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Research Paper

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Natural Antifungal: Garlic Extract's Activity Against *Candida parapsilosis* in Otomycosis

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Abstract

Background: Otomycosis is a common ear infection with a high recurrence rate due to resistance to standard antifungal drugs. Alternative therapies are needed, one of which is using garlic (*Allium sativum*) which is known to have antifungal effects. **Objectives:** [This study aims to evaluate the activity of garlic extract (*Allium sativum*) as an antifungal against the growth of *Candida parapsilosis* fungus from otomycosis patients in vitro. **Methods:** [A true experimental study with a posttest only control design using the microdilution method to determine the minimum inhibitory concentration (MIC) and the minimum fungisidal concentration (MFC). The sample consisted of 9 treatment groups, including garlic extract concentrations (6.25 mg/mL, 12.5 mg/mL, 25 mg/mL), ketoconazole positive control, 10% DMSO control, and negative control. Data analysis uses the Kruskal Wallis and Post-Hoc Mann Whitney tests. **Key findings:** The results of univariate analysis showed significant differences in each treatment group with bivariate analysis showing a significant relationship between extract treatment on fungal growth inhibition in the growth inhibition of *Candida parapsilosis* fungus with p-value P=0.021 (P<0.05). The value of MIC was found at a concentration of 6.25 mg/mL, while MFC was at a concentration of 12.5 mg/mL with an inhibition percentage of 99.67%. **Conclusions:** Garlic extract has the potential as an alternative antifungal therapy against infections caused by *Candida parapsilosis*.

Keywords: *Candida parapsilosis*, Garlic extract, MFC, MIC, Otomycosis

Introduction

Fungal infections are a common health problem in Indonesia, including otomycosis, which is a fungal infection of the external auditory canal with a high prevalence in tropical and subtropical regions [1]. One of the causes of otomycosis is Candida parapsilosis, which has a global prevalence rate of 14.96% and a mortality rate of 20-45% [2]. *Candida parapsilosis* is difficult to treat due to its ability to form biofilms that hinder antifungal penetration and cause drug resistance, particularly to the azole and echinocandin classes [3].

Herbal treatments, such as the use of garlic (Allium sativum) extract, have become a promising alternative. The bioactive compound allicin found in garlic has been proven to have antifungal effects by inhibiting biofilm formation and increasing oxidative stress in the fungus [4]. Previous studies have shown the effectiveness of garlic against Candida albicans, but research on *Candida parapsilosis* is still limited. Therefore, further studies using the microdilution method are needed to evaluate the antifungal activity of garlic extract against *Candida parapsilosis* at concentrations of 6.25%, 12.5%, and 25%. The results of this research are expected to provide valid data to support the use of garlic as an alternative therapy for otomycosis.

Materials and Methods

This study uses a true experimental design method with a posttest only control design. The research will compare a control group that does not receive treatment with a test group that receives treatment. The treatment involves administering garlic extract. The control groups consist of a negative control, positive control, media control, and solvent control, while the treatment groups include several treatments that will be carried out related to the concentration level of administration and the type of solvent used. The garlic extract treatment groups will be compared to the negative control group to evaluate fungal inhibition and eradication. Phytochemical screening of the extract is based on color reaction and the inhibitory effect of garlic extract (*Allium sativum*) against *Candida parapsilosis* using the microdilution method.

Tools and Materials

The equipment used in this study includes an oven (Binder GmBH), incubator (Memmert incubator), autoclave (All American Model No 25 X), evaporator, petri dishes (Normax, Pyrex), stirring rod, inoculating loop (Germany handle), bunsen burner, sterile cotton swab, alcohol burner, matches, micropipette (Finnipipette), bluetip, erlenmeyer

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flask (Schott Duran), racks and test tubes (Iwaki), vortex (Vortex mixer K), measuring glass (Iwaki, Therma), 96-well microplate, centrifuge, digital scale (Ohaus), label paper, tissue, aluminum foil, object glass, cover glass, densitometer.

The materials used in this study are 96% ethanol garlic extract, Sabouraud Dextrose Agar, Roswell Park Memorial Institute 1640 (RPMI 1640) media, *Candida parapsilosis* fungal culture, distilled water, crystal violet, safranin, acetone alcohol, garlic simplicia, potassium iodide, Phosphate Buffer Saline (PBS), Dimethyl sulfoxide (DMSO 100%), ketoconazole suspension, and garlic simplicia commercially obtained from PT. Phyto Chemindo Reksa, a factory certified by the National Agency of Drug and Food Control (BPOM) for CPOTD for the use of dried extracts.

Research Procedure

The research begins with the extraction of garlic (500 grams simplicia) using the maceration method and 96% ethanol (6.339 L) as the solvent, followed by phytochemical screening using the Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS) method. The extract is then diluted with 10% DMSO to the desired concentration. Antifungal activity testing is carried out using 96-well microplate media for MIC measurements and SDA media for MFC measurements. The working suspension of Candida parapsilosis, resulting from subculturing and prepared according to the 0.5 McFarland standard, is then inoculated into the 96-well microplate, with four repetitions, and incubated for 24 hours. The MIC is measured by assessing the clarity of each well. MFC is determined by planting each treatment onto SDA media and incubating with measurements of colony growth and inhibition percentage at 24 hours and 48 hours. The number of colonies and inhibition percentage can be calculated with the following formula:

$$\frac{\text{number of colonies}}{\text{mL}} = \frac{\text{A x } 10^{a}}{\text{n}}$$

A = number of Candida albicans colony
a = dilution factor
n = the volume of fungal suspension poured onto the agar

(a)

$$\text{inhibition percentage} = \frac{\text{number of treatment colony 0\%-number of each concentration colony}}{\text{number of treartmen koloni 0\%}} \text{ x } 100\%$$

(b)

Figure 1 Formula for Calculating the Number of Colonies and Inhibition Percentage. Explanation: a. Formula for calculating the number of colonies b. Formula for the percentage of colony inhibition

Data Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) version 29. The analysis began with a univariate descriptive analysis, followed by a bivariate analysis. Normality was tested using the Shapiro-Wilk test and homogeneity of variance was assessed with Levene's Test, followed by the Kruskal-Wallis test to compare the average number of colonies between treatment and control groups. The Post-Hoc Mann-Whitney test was used to assess significant differences between groups in pairs. A significant difference with a p-value <0.05 indicates a distinct effect among the groups in antifungal activity against Candida parapsilosis.

Results

The morphological identification of *Candida parapsilosis* colonies can be recognized by their white to cream color, smooth and shiny appearance, and round shape. The Germ tube test was also performed, resulting in a negative Germ tube interpretation, indicated by the presence of germination in the yeast cells with constriction and short pseudohyphae, while Gram staining revealed oval-shaped yeast cells. The results of the phytochemical tests in this study showed that the garlic extract used contains bioactive antifungal compounds, as shown in Table 1. Several identified compounds are derivatives from the allicin and saponin groups. The maximum area value indicates the abundance of compounds in the sample (NCBI, 2024).

Table 4. In colony plate 1, a mean standard deviation of 2.00 (± 1.826) was observed; in colony plate 2, it was 1.00 (± 0.816); in colony plate 3, it was 3.50 (± 2.887); in colony plate 4, it was 5.00 (± 1.826); in colony plate 5, it was 9.00 (± 7.528); in colony plate 6, it was 1.50 (± 1.291); in colony plate 7, it was 83.50 (± 68.937); in colony plate 8, it was 90.5 (± 66.756); and in plate 9, the colony count was 456.50 (± 375.47). The differences in the mean (SD) values are due to variations in treatment and the density of fungal colonies inoculated on each plate. Table 5. Based on the results of the normality test using the Shapiro-Wilk test, the data distribution is normal because the significance level meets the requirements with p>0.05.

Table 6. The results of the homogeneity test showed p = 0.000 (p < 0.05), indicating that the data were not homogeneous. The data were then subjected to a transformation process using the Log10 formula, but there was no significant change. Therefore, the analysis was continued with the non-parametric Kruskal-Wallis test to determine the differences between groups. The results of the Kruskal-Wallis test showed a significance value of P =

0.003 (P < 0.05), indicating a significant difference between groups, and the data testing could proceed with a Post Hoc test, namely Mann Whitney (Table 6), to identify the most significant group. The results of the Post-Hoc Mann-Whitney test in Table 6 showed a significance value of P = 0.000 (P < 0.05) for the negative control, indicating a significant difference between each group tested and the negative control in the study of the effectiveness of garlic extract in inhibiting the growth of *Candida parapsilosis*.

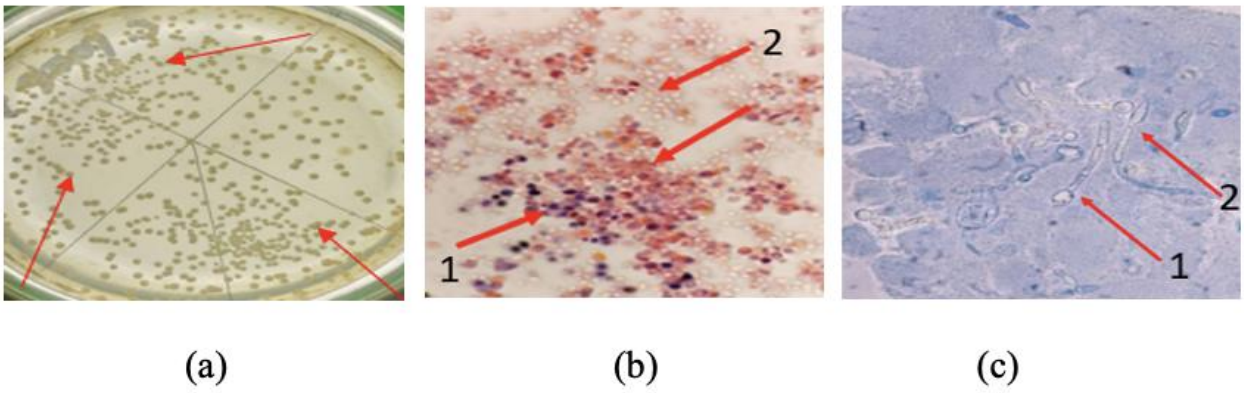

Figure 2 Results of Colony and Cell Identification of Candida parapsilosis**.** Description: a. Colony of Candida parapsilosis on SDA Medium: Cream-colored, Shiny (b) Candida parapsilosis Gram Staining: (1) Yeast Cells (2) Budding Stage; (c) Candida parapsilosis Germ Tube Test: (1) Cell Germination (2) Short Pseudohyphae

Discussion

Candida parapsilosis is a type of yeast that is not an obligate pathogen for humans. Colonies of *Candida parapsilosis* can be grown on SDA medium, and the identification results obtained in this study on SDA medium showed that the colony morphology of *Candida parapsilosis* can be identified by its white-cream color, smooth and shiny appearance, and round shape. In the cell morphology test using the germ tube test and Gram staining, the interpretation result was germ tube negative, with germination observed in the yeast cells marked by temporary constriction. Gram staining showed oval-shaped yeast cells [5].

Table 1 Compound content results of LC-HRMS test

Compound found	Chemical structure	Group of compounds	Area size
S-allylcysteine	C11-H18-N2-O5-S	*Allicin*	4,45E+09
Diallyl disulfide	C7-H12-O-S3	*Allicin*	5673140,499
gitogenin	C27-H44-O4	*Saponin*	1,22E+08
5β-Spirostan-3β-yl β-D-glucoside	C33-H54-O8	*Saponin*	5,1E+08

Table 2 Results of microplate observation of minimum inhibitory concentration

Microplate bar	Concentration (mg/mL)	Turbidity level
1	Ketoconazole 16 µg/mL	Clear
2	Ketoconazole 8 µg/mL	Clear
3	Ketoconazole 4 µg/mL	Clear
4	Ketoconazole 2 µg/mL	Clear
5	Ekstrak 25 mg/mL	Clear
6	Ekstrak 12,5 mg/mL	Clear
7	Ekstrak 6,25 mg/mL	Medium
8	DMSO 10%	Cloudy
9	Negative	Cloudy

This study aims to test the effectiveness of garlic extract at concentrations of 6.25 mg/mL, 12.5 mg/mL, and 25 mg/

mL in inhibiting the growth of the fungus Candida parapsilosis, based on the measurement of minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). The results of this study showed that a garlic extract concentration of 6.25 mg/mL can be determined as the MIC because, at that concentration, it was already able to inhibit the growth of *Candida parapsilosis* by 81.69%.

Table 3 Observation results of fungal colony count and inhibition percentage of garlic extract against *Candida parapsilosis* fungus

Plate	Number of Fungal Colonies	TPC (CFU/mL)	Percentage of inhibition
1	2	200	99,56%
2	1	100	99,78%
3	3,5	350	99,23%
4	5	500	98,91%
5	9	900	98,03%
6	1,5	150	99,67%
7	83,5	8.350	81,69%
8	90,5	9.050	80,14%
9	456,5	45.650	0%

Table 4 Results of univariate analysis

Colony group	Number of colonies	
	Average(±SD)	Minimum-maximum
Plate 1	2.00 (± 1.826)	0 - 4
Plate 2	1. 00 (± 0.816)	0 - 2
Plate 3	3.50 (± 2.887)	0 - 7
Plate 4	5.00 (± 1.826)	3 - 7
Plate 5	9.00 (± 7.528)	0 - 18
Plate 6	1.50 (± 1.291)	0 - 3
Plate 7	83.50 (± 68.937)	0 - 167
Plate 8	90.5 (± 66.756)	9 – 172
Plate 9	456.50 (± 375.47)	191 - 722

Table 5 Saphiro-Wilk data normality test results

Variable	*p-value*	Interpretation
Positive Control (Ketoconazole 16 mg/mL)	0,714	Normally distributed
Positive Control (Ketoconazole 8 mg/mL)	0,683	Normally distributed
Positive Control (Ketoconazole 4 mg/mL)	0,962	Normally distributed
Positive Control (Ketoconazole 2 mg/mL)	0,714	Normally distributed
Garlic Extract 25 mg/mL	0,999	Normally distributed
Garlic Extract 12,5 mg/mL	0,972	Normally distributed
Garlic Extract 6,25 mg/mL	0,968	Normally distributed
Solvent control DMSO 10%	0,874	Normally distributed
Negative control	0,118	Normally distributed

These results are consistent with research conducted by Barbu et al. (2023), which stated that garlic extract at a concentration of 6.25 mg/mL already exhibits antifungal activity by inhibiting the growth of *Candida parapsilosis* [6]. A garlic extract concentration of 12.5 mg/mL tested in this study was determined to be the MFC, as it was able to kill 99.67% of fungal cells, in accordance with research by Barbu et al. (2023), which stated that garlic extract at a concentration of 12.5 mg/mL is already able to kill *Candida parapsilosis* fungal cells [6].

The results of this study indicate that the higher the concentration of garlic used, the fewer colonies of *Candida parapsilosis* there are, and the higher the percentage of inhibition of *Candida parapsilosis* growth. The MFC value was obtained at a concentration of 12.5 mg/mL, and the MIC at a concentration of 6.25 mg/mL, resulting in an MFC to MIC ratio of 2 (MFC/MIC≤4). According to Mogana et al. (2020), this ratio indicates that the garlic extract used is fungicidal [7].

Data obtained from the measurement of MIC and MFC

were analyzed using statistical tests to determine the significance of differences between each group. The results of the univariate analysis showed differences in the mean values (SD) due to differences in treatment and density of fungal colonies grown on each plate. Plates 1-4 were the treatment groups using antifungal drugs, and plates 5-7 were the treatment groups using garlic extract at concentrations of 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL, respectively. Treatments on plates 1-6 showed differences in mean values that were not too significant due to inhibited growth of the observed fungal colonies, while plate 8 was treated with 10% DMSO as the extract solvent, and plate 9 was a negative control group without treatment. This caused a significant difference in mean values between plate 9 and plates 1-7, as the colonies observed on plate 9 could grow without any inhibitory effect.

Table 6 Bivariate analysis results of all treatment groups

Test	Variable	*p-value*	Interpretation
Lavene's Test	All groups	0,000	The Variance is Not Homogeneous
Kruskall-Wallis	All Groups	0,003	Significant differences between groups
Post-Hoc Mann-Whitney U	Negative control with another group	0,000*	Significant differences were observed; the negative control showed notable fungal growth activity compared to the other groups.
	Between groups other than the negative control	1,000	There is no significant difference, no noticeable fungal growth activity

The data obtained from the univariate analysis was then tested for normality using the Shapiro-Wilk test. The results of this test (Table 4) showed that the data from this study were normally distributed, as the significance levels of each group met the requirement ($p>0.05$). The data was subsequently tested for homogeneity among the groups using Levene's Test in table 4.4. The significance value (Sig.) obtained was 0.000 for all calculation methods (Mean, Median, Median with adjusted df, and Trimmed Mean). Since the significance value (Sig.) was < 0.05, it can be concluded that the data did not fulfill the assumption of homogeneity of variance between groups, as there were significant differences in variance among the tested colony groups. Therefore, data transformation using the Log10 formula was performed, but no significant changes were found. Bivariate analysis continued with the non-parametric Kruskal-Wallis and Post-Hoc Mann-Whitney tests to evaluate the significance of the differences between each group.

The results of bivariate analysis using the Kruskal-Wallis test and the Mann-Whitney Post-Hoc test showed a significant relationship between each treatment group, including the group receiving garlic extract, with inhibitory effects on the growth of the fungal species Candida parapsilosis. This was indicated by a significance value of P=0.021, which is below the threshold of P=0.05. This suggests that the compounds in garlic extract have the potential to inhibit the growth of *Candida parapsilosis* with an effectiveness almost comparable to the standard antifungal drug. The antifungal used in this study was an azole-class drug, ketoconazole, which also had a significance value of P=0.021 (P<0.05) and demonstrated inhibitory effects on the growth of Candida parapsilosis.

The inhibitory effect of garlic extract may occur because garlic contains active compounds such as allicin, flavonoids, and saponins, which act as antifungal agents. Allicin works by disrupting the structure of the fungal cell wall and plasma membrane, while saponins alter the permeability of microbial cell wall structures, affecting the growth and biofilm formation of fungal cells as a cellular defense mechanism. Allicin derivatives found in garlic—S-allylcysteine and Diallyl disulfide—have shown antifungal activity against *C. parapsilosis*. S-allylcysteine and Diallyl disulfide are precursor compounds to allicin and work by inhibiting the formation of chitin in bacterial cell walls, thereby disrupting the structure of the cell wall and organelle membranes such as mitochondria, which leads to organelle damage and cell death [8].

Saponin compounds work by disrupting cell membrane permeability and causing leakage of cellular components. Saponin derivatives present in garlic include gutein and 5β-Spirostan-3β-yl β-D-glucoside. The mechanism of gutein and 5β-Spirostan-3β-yl β-D-glucoside involves disrupting the fungal cell membrane by increasing its permeability, resulting in cellular leakage and ultimately cell death. These compounds also inhibit the ergosterol biosynthesis pathway, which is an important component of the fungal cell membrane, thus compromising the integrity of the cell wall [9].

The garlic extract in this study was prepared using the maceration method, by soaking the material in 96% ethanol solvent. The maceration extraction method with 96% ethanol was chosen because it is a universal extraction method suitable for both polar and non-polar compounds, and for easily degradable compounds such as allicin, flavonoids, and saponins. During the soaking process, the

cell walls and membranes break down due to differences in pressure inside and outside the cells, causing secondary metabolites in the cytoplasm to be released and mix with the solvent used to make the garlic extract [10].

The solvent chosen for this study was 10% dimethyl sulfoxide (DMSO) because garlic extract cannot be dissolved with ordinary solvents such as distilled water. Ten percent DMSO can dissolve both polar and non-polar compounds, is clear, and non-toxic. DMSO used as a solvent should not affect fungal growth activity. However, in this study, the 10% DMSO used as the extract solvent actually showed inhibitory effects on the growth of Candida species fungi, similar to the results reported by de Sousa et al. (2020), which mentioned that the higher the concentration of DMSO used as a solvent, the greater the inhibitory effect on Candida species fungi, including *Candida parapsilosis* as the test fungus [11]. According to Marfan et al. (2024), if the use of 10% DMSO produces inhibitory effects, the concentration should be reduced to 1%, since at this concentration, it has not shown inhibitory effects on the growth of Candida species [12].

Conclusion

Based on the research results, it was found that there is a significant relationship between the administration of garlic extract and the inhibition of *Candida parapsilosis* growth. The minimum inhibitory concentration (MIC) of garlic (*Allium sativum*) extract against *Candida parapsilosis* is 6.25 mg/mL, and the minimum fungicidal concentration (MFC) of garlic (*Allium sativum*) extract against *Candida parapsilosis* is 12.5 mg/mL.

Supplementary Material

None

Author Contributions

DKF : Conceptualization, Methodology, Writing-Original Draft. **APKNW** : Data Curation, Formal Analysis, Visualization. **WDK** : Supervision, Writing- Review & Editing.

Conflict of Interest

A The authors have no financial conflicts of interest to declare.

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