaked in 1 L ethanol solvent in the ratio of 1:5 (w/v) solvent made again soaking again with the same ratio. Immersion is carried out for 6 days with several stirring. Maceration results collected and filtered. Concentration is done by rotary evaporator with a temperature of 55^{o} C and rotation speed of 30-80 rpm

Soursop leaf extract fractionation

Fractionation is done by concentrating extracts of soursop leaves dissolved in water, filtered then fractionated with chloroform of 1:1 ratio. Left to form 2 layers, namely chloroform and water layers. This treatment is repeated

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several times until the chloroform layer looks clear. The results of the chloroform fraction were then evaporated with a rotary evaporator and their compounds were identified by a UV-VIS spectrometer, FTIR.

Test Of Antimicosis Activity

Antimycosis activity testing is done by using agar diffusion method wells 6 mm in diameter. A microbial suspension of 0.2 ml was put into a petri dish. Then add 20 ml of SDA media, shake it and shake it allowed to solidify. With the help of a sterile perforator, 3 holes were made in the pit. Furthermore, each well was filled with 0.020 ml extracts of 10%, 20%, 30% concentration, DMSO (negative control) and ketoconazole solution, Nystatin 1 mg / ml (positive control). Pre-incubation is carried out for 30 minutes.

Test of Minimum Inhibition Concentration (MIC) and Minimum Kill Concentration (MKC)

MKC test is done by growing the culture on a positive tube MIC in a pour plate on sterile SDA media. Taken 1 mL of the concentration that showed positive MIC grown on pourA SDA media by pour plate, incubated at 37ºC for 24 hours. MKC is indicated by the absence of a growing fungus colony on the media.

RESULTS AND DISCUSSION

Soursop leaf extraction

At this stage, the extraction of soursop (*Annona muricata* L.) leaves was done by the maceration method. This method was chosen because the method of work and equipment used is simple and maximizes contact between the solvent and the material and can be used for substances that are resistant or not resistant to heating. The maceration process is carried out in a dark glass container and not exposed to direct sunlight to avoid the reaction that is catalyzed by light and discoloration.[8] The solvent used in the maceration process is ethanol 96%. Ethanol solvents can damage cell walls in samples so that polar compounds can be dissolved in ethanol.[9]

During the maceration process, a diffusion process occurs. The advantage of extraction using maceration methods is that the procedures and equipment used are relatively simple, the operational costs are relatively low and are carried out at room temperature without a heating process.[10] The use of ethanol 96% makes it easier to separate compounds because the higher the concentration of ethanol, the easier it is to separate the secondary metabolite from the sample.[11] Soursop leaves of 500 grams were extracted maceration using 1 L 96% ethanol by soaking for 24 hours while stirring occasionally. This process is repeated up to 5 times to get maximum extraction results. The concentration of the extract with a rotary evaporator to produce a concentrated green extract.

Fractionation of soursop leaf extract with chloroform solvent

Concentrated extract of soursop leaves then carried out the fractionation process by liquid-liquid extraction. The purpose of the fractionation step is to separate compounds based on different levels of polarity in two solvents that have different levels of polarity. Fractionation by liquid-liquid extraction is carried out by shaking using a separating funnel.[12] The principle of separation in the fractionation process is based on differences in the level of the polarity of the solvent and the difference in specific gravity between the two fractions. Fractionation was carried out between concentrated extracts with a chloroform

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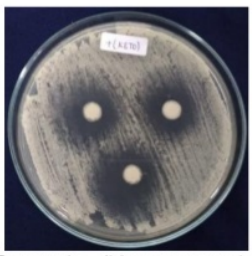
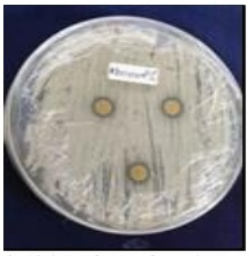
solvent of 1: 1 and formed two layers, namely the water fraction (top) and the Chloroform fraction (bottom). Furthermore, the chloroform fraction was concentrated with a rotary evaporator to produce a concentrated fraction. Subsequent fractionation results were tested for antifungal activity and identified using UV-VIS and FT-IR spectrophotometry.

Inhibitory Test of Soursop leaf chloroform fraction

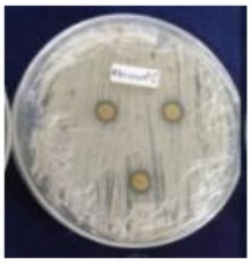
The chloroform fraction was tested in vitro for *Candida albicans*. Antifungal activity is carried out by the method of disk diffusion using disc paper. The inhibition zone is measured by looking at the clear zone around the disc paper that has been dropped by the chloroform fraction. Inhibition zone measurements were carried out using the bar to determine the strength of the fraction inhibition against the growth of the fungus *Candida albicans*.[13]



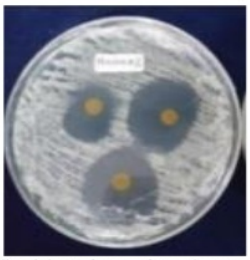
a) Control + (Ketoconazole)



(b) chloroform fraction 10%



(c) chloroform fraction 20%



(d) chloroform fraction 30%

Figure 1. Results of inhibition zones Soursop leaf chloroform fraction (Source: Rustanti, 2019)

In the inhibition test variations of 10%, 20%, and 30% are made, the aim is to find the concentration of an effective chloroform fraction as an antimycotic. Fungal colonies that grew in each treatment showed a noticeable difference from the inhibition zone after 24-hour incubation. Inhibition zone diameters can be seen in table 1.

Table 1. Results of inhibitory test of Soursop leaf chloroform fraction

Treatment group	Inhibitory Zone Diameter (mm)
chloroform fraction 10%	10.15
chloroform fraction 20%	9.15
chloroform fraction 30%	25,4
Control + (Nystatin)	15,9
Control + (Ketoconazole)	22,5
Control - (DMSO)	1,88

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Based on the inhibition zone diameter in Figure 1. and Table 1. When compared with the colony resistance response category it can be seen that the highest inhibition zone is found in the Chloroform Faction concentration of 30% with a very strong average resistance category of 25.4 mm. The test fraction used contained active compounds with the most effective concentrations thus providing large clear zone because it can diffuse well into the SDA media so that it can inhibit the growth of the fungus *Candida albicans*.[14]

Test Of Minimum Inhibition Concentration (MIC) and Minimum Kill Concentration (MKC)

In this study, the determination of the MIC and CBC were carried out using the liquid dilution method. Determination of the MIC value itself is done using UV-VIS spectrophotometry and the MKC price is done by planting the MIC results into agar media. The principle of liquid dilution uses spectrophotometry, namely measurement of levels of turbidity levels of antifungal substances to obtain the MIC. The turbidity value is indicated by the absorbance or optical density (OD) seen from the spectrophotometer.[15] The positive control used by media and test mushrooms. While the negative controls used are the media and test extracts. Determination of Minimum Inhibitory Levels (MIC) and MKC is done by looking at the difference in absorbance before and after incubation. The lowest concentration that can inhibit the growth of fungi is shown by the difference in absorbance after and before incubation ≤ 0, the MIC is obtained.[16] A value of ΔOD ≤ 0 indicates that there is no fungus growth, meaning that the ability of the fungus to divide itself is inhibited. A value of ΔOD> 0 indicates no inhibition of the growth process of fungus. Whereas in the MKC test the success rate was indicated by the absence of a growing fungus colony. The lowest concentration that is able to kill the test fungus is stated as MKC.[17]

Table 2. Test of the Results MIC and MKC

Concentration extract (mg / mL)	The average OD value of MIC			Value of MKC (Grow/not grow)	Cell count growing fungus
	Before incubation	After incubation	ΔOD		
control (+)	0,290	1,293	1,003	Grow	6,08 x 107
control (-)	0,360	0,784	0,424	Grow	2,70 x 107
10	0.096	1,160	1,064	Grow	6,63 x 107
20	0,090	0,976	0,886	Grow	5,38 x 107
30	0,259	0,868	0,609	Grow	4,08 x 107

Based on Table 2. it can be observed that there is a decrease in OD values along with the increase in extract concentration. This is thought to be due to an inhibition of the growth of the fungus *Candida albicans* although not significant enough as evidenced by the value of the number of growing fungal cells. Where there is a decrease in the number of fungal cells along with an increase in the concentration of extract used. This is by the statement of research, where the higher the concentration of the extract, the more active antimicrobial compounds contained so that the ability to inhibit microbial growth is also higher.[18] OD values that have not yet reached this target (ΔOD ≤ 0) are possible because the concentration of the chloroform fraction used is not high enough, so the fraction has not been able to inhibit the growth of the fungus *Candida albicans* optimally. The active compounds contained in the fraction have

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not been able to offset the growth of the fungus *Candida albicans* so that the MIC and MKC values have not been found.

The fraction that has been known to have the most optimal antimycotic activity against *Candida albicans* is the 30% chloroform fraction. Content analysis of its compounds using UV-VIS and FTIR. identification of active compounds by UV-VIS spectrophotometry was used to measure the absorption of light in the UV region and visible light by a compound, while FTIR was used to determine compounds based on functional groups. The results of UV-VIS uptake in methanol solvents show the wavelength uptake of 203 nm, shown in Figure 2.

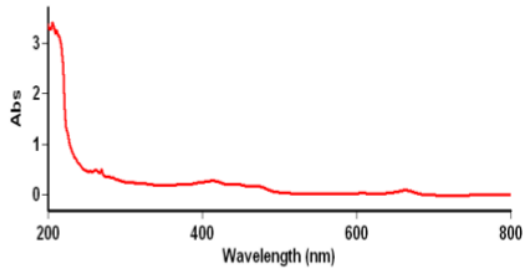

Figure 2. UV-VIS Spectrum of the Chloroform Fraction (Source: Rustanti, 2019)

The appearance of uptake at 203 nm wavelengths is one of the characteristics of triterpenoid compounds where the uptake that appears is the absorption of cut off solvent used. Methanol absorbs at wavelengths shorter than 185 nm and is therefore commonly used for solvents in the UV region. When methanol is used as a solvent, strong uptake extends into the near UV region, resulting in a cut-off in the 200-220 nm region.[19] This is confirmed by the results of the analysis with the FTIR spectrophotometer in Figure 3 which shows the functional groups contained in the isolates are as follows:

Figure 3. The Spectrum of FTIR of Chloroform fraction (Source: Rustanti, 2019)

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The results of the FT-IR absorption pattern analysis show that there is absorption at wave number 3421.16 cm-1 which indicates the existence of stretch vibrations in the hydroxy group (OH) strengthened by the presence of O-H absorption at wave number 667.23 cm-1. The existence of aliphatic C-H stretch absorption is indicated by the sharp absorption with strong intensity at wavenumbers 2924.50 cm-1 and 2855.29 cm-1. This gives a clue to the possibility of methyl (CH3) and methylene (CH2) groups which are reinforced by the existence of C-H buckling vibrations at wave number 844.82 cm-1. This assumption is reinforced by the absorption of the wave numbers 1458.54 cm-1 and 1383.11 cm-1 which is the vibration vibrations of -CH2 and -CH3 which indicate the presence of terpenoid compounds (atmoko). Strong absorption in the region of wave number 1741.18 cm-1, presumably due to the presence of the functional group C = O of the ester is amplified at wave number 1078.64 cm-1 which is a stretching vibration of C-O. The absorption at wave number 1649.67 cm-1 indicates the presence of C = C alkenes. According to the results of the FT-IR absorption pattern analysis of compounds that have O-H, C-O, C-H, C = C, and C = O functional groups indicate the presence of terpenoid compounds but need further identification using NMR and MS spectrometry.[20]

CONCLUSIONS

Chloroform fraction of soursop leaf extract has the antifungal activity to the growth of *Candida albicans*. The minimum inhibitory concentration (MIC) of the Chloroform fraction is a 30% concentration with an inhibition zone of 25.4 mm which is greater than ketoconazole. The results of the identification of the Soursop leaf chloroform fraction that functioned as an antimycotic were thought to be terpenoid compounds.

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