Pharmacology and Herbal Medicine Journal, 2026, **3**, 81–86
DOI: 10.20884/1.phmj.2026.1.3.19652
Research Paper

PHMJ PHARMACOLOGY AND HERBAL MEDICINE JOURNAL

In Vitro Antifungal Activity Test of Garlic Extract (*Allium sativum* L.) Againts *Aspergillus niger*

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Abstract

Background: Aspergillosis that caused by *Aspergillus niger* is one of the leading causes of death in Aspergillosis cases, with the highest mortality rate. Resistance of *A. niger* to various antifungal drugs continues to increase each year. Garlic (*Allium sativum* L.) is a potential alternative antifungal therapy due to its bioactive compounds such as allicin, saponin, flavonoid, and tanin. **Objectives:** To determine the antifungal activity of garlic (*Allium sativum* L.) extract at concentrations of 40%, 60%, 80%, and 100% against *A. niger* isolates in vitro. **Methods:** This study used a true experimental posttest-only control group design. The antifungal activity was assessed using the well diffusion method with garlic extract concentrations of 40%, 60%, 80%, and 100%, along with positive, negative, and 10% DMSO controls. Data were analyzed using the Kruskal-Wallis test due to the non-normal and non-homogeneous distribution, followed by the Games-Howell post hoc test. **Key findings:** The garlic extract produced inhibition zones that were considerably smaller than those in the positive control group. For this research, wells with a diameter of 6 mm were used. The inhibition zones for 40%, 60%, 80%, and 100% concentrations were 7.25 mm, 6.5 mm, 6 mm, and 6.75 mm, respectively. The positive control (itraconazole 8 µg/mL) produced an inhibition zone of 17 mm. **Conclusions:** Garlic extract did not exhibit significant antifungal activity against *A. niger*, as indicated by the minimal inhibition zones compared to the positive control.

Keywords: *Allium sativum* L., *Aspergillus niger,* Antifungal, Garlic

Introduction

Fungal infections occur in more than 150 million cases worldwide annually, with a mortality rate of 1.7 million people [1]. In Indonesia, which has a tropical climate, more than 6 million people are affected by fungal infections every year [2]. Fungal infections caused by *Aspergillus* spp. in cases of invasive aspergillosis have affected more than 2,113,000 people worldwide, with an annual incidence reaching 1,837,272 cases, and 340,000 (18.5%) of these resulting in death [3-4]. Aspergillosis caused by *Aspergillus niger* has a poorer prognosis and a high mortality rate of 75%. In Banyumas itself, the prevalence of *Aspergillus niger* infections causing otomycosis reaches 16.1% [5-7].

In a study conducted by Ali et al. (2018), *A. niger* showed the highest sensitivity to voriconazole at 98%, followed by amphotericin B at 92%, terbinafine at 73%, and itraconazole at 55% [8]. Conversely, resistance has begun to occur against clotrimazole (30%), ketoconazole (55%), and is highest against fluconazole at 100%. The first-line drug for *A. niger*, voriconazole, is very expensive, costing up to 20 times more than other azole groups such as fluconazole or itraconazole [7]. Therefore, the use of herbal medicine as an alternative treatment that is more affordable yet highly sensitive needs to be considered.

Garlic (*Allium sativum* L.) is a natural ingredient that possesses anti-inflammatory, antidiabetic, antimicrobial, and anticancer effects. Garlic also has antifungal properties [9]. The compounds found in garlic include allicin, tannins, saponins, and flavonoids. These four compounds work together to damage the fungal cell membrane, while allicin also functions to denature the nucleic acids in fungi [10-11].

This research was conducted because the author identified a gap in the methods that have not yet been studied. This study aims to determine the activity of garlic extract obtained through the maceration extraction method and antifungal activity testing using the well diffusion method. Maceration was chosen because it can yield a higher and purer content of active compounds compared to direct pressing. Meanwhile, the well diffusion method was selected because the diffusion process of the extract can spread more evenly from the surface to the bottom of the culture media [12]. The use of the maceration method to obtain garlic extract and the well diffusion method is expected to provide an additional contribution to previous research. The inhibitory power of garlic extract was assessed at concentrations of 100%, 80%, 60%, and 40%, aiming to observe the activity of garlic extract in inhibiting the growth of *A. niger*.

Received: November 10, 2025. **Revised**: December 17, 2025. **Accepted**: 21 January 2026
© 2026 Faculty of Medicine, Jenderal Soedirman University. *PHMJ* 2026; 3:81-86; doi: 10.20884/1.phmj.2026.1.3.19652

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Materials and Methods

This research employs a true experimental method with a post-test only control group design approach. This study includes both treatment groups and control groups. The treatment groups consist of varying concentrations of garlic extract (40%, 60%, 80%, and 100%), while the control groups consist of a positive control (itraconazole 8 µg/mL), a negative control (distilled water), and a solvent control (10% DMSO).

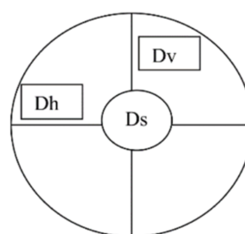
Tools and Materials

Tools used in this research include: micropipettes, vortex, Bunsen burners, lighters, oven, inoculation loops (ose), petri dishes, vernier calipers, Thin Layer Chromatography (TLC), cork borer, scales, autoclave, trays, timers, incubator, labels, stationery, camera, tissues, pipettes, maceration vessels, rotary evaporator, hot plate heater, measuring cylinders, Erlenmeyer flasks, test tube racks, refrigerator, test tubes, tweezers, glass objects, rubber bands, magnetic stirrer, and a water bath heater.

Materials used in this study are: Mueller Hinton Agar (MHA) media (Oxoid brand), Sabouraud Dextrose Agar (SDA) media (Oxoid brand), 500 grams of garlic simplicia obtained commercially from PT. Phytochemindo Reksa (BPOM certified), *Aspergillus niger* cultures from otomycosis patients at the Microbiology Laboratory of FK Unsoed, lugol, spiritus, 10% KOH, Lactophenol Cotton Blue (LPCB) solution, 96% ethanol, 10% Dimethyl Sulfoxide (DMSO), 1 strip of itraconazole capsules, sterile distilled water, physiological NaCl, yellow tips, blue tips, plastic wrap, sterile cotton swabs, aluminum foil, and filter paper.

Research Procedures

The research began with the extraction of 500 grams of garlic simplicia using the maceration method with 6.339 liters of 96% ethanol solvent. The extraction results were then phytochemically tested using Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) and subsequently dissolved in 10% DMSO to achieve the desired concentrations. The antifungal activity test was performed on MHA media inoculated with *Aspergillus niger* spores adjusted to a standard McFarland 0.09–0.13, using wells with a diameter of 6 mm. Each treatment was repeated four times and incubated for observation at 24 and 48 hours. The measurement of the clear zone formed was conducted using the following formula.



$$x = \frac{(Dh - Ds) + (Dv - Ds)}{2}$$

Description:
Dh: Horizontal diameter
Dv: Vertical diameter
Ds: Well diameter
x: Inhibition zone diameter

Figure 1. Inhibition Zone Measurement Formula [13]

Data Analysis

Data were analyzed using SPSS software to determine whether there were significant differences in antifungal activity between the negative control, solvent control, positive control, and various concentrations of garlic extract in inhibiting the growth of *Aspergillus niger*. The obtained research data were analyzed using the Shapiro-Wilk normality test and the Levene's test for homogeneity. If the resulting data were not normally distributed, the non-parametric Kruskal-Wallis test was applied. Following this, a post-hoc test was conducted to determine the significance of the data obtained. The Games-Howell post-hoc test was utilized because the data were not homogeneous. Data are considered significant if the p-value is < 0.05, indicating a 95% confidence level, allowing the research hypothesis to be accepted.

Results and Discussion

The identification results of the compounds from the garlic extract found in this study are shown in Table 1.

Table 1. Identification of Antifungal Compounds in *A. sativum* L. Extract

Compound	Group	RT (minutes)	Maximum Area
γ-Glutamyl-S-Allylcysteine	Allicin	2.413	4451891217.70111
S–Allyl–L-cysteine	Allicin	2.418	120471379.148629
γ–Glutamyl–3-[(1E)-1-propen–1-ylsulfinyl]alanine	Allicin	1.019	45633521.9329485
(1E)–1-(Allyldisulfanyl)–3-(methylsulfinyl)-1–propene	Allicin	7.72	5673140.49949431
(25S)-5β-spirostan-3β-yl β-D-glucoside	Saponin	9.344	509577801.043937
Gitogenin	Saponin	9.615	86777956.8159613
Diosgenin	Saponin	12.597	47850264.0647479
(3β,5β,25R)-Furost-20(22)-en-3,26-diol	Saponin	10.521	28771624.8314826

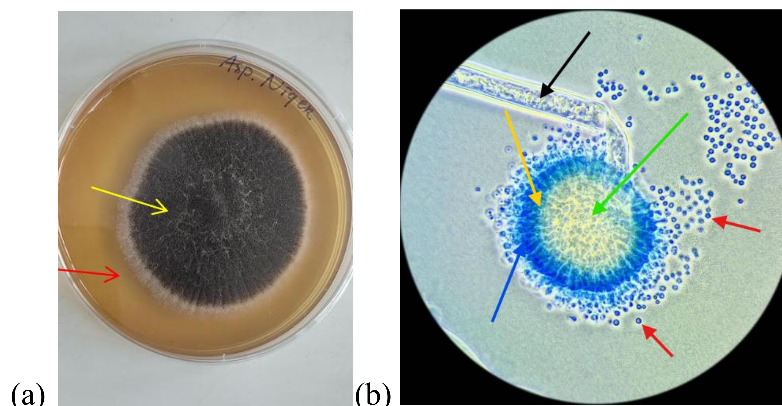


Figure 2. Morphological Identification of *Aspergillus niger*

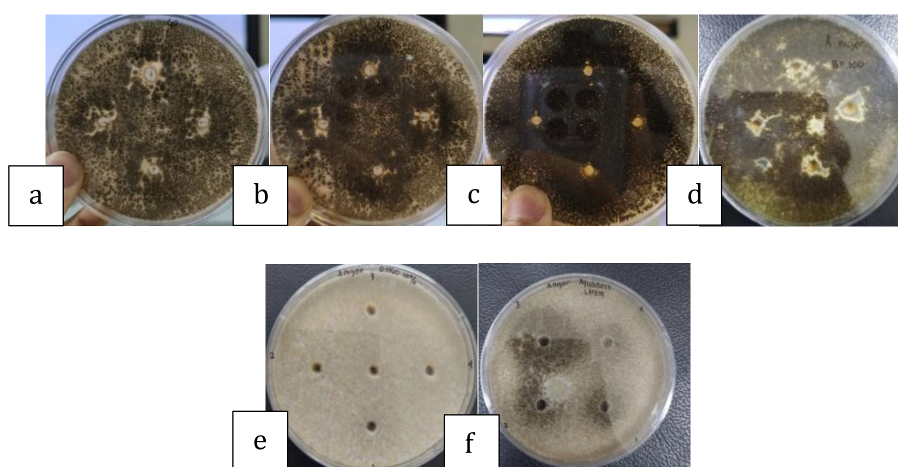


Figure 3. Antifungal Activity Test Results of Treatment Groups

Figure 4. Antifungal Activity Test Results of Positive Control Group

Retention time (RT) is the interval between sample injection and the maximum peak, while the maximum area indicates the quantity of the compound released by the sample [14]. In these phytochemical analysis results, the presence of sulphur compounds such as allicin derivatives and several saponin derivatives dominated, aligning with the general characteristics of secondary metabolites in garlic.

Fungal isolates were grown on Sabouraud Dextrose Agar (SDA) media. On SDA media, the colonies exhibited a black surface (indicated by yellow arrows) and white margins (indicated by red arrows) in Figure 2 (a). The cell morphology of *A. niger* was observed using LPCB staining. *A. niger* displays conidiophores (black arrows), spherical vesicles (green arrows), biseriate phialides (yellow arrows), metulae (blue arrows), and black conidia (red arrows), which are characteristic morphological features of *A. niger*. The cell morphology is visible in Figure 2 (b).

Figure 3 shows the observation of the inhibition zones for garlic extract at concentrations of 40% (a), 60% (b), 80% (c), and 100% (d). The solvent control (e) and negative control (f) barely formed any clear zones around the wells. The inhibition zone diameters for all these treatments were approximately 6–8 mm, while the well diameter itself was 6 mm, indicating no significant antifungal activity.

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Different results were observed in the positive control group, showing an inhibition zone with an average diameter of 17.875 mm after 24 hours of incubation and 17 mm after 48 hours. This indicates that the positive control group using Itraconazole 8 µg/mL is sensitive against *A. niger*.

Based on the Itraconazole inhibition zone criteria according to the epidemiological cut-off by Espinel-Ingroff et al. (2007), an inhibition zone ≥17 mm is categorized as "susceptible (S)", 14–16 mm as "intermediate (I)", and ≤13 mm as "resistant (R)" [15]. According to these criteria, the garlic extract at concentrations of 40%, 60%, 80%, and 100% did not form effective inhibition zones, placing them in the "resistant" (R) category. Thus, the initial hypothesis stating that the garlic extract has antifungal activity at these concentrations was not proven.

Table 2. Inhibition Zone Diameters of *Aspergillus niger*

Treatment	Diameter of *Aspergillus niger* Isolate Replications (mm)								Mean Diameter (mm)	
	1		2		3		4			
	24	48	24	48	24	48	24	48	24	48
Extract 40%	10	8	7	7	10	8	7	6	8,5	7,25
Extract 60%	8	7	8	7	6	6	6	6	7	6,5
Extract 80%	6	6	6	6	6	6	6	6	6	6
Extract 100%	6	6	9	8	8	7	6	6	7,25	6,75
Positive Control	17,5	16	20	19	16	15,5	18	17,5	17,875	17
Negative Control	6	6	6	6	6	6	6	6	6	6
Solvent Control	6	6	6	6	6	6	6	6	6	6

Table 3. Bivariate Analysis of All Treatment Groups

Test	Variable	p-value	Interpretation
Saphiro-Wilk	All Groups	0,024	Not normally distributed
Levene's Test	All Groups	0,000	Not homogeneous
Kruskall-Wallis	All Groups	0,000	Significant difference between groups
Post Hoc Games Howell	Positive control vs others	0,002-0,008	Significant difference; positive control active
	Between non-positive groups	0,443-1,190	No significant difference; no significant activity

Based on Table III, it can be concluded that the positive control showed significant antifungal activity, while the garlic extract at concentrations of 40%, 60%, 80%, and 100% did not show significant antifungal activity against *A. niger*. The small inhibition zones in the extract groups indicate that the garlic extract was ineffective at the concentrations tested.

This study aimed to determine the activity of garlic extract at concentrations of 40%, 60%, 80%, and 100% as an antifungal against *A. niger* isolates in vitro. After 48 hours of incubation, inhibition zones were obtained for each concentration: 40% (7.25 mm), 60% (6.5 mm), 80% (6 mm), and 100% (6.75 mm). While the Kruskal-Wallis test showed a significant result, the Games-Howell post-hoc test revealed that meaningful differences only existed between the positive control group (Itraconazole) and the other groups. This indicates that the significance was solely driven by the positive control, while no significant differences were found between the various extract concentrations.

A study by Hafifah et al. (2021) revealed that fresh garlic juice had much higher inhibition zones at concentrations of 40% (28.3 mm), 60% (35.3 mm), 80% (36.1 mm), and 100% (43.5 mm), indicating high sensitivity [10]. Similar effects were found by Yasmeen and Mazhar (2021) [16]. The discrepancy in this study is

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likely because the active compounds in fresh garlic (allicin, tannins, flavonoids, saponins) tend to decrease after industrial processing into simplicia powder. The primary factors for this decline are thermal exposure and oxidation during drying, which can damage the chemical structure or inactivate the enzymes required to produce active components. Allicin formation is easily disrupted by high temperatures and can decompose under heat. While manufacturers attempt to minimize loss through low-temperature drying and airtight packaging, garlic powder cannot fully match the potency of fresh garlic because much of the allicin and other phytochemicals are lost or altered during processing [17].

Phytochemical screening using LC-HRMS in this study detected *γ-Glutamyl-S-Allylcysteine* (γ-GSA), the precursor for allicin, and steroid saponin derivatives such as *(25S)-5β-spirostan-3β-yl β-D-glucoside*, gitogenin, and diosgenin. While γ-GSA is a precursor, it lacks significant antifungal activity compared to allicin itself. The failure to form active allicin in the garlic extract led to the reduced antifungal activity observed [18-19]. Furthermore, tannins and flavonoids were not detected in any form in this study.

Allicin is formed immediately after garlic tissue is crushed through the action of the enzyme alliinase. Physically, allicin is moderately lipophilic (logP = 1–1.35), making it more soluble in organic solvents than water. However, due to its sulfinate group, allicin is highly reactive and easily decomposed, requiring controlled extraction conditions. Cold and rapid extraction methods are recommended to retain allicin [20-21].

Ethanol was chosen as the solvent due to its ability to dissolve various active compounds like alkaloids, phenols, and tannins. Theoretically, ethanol extraction yields high levels of allylcysteine and its derivatives [22-23]. While maceration is simple and low-cost, newer methods like ultrasonic-assisted extraction (UAE) or microwave-assisted extraction (MAE) often provide higher bioactive yields and better allicin retention compared to conventional maceration [20,24].

In conclusion, this research suggests that garlic extract derived from simplicia raw materials lacks the active antifungal substances necessary to inhibit *A. niger*. Previous publications using fresh garlic juice were more effective [10]. The use of industrial simplicia as an antifungal agent should be reconsidered, as LC-HRMS results did not show the required active substances.

Conclusion

Garlic extract (*Allium sativum* L.) at concentrations of 40%, 60%, 80%, and 100% does not show significant antifungal activity against *A. niger*, characterized by very small inhibition zones (>13 mm, which is the minimum value for the intermediate category). This may be caused by differences in the extract content compared to other studies, differences in the concentrations and levels of active compounds in the extract, differences in extraction methods, and the degradation of compounds that may have occurred during the research process.

Supplementary Material

None

Author Contributions

MFATY : Conceptualization, Methodology, Writing-Original Draft. **NK** : Data Curation, Formal Analysis, Visualization. **APKNW** : Supervision, Funding Acquisition, Writing- Review & Editing. **S** : Supervision, Funding Acquisition, Writing- Review & Editing. **SI** : Supervision, Funding Acquisition, Writing- Review & Editing.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

Acknowledgement

None

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