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ANTIFUNGAL ACTIVITY TEST OF SINGLE CLOVE GARLIC AND SINGLE CLOVE BLACK GARLIC (*Allium sativum* L.) AGAINST *Trichophyton rubrum*

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Abstrak

Dermatofitosis adalah infeksi jamur pada permukaan kulit dengan penyebab terbanyak di Indonesia, yaitu *Trichophyton rubrum*. Bawang putih siung tunggal diketahui memiliki aktivitas antimikroba yang lebih tinggi dibandingkan bawang putih biasa. Inkubasi bawang putih siung tunggal pada temperatur dan kelembapan tertentu (70°C, 90%) mengubah warna bawang menjadi hitam dan meningkatkan aktivitas antimikroba. Penelitian ini bertujuan untuk mengetahui aktivitas antijamur ekstrak bawang putih siung tunggal dan bawang hitam siung tunggal terhadap *Trichophyton rubrum*. Aktivitas antijamur diuji dengan metode difusi cakram pada konsentrasi 25%, 50%, 75%, dan 100%. Zona hambat tidak terbentuk pada kelompok ekstrak bawang putih siung tunggal pada konsentrasi 25%, 50%, 75%, dan 100%, serta kelompok ekstrak bawang hitam siung tunggal pada konsentrasi 25%, sedangkan pada kelompok ekstrak bawang hitam siung tunggal pada konsentrasi 50%, 75%, dan 100% terbentuk diameter zona hambat yang berkisar antara 16,4 – 21,8 mm (aktivitas kuat – sangat kuat). Ekstrak bawang putih siung tunggal tidak memiliki aktivitas antijamur terhadap *Trichophyton rubrum*, sedangkan bawang hitam siung tunggal memiliki aktivitas antijamur terhadap *Trichophyton rubrum* pada konsentrasi 50%, 75%, 100% dengan konsentrasi efektif yaitu 50%. Temuan ini menunjukkan bahwa ekstrak bawang hitam dapat berfungsi sebagai penanganan alternatif potensial untuk infeksi jamur dermatofita, terutama yang disebabkan oleh *Trichophyton rubrum*.

Kata kunci: Bawang putih siung tunggal, Bawang hitam siung tunggal, Antijamur, *Trichophyton rubrum*

Abstract

Dermatophytosis is a fungal infection of the skin surface which mostly caused by Trichophyton rubrum *in Indonesia. Single clove garlic (Allium sativum) exhibits superior antimicrobial properties compared to conventional garlic. When incubated at a specific temperature and humidity (70°C, 90%), single clove garlic transforms into black garlic, further enhancing its antimicrobial efficacy. This research aims to evaluate the antifungal properties of extracts from both single clove garlic and single clove black garlic against* Trichophyton rubrum. *The antifungal activity was tested by disc diffusion at concentrations of 25%, 50%, 75% and 100%. No inhibition zones were observed in the group treated with single clove garlic extract at concentrations of 25%, 50%, 75%, and 100%, nor with single clove black garlic extract at a concentration of 25%. However, single clove black garlic extract at concentrations of 50%, 75%, and 100% demonstrated inhibition zones ranging from 16.4 to 21.8 mm, indicating strong to very strong antifungal activity. Single clove garlic exhibited no*

antifungal activity against Trichophyton rubrum. *In contrast, single clove black garlic demonstrated antifungal activity against* Trichophyton rubrum *at concentrations of 50%, 75%, and 100%, with an effective dose starting at 50%. These findings indicate that black garlic extract may serve as a potential alternative treatment for dermatophyte fungal infections, particularly those caused by* Trichophyton rubrum.
Keywords: *Single clove garlic, Single clove black garlic, Antifungal,* Trichophyton rubrum

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INTRODUCTION

Indonesia experiences an almost entirely tropical climate characterized by high temperatures and humidity, which contribute to a high incidence of fungal infections. The prevalence of fungal infections in Indonesia from 2016 to 2020 ranges from 0,9% to 25,61%, with dermatophytosis being the second most common fungal infection after pityriasis versicolor (Nurwulan et al., 2019; Prakoeswa et al., 2022).

Dermatophytosis is a fungal infection of the epidermis caused by keratinophilic fungi known as dermatophytes. Dermatophytes are classified into three genera: Trichophyton, Microsporum, and Epidermophyton (Hidayah et al., 2021). They can be differentiated by their macroconidia and microconidia. Trichophyton rubrum exhibits few macroconidia, which are smooth and thick-walled. In contrast, the microconidia are more abundant, typically teardrop or peg-shaped, and generally found along the sides of the hyphae (Murray et al., 2016). *Tricophyton rubrum* is the most common species that cause dermatophytosis in Indonesia with a percentage of 37.3% (Sarumpaet & Wahyuni, 2021).

Topical medications are frequently employed to treat dermatophytosis, with terbinafine as the first-line treatment and itraconazole as the alternative. However, these synthetic drugs are often associated with high costs, extended treatment durations, adverse effects, and potential drug interactions. Common side effects of terbinafine and itraconazole include gastrointestinal upset, headache, taste alterations, abnormal liver function tests, and rash. In rare instances, terbinafine can cause blood disorders and hepatitis, while itraconazole can lead to hypokalemia, arrhythmias, and heart failure. (Anuradha et al., 2019). Moreover, there have been numerous reports of antifungal resistance. A study published by (Sacheli & Hayette, 2021) has shown that some strains of dermatophytes are resistant to terbinafine and itraconazole. Consequently, there is a need for affordable and low-side-effect alternatives, such as natural substances.

Garlic is one of the natural substances known for its antifungal properties. It contains 33 bioactive organosulfur compounds, along with minerals, vitamins A, B1, and C, fiber, water, 17 amino acids, oligosaccharides, peptides, steroids, terpenoids, alkaloids, flavonoids, tannins, saponins, and phenolic carbohydrates (sucrose and glucose) (Mahi et al., 2021). In Indonesia, single clove garlic (Allium sativum Linn) is more commonly employed for therapeutic purposes. Single clove garlic is reported to contain five to six times more active compounds compared to conventional garlic, as all the bioactive substances are concentrated within a single clove (Gofur et al., 2019).

The active compounds in single clove garlic with antimicrobial properties include allicin, s-allyl-cysteine, alkaloids, flavonoids, tannins, phenolics, saponins, ajoene, diallyl sulfide (DAS), diallyl disulfide (DADS), and diallyl trisulfide (DATS). However, single clove garlic is less favored for oral use due to its high allicin content, which imparts a distinctive odor and bitter taste. To mitigate

these characteristics, various processing techniques are employed, such as heating. Heating single clove garlic induces a Maillard or browning reaction, leading to the garlic's blackening (Agustina et al., 2020).

Black garlic has a milder flavor compared to fresh garlic because the allicin content is reduced and converted into antioxidant compounds, including bioactive alkaloids and flavonoids (Agustina et al., 2020). Additionally, after heating, the concentration of flavonoids in black garlic significantly increased, and the total polyphenol content rose sixfold (Kimura et al., 2017). Therefore, with higher antimicrobial compounds, it is expected that the single clove black garlic can be more effective in treating pathogenic fungi that cause dermatophytosis than the fresh one.

METHODS

This study was conducted at the Non-Microscopic Laboratory, Pharmaceutical-Biology Laboratory, and Microbiology Laboratory, Faculty of Medicine, Tanjungpura University.The study included ten groups of *Trichophyton rubrum*, divided into eight experimental groups and two control groups. The experimental groups were treated with single clove garlic extract at concentrations of 25%, 50%, 75%, and 100%, and single clove black garlic extract at the same concentrations to determine the effect of variations in treatment concentration on the antifungal activity. The selection of varying concentrations is necessary due to the lack of prior research; thus, it is essential to observe the response to a range of concentrations, from low to high, on fungal growth. The positive control group received 8 µg of itraconazole, while the negative control group received 1% DMSO. The experiments were performed in triplicate according to Federrer's formula.

Single clove garlic, weighing up to 5 kg, was heated in an oven at 70°C for 21 days until it turned black. Both single clove garlic and single clove black garlic were separated from their cloves, peeled, and then separately minced. Each type of garlic was macerated with 3 × 2400 ml of 96% ethanol for 3 days. The macerates were then concentrated at 40°C using a rotary evaporator to achieve a 100% concentration. Phytochemical screening was conducted to identify the presence of alkaloids, flavonoids, phenolics, tannins, and saponins. The extracts of single clove garlic and single clove black garlic were diluted with 1% DMSO to prepare solutions at concentrations of 25%, 50%, 75%, and 100% (Atika et al., 2021; Saejung & Chimsook, 2019).

The *Trichophyton rubrum* isolate was subcultured onto Sabouraud Dextrose Agar (SDA) and incubated at 28°C for 7 days (Kadhim & Al-hamadani, 2015). A 7-day-old colony was collected using an inoculation loop and suspended in a tube containing 0.9% sterile saline solution. The suspension's density was adjusted to match the 0.5 McFarland turbidity standard, corresponding to a concentration of 1.5 × 10^8 CFU/ml. A sterile swab was then used to transfer the suspension onto Sabouraud Dextrose Agar (SDA) plates using a three-way streaking method (Alioes & Kartika, 2019; CLSI, 2021).

Sterile blank discs were immersed in each extract concentration, as well as the positive and negative controls, for 15 minutes. The discs were then placed onto the surface of the agar medium and gently pressed to ensure full contact with the agar. Within 15 minutes of application, the agar plates were sealed and incubated at 28°C for 7 days. After incubation, the inhibition zones on each agar plate were measured with calipers to assess the antifungal activity (Chandra et al., 2022).

RESULTS AND DISCUSSION

Results

Qualitative phytochemical screening revealed that both extracts-single clove garlic and single clove black garlic—contain alkaloids, flavonoids, phenolics, saponins, and tannins.

Table 1. Results of Phytochemical Screening

Phytochemical	Reagent	Type of Extract	Result	Interpretation
Alkaloids	Wagner	SBG	Reddish brown deposits	Positive
		SBBG	Reddish brown deposits	Positive
Flavonoids	Magnesium powder, HCl	SBG	Yellow color	Positive
		SBBG	Brown color	Positive
Phenolics	10% of $FeCl_3$	SBG	Slightly greenish color	Positive
		SBBG	Dark blue color	Positive
Saponins	Aquadest	SBG	Presence of foam	Positive
		SBBG	Presence of foam	Positive
Tannins	NaOH 10%	SBG	Presence of emulsion	Positive
		SBBG	Presence of emulsion	Positive

SBG = Single clove garlic SBBG = Single clove black garlic

The antifungal test results after 7 days of incubation indicated that none of the concentrations (25%, 50%, 75%, and 100%) of single clove garlic extract produced an inhibitory zone (Figure 1 and Table 2).

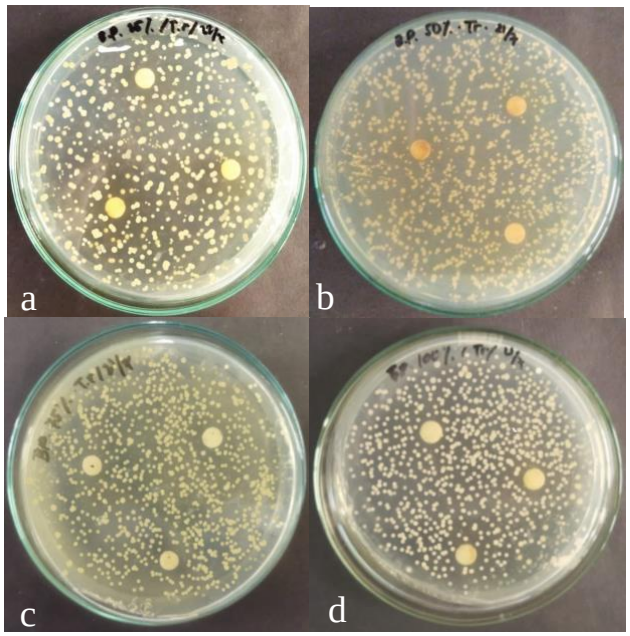


Figure 1. Results of the Antifungal Activity Test for Single Clove Garlic Extract at the Following Concentrations: (a) 25%, (b) 50%, (c) 75%, (d) 100%

Single clove black garlic extract at a concentration of 25% did not form an inhibitory zone. However, inhibitory zones were observed with single clove black garlic extract at concentrations of 50%, 75%, and 100% (Figure 2 and Table 2)

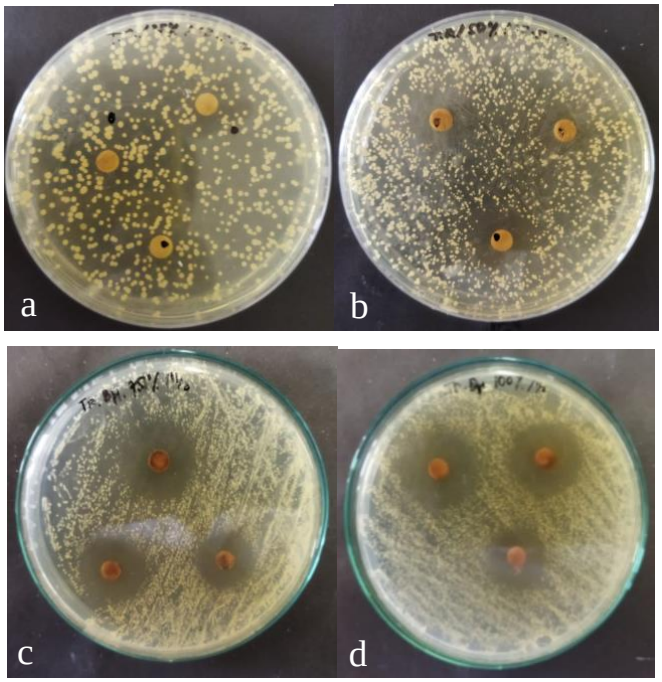


Figure 2. Results of Antifungal Activity Test of Single Clove Black Garlic Extract At The Concentrations of
(a) 25%, (b) 50%, (c) 75%, (d) 100%

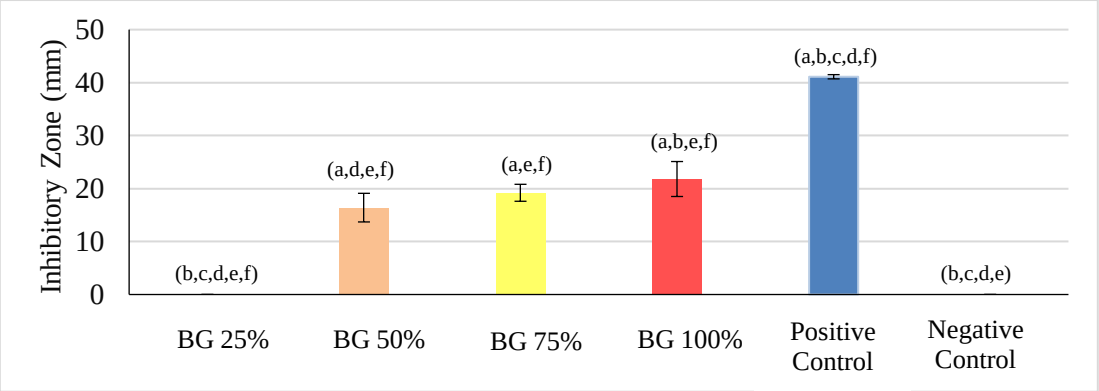
Table 2. Results of the Antifungal Activity Test

Concentration	Single Clove Garlic		Single Clove Black Garlic	
	Zone of inhibition in diameter (mm)	Interpretation	Zone of inhibition in diameter (mm)	Interpretation
25%	0	No inhibition zone	0	No inhibition zone
50%	0	No inhibition zone	16,4±2,7	Strong
75%	0	No inhibition zone	19,2±1,6	Strong
100%	0	No inhibition zone	21,8±3,3	Very strong
Positive Control		41,1±0,4		Sensitive
Negative Control		0		No inhibition zone

Single clove black garlic extract at a concentration of 25% did not exhibit an inhibitory response to fungal growth. The diameters of the inhibition zones for single clove black garlic extract at concentrations of 50% (16.4 mm) and 75% (19.2 mm) indicated strong antifungal activity, while the 100% concentration (21.8 mm) demonstrated very strong antifungal activity. These three concentrations effectively inhibited the growth of *Trichophyton rubrum*, with the minimum effective concentration being 50%.

The Shapiro-Wilk normality test, Homogeneity of Variances test, and Levene test confirmed that the data were normally distributed and homogeneous ($p>0.05$). Statistical analysis using One-Way ANOVA revealed significant differences in the diameter of the inhibition zones between the groups treated with single clove black garlic at concentrations of 25%, 50%, 75%, and 100%, and the control group. Further analysis using Post Hoc Least Significant Difference (LSD) test (Figure 3) indicated that a 50% concentration of single clove

black garlic extract did not differ significantly from the 75% concentration (p=0.097), but showed a significant difference when compared to the 100% concentration (p=0.04). In contrast, the 75% concentration did not exhibit a significant difference from the 100% concentration (p=0.117). The single clove black garlic extract groups at concentrations of 50%, 75%, and 100% demonstrated significant differences compared to both the negative control and positive control groups (p=0.000).



(a) significantly different from 25% concentration, (b) significantly different from 50% concentration, (c) significantly different from 75% concentration, (d) significantly different from 100% concentration, (e) significantly different from positive control, and (f) significantly different from the negative control. $p<0.05$

Figure 3. Concentration of Extract Against Diameter of Inhibition Zone

Discussions

The results of this study indicated that single clove garlic exhibited no antifungal activity against *Trichophyton rubrum*. In contrast, the single clove black garlic extract demonstrated antifungal activity at concentrations of 50%, 75%, and 100%, with activity levels ranging from strong to very strong.

The results of this study were slightly different from the results of a study conducted by (Sabila et al., 2019) and (Indraswari et al., 2022) which examined the antifungal activity of garlic and black garlic extracts against *Candida albicans*. The results of the study by (Sabila et al., 2019) showed the formation of inhibition zone diameters in both the garlic and black garlic extract groups at 25%, 50%, 75%, and 100% concentrations with all groups having weak categories, while the study conducted by (Indraswari et al., 2022) showed the formation of inhibition zone diameters in both the garlic and black garlic extract groups at 75%, and 100% concentrations with results ranging from weak to moderate. The variations in the results may be attributed to differences in fungal types or strains, garlic varieties, heating duration and temperature, environmental pH, media components, and microorganism incubation time.

Nevertheless, the black garlic extract gave a better response of inhibiting the growth of *Trichophyton rubrum* as indicated by the larger diameter of the inhibition zone compared to the garlic extract. This finding aligns with the theory that black garlic extract exhibits higher antifungal activity than regular garlic. This increased activity is attributed to the Maillard reaction, which induces changes in the compounds within the garlic, including those involved in antifungal activity (Kimura et al., 2017).

The Maillard reaction transforms the allicin compound in garlic into antioxidant compounds, such as bioactive alkaloids, flavonoids, S-allylcysteine (SAC), and tetrahydro-β-carboline, which possess antifungal properties (Agustina et al., 2020). Alkaloid compounds intercalate between the cell wall and DNA, thereby preventing fungal DNA replication and disrupting the growth of

Trichophyton rubrum. Flavonoid compounds inhibit fungal growth by altering the permeability of fungal cell membranes. The hydroxyl groups present in flavonoids induce changes in organic components and nutrient transport, leading to toxic effects on fungi. Additionally, flavonoids inhibit mitochondrial electron transport, which reduces mitochondrial membrane potential and ultimately causes fungal cell death (Komala et al., 2019). Tetrahydro-β-carboline functions by compromising the integrity of the fungal cell membrane and degrading the cell surface. (Asali et al., 2018).

Additionally, the heating process can enhance S-allylcysteine (SAC) levels by a factor of six compared to raw garlic. According to research by (Ryu & Kang, 2017), heating reduced the allicin content in garlic from 345 mg/100 mg to 20 mg/100 mg, while SAC content increased from 2.4 mg/100 mg to 19.4 mg/100 mg. Although the reduction in allicin was greater than the increase in SAC, the elevated SAC levels can improve the absorption of allicin by fungi. This enhanced absorption contributes to a greater antifungal activity in black garlic, as the remaining allicin is more effectively utilized by the fungi.

Allicin has been demonstrated to inhibit the growth of hyphae in *Trichophyton rubrum* and biofilms in Candida albicans (Aala et al., 2013). In addition to facilitating allicin absorption, S-allylcysteine (SAC) interferes with cell metabolism by inactivating proteins, competitively inhibiting sulfhydryl compounds, and participating in non-competitive inhibition of enzyme activity through oxidation. These mechanisms collectively inhibit the synthesis of DNA and proteins. (Tran et al., 2020).

The lack of antifungal activity observed in the four groups treated with single clove garlic extract may be attributed to several factors. Phytochemical screening in this study revealed that single clove garlic extract contained very few phenolic compounds, as indicated by minimal discoloration when FeCl3 was added. This absence of phenolic compounds could explain the lack of antifungal activity across all tested concentrations of single clove garlic extract. Phenolic compounds, which include both phenols and polyphenols, are significant natural antioxidants in plants. They possess one or more phenol rings and hydroxy groups attached to aromatic rings, which facilitates their oxidation through hydrogen atom donation to free radicals. The ability of phenolic compounds to form stable phenoxy radicals during oxidation reactions enhances their potential as antimicrobial agents. Additionally, certain phenolic compounds exert their antifungal effects through apoptotic mechanisms by increasing Reactive Oxygen Species (ROS) levels and inducing the expression of the CaMCA1 gene in various fungi (Pudiarifanti & Farizal, 2022).

Single clove black garlic has been demonstrated to contain higher levels of phenolic compounds compared to single clove garlic. The heating process induces the Maillard reaction, which alters the original compounds present in garlic, including phenolic compounds. Research indicates that the concentration of phenolic compounds in black garlic increases by a factor of 4 to 10, while in single clove black garlic, the increase is up to 11 times (Zhang et al., 2016).

Additionally, the substantial thickness of the fungal cell wall may contribute to the lack of inhibitory effect on *Trichophyton rubrum* observed in this study. The thick cell wall can function as a barrier, preventing the permeation of single clove garlic extract. Consequently, higher concentrations of secondary metabolites, such as flavonoids, phenolics, and organosulfur compounds (e.g., S-allylcysteine [SAC] and tetrahydro-β-carboline), are required to achieve effective antifungal activity, which is a characteristic of single clove black garlic extract. (Fitria and Setiawati 2020).

The positive control test results in this study demonstrated that *Trichophyton rubrum* was sensitive to the first-line dermatophytosis therapy. This sensitivity is characterized by an inhibition zone of ≥15 mm, while an inhibition zone of 10−14 mm indicates intermediate sensitivity, and ≤10 mm indicates resistance. In contrast, the negative control test using a 1% DMSO solution showed no inhibition zone around the disc, as DMSO is an organic solvent without antifungal properties. Consequently, no inhibition zone was observed in the negative control test (Petrucelli et al., 2020).

The results of the antifungal activity test in this study were compared to research conducted by (Sabila et al., 2019) and (Indraswari et al., 2022) which tested the antifungal activity of garlic and

black garlic extracts against *Candida albicans*. The study by (Sabila et al., 2019) reported that the inhibition zone diameters for both garlic and black garlic extract at concentrations of 25%, 50%, 75%, and 100% were categorized as weak. In contrast, research by (Indraswari et al., 2022) found that inhibition zone diameters for garlic and black garlic extract at concentrations of 75% and 100% were categorized as weak to moderate. Variations in these results may be attributed to differences in fungal types or strains, garlic varieties, heating conditions (time and temperature), environmental pH, media components, and microbial incubation times.

According to Choi et al., 2014, The duration of heating influences the appearance and content of black garlic. When garlic is heated at 70°C for 0-14 days, the resulting color is not homogenous. By 21 days, the color becomes uniformly black. However, after heating for 28 and 35 days, the garlic appears dry due to the Maillard reaction, a non-enzymatic browning process.

Additionally, antioxidant levels in garlic, such as total polyphenols and flavonoids, increase significantly up to the 21st day of heating. This occurs because the heat treatment of phenolic compounds in garlic increases the free fraction of phenolic acids while decreasing the ester, glycoside, and ester-bound fractions, resulting in a higher free phenol form. However, heating beyond 21 days can reduce the total amount of phenols and flavonoids (Choi et al., 2014).

The antifungal activity of single clove black garlic extract was observed at concentrations of 50%, 75%, and 100%. In this study, effective concentration is defined as the concentration that exhibits a biological effect, specifically antifungal activity against Trichophyton rubrum. Thus, these three concentrations are deemed effective, with the minimum effective concentration identified as 50%. The concentration with the largest inhibition zone was 100%.

Post hoc LSD test results (Figure 4.9) at a 95% confidence level indicated that the 50% concentration of single clove black garlic extract did not differ significantly from the 75% concentration but did show a significant difference compared to the 100% concentration. However, the 75% concentration did not significantly differ from the 100% concentration. The 100% concentration of single clove black garlic extract exhibited a significant difference compared to both the negative control and positive control groups. This indicates that the 100% concentration provided antifungal activity, though the 50% and 75% concentrations did not have the same effect as itraconazole.

CONCLUSION

The study demonstrated a distinct difference in antifungal activity between single clove garlic extract and single clove black garlic extract against *Trichophyton rubrum*. Single clove garlic extract exhibited no antifungal activity, whereas single clove black garlic extract showed significant antifungal activity, with inhibition zones ranging from 16.4 to 21.8 mm, indicating strong to very strong activity. Among the concentrations of single clove black garlic extract tested, a 50% concentration was identified as the minimum effective dose inhibiting Trichophyton rubrum growth, while a 100% concentration produced the largest inhibition zone. These findings indicate that black garlic extract may serve as a potential alternative treatment for dermatophyte fungal infections, particularly those caused by Trichophyton rubrum. For future research, quantitative phytochemical screening is necessary to determine and compare the total content of secondary metabolite compounds in single clove garlic and black garlic.

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