

***In vitro* antifungal effects of aqueous garlic extract alone and in combination with azoles against dermatophytic fungi**

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Dermatophytes are fungi able to invade keratinized tissues, causing dermatomycosis. Azole antifungal drugs are used in the treatment of dermatomycosis, but have side effects and resistance may develop against them. In this study, garlic, which is a medicinal plant with antifungal activity, was tested. The study evaluated the *in vitro* efficacy of garlic extract alone against 10 isolates of *Trichophyton rubrum* and it was found that the MICs ranged from 0.0156-0.5 mg/ml, whereas the MIC values for 6 other dermatophyte isolates ranged from 0.0156-1.0 mg/ml. In conclusion, when used alone, aqueous garlic extract showed very good potential as an antifungal compound, and in combination of garlic extract with ketoconazole or fluconazole showed synergistic or additive interaction against dermatophytes.

Keywords: Antifungal drugs, aqueous garlic extract, dermatophytes, MIC (minimal inhibitory concentration).

INTRODUCTION

Dermatophytosis ranks among the most prevalent infectious diseases worldwide caused by dermatophytes (Barros, Santos, Hamdan, 2007). The azoles group of antifungal drugs such as ketoconazole and fluconazole have been used for the treatment of dermatomycosis, but they are known to cause side effects and toxicity in patients (Al-Mohsen and Hughes, 1998; Odds, Brown, Gow, 2003; Pyun and Shin, 2006). Hence this study investigated the use of a plant-based, biodegradable natural product as an alternative. Garlic (*Allium sativum*), is an edible plant, which has therapeutic effects because of sulphur-containing compounds, and this structure seems to be necessary for the antifungal properties of garlic (Shokouhi Sabet Jalali, Saifzadeh, Tajik, Hobbi, 2008; Woods-Panzaru, Nelson, McCollum, Ballard, Millar, 2009).

The aim of this study was to evaluate the *in vitro* antifungal activity of aqueous garlic extract alone and in combination with ketoconazole or fluconazole against sixteen isolates of dermatophytic fungi, as a potential plant-based alternative treatment of dermatomycosis.

MATERIALS AND METHODS

Study design

Firstly 10 isolates of *Trichophyton rubrum*, and then 6 isolates of other dermatophytic fungi were tested for their interactions with aqueous garlic extract, ketoconazole and fluconazole following the protocol NCCLS M38-A. *T. rubrum* (ATCC-10218) was used as a control strain. Isolates were selected from the culture collection of clinical isolates preserved at the laboratory of Medical Mycology Department in Tehran University of Medical Sciences, Iran.

Preparation of aqueous garlic extract

Garlic cloves were separated, hulled and washed with distilled water. Then peeled garlic was chopped and blended with sterile distilled water in a Waring blender. Then was centrifuged at 14000 rpm at 4 °C for 30 min in refrigerated centrifuge. The supernatant was collected and sterilized by filtration using 0.22 µm pore-size filter. Then the aqueous garlic extract was dried and changed to garlic powder by using freeze dryer. Finally, 8 mg of the powder dissolved in 1 ml of sterile saline (0.85%) v/v NaCl solution and then sterilized by passing through Millipore filter (0.22 µm) and stored at -70°C until use.

Inoculum preparation

All isolates were cultured in potato dextrose agar and incubated at 28 °C for 7 days. The seven-day-old colonies were suspended in 5 ml of sterile saline (0.85%) v/v NaCl. The resulting mixture was filtrated with a Whatman's filter no. 40 (pore size: 8 µm). The densities of the conidial suspensions were adjusted with spectrophotometric method at 520 nm wavelength. The inoculum concentrations ranged from $2-4 \times 10^6$ conidia/ml.

Antifungal compounds

Two antifungal drugs, namely Ketoconazole and fluconazole were dissolved separately in dimethyl sulfoxide (DMSO) at 1.5 and 5 mg/ml. Aqueous garlic extract, ketoconazole and fluconazole drug dilutions ranged from 0.0156-8.0 mg/ml, 0.03-16.0 µg/ml, and 0.03-64.0 µg/ml, respectively.

Broth microdilution method (NCCLS M38-A protocol) for determination of MIC

U-bottom 96 well microdilution plates were used. All microdilution trays were incubated at 28 °C and read visually after 7 days and 10 days incubation too. Then the plates were read with a spectrophotometer at 520 nm wavelength. The MICs were determined as the lowest concentration of the drug that gave a 50 and 90% reduction in optical density when compared with the turbidity of the growth control well (Rex, Pfaller, Walsh, 2001; Cai, Wang, Pei, Liang, 2007). Spectrophotometric readings were taken by measuring the OD at this wavelength, as suggested by Pelletier, Loranger, Marcotte and Carolis (2002); Swinne, Watelle, Nolard (2005).

Evaluation of the MIC, MFC and FICI

To compare the actions of the tested drugs the MIC of all drugs were acquired, including readings of the MIC₅₀ and MIC₉₀; that is, the lowest concentration of the medicines (alone or in combination) at which 50% or 90% of the growth of a microorganism was inhibited (Santos and Hamdan, 2005; Santos, Barros and Hamdan, 2006).

High concentrations of garlic extract, have been revealed to have both fungistatic and fungicidal activities *in vitro* and *in vivo* (Yamada and Azuma, 1977; Yoshida et al., 1987; Davis, Shen and Cai, 1990). The contents of the wells (100 µl of solution) without turbidity or visible fungal growth in the MIC test were subcultured into potato dextrose agar medium at 28 °C for 7 days to examine for the fungicidal property of the extract. The *in vitro* fungicidal activities (MFCs) were determined as the lowest extract concentration that displayed either no fungal growth or three or fewer colonies per plate to acquire nearly 99 to 99.5% killing action (Espinel-Ingroff, Fothergill, Peter, Rinaldi, Walsh, 2002; Fontenelle, Morais, Brito, Kerntopf, Brilhante, 2007; Mann, Banso, Clifford, 2008).

The fractional inhibitory concentration index (FICI) can be classified as synergistic, additive, and indifferent (autonomous) or antagonistic based on the formula for calculation of FICI: (MIC of drug A in combination/ MIC of drug A alone) + (MIC of drug B in combination/ MIC of drug B alone). The drug interaction was defined as synergistic when the FICI value was < 1.0, additive when the FICI was 1.0, indifferent when the FICI was 1.0 < FICI < 2.0 and antagonistic when the FICI was > 2.0 (Mosquera, Sharp, Moore, Warn, Denning, 2002; Ozawa, Okabayashi, Kano, Watanabe, Hasegawa, 2005).

RESULTS

The results of the study, for ten isolates of *T.rubrum*, showed that the MIC₅₀ for garlic extract ranged from 0.0156-0.125 mg/ml and the MIC₉₀ with 0.0625-0.5 mg/ml, but MFC value ranged from 2.0-8.0 mg/ml at 28 °C for 7 and 10 days incubation too. In this study, the results on FICI revealed that 85% of the treated samples with combination of garlic extract /ketoconazole and garlic extract /fluconazole after 7 days incubation at 28 °C had synergistic or additive properties (Table 1). Moreover, 77.5% of the treated samples with drug combinations after 10 days incubation at 28 °C had synergistic or additive properties (Table 2).

The results of the study, for six other isolates of dermatophytes, displayed that the MIC₅₀ for garlic

Table 1. Antifungal activities of garlic extract, ketoconazole, and fluconazole alone and in combination on *Trichophyton rubrum* at 28°C at 7 days incubation.

Dermatophyte isolates	Garlic extract			Ketoconazole		Fluconazole		G + K ¹		G + F ²	
	MIC ₅₀ ³	MIC ₉₀ ⁴	MFC ⁵	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	FICI ₅₀ ⁶	FICI ₉₀	FICI ₅₀	FICI ₉₀
<i>T. rubrum</i> (1)	0.0312	0.125	4.0	2.0	4.0	4.0	8.0	0.5	0.75	1.0	1.0
<i>T. rubrum</i> (2)	0.0156	0.0625	2.0	1.0	2.0	2.0	4.0	0.25	0.375	0.375	0.5
<i>T. rubrum</i> (3)	0.125	0.5	8.0	4.0	8.0	8.0	32.0	1.0	1.0	1.0	1.5
<i>T. rubrum</i> (4)	0.0156	0.0625	4.0	0.25	0.5	1.0	4.0	0.5	0.75	0.75	1.0
<i>T. rubrum</i> (5)	0.0625	0.25	8.0	2.0	4.0	4.0	8.0	1.0	1.0	1.5	1.0
<i>T. rubrum</i> (6)	0.125	0.5	8.0	1.0	2.0	8.0	32.0	1.0	1.0	1.0	1.5
<i>T. rubrum</i> (7)	0.0312	0.125	2.0	0.5	2.0	2.0	8.0	0.5	0.75	0.5	1.0
<i>T. rubrum</i> (8) (ATCC)	0.0312	0.25	4.0	1.0	2.0	2.0	8.0	0.75	1.0	1.5	1.5
<i>T. rubrum</i> (9)	0.0156	0.0625	2.0	0.5	1.0	1.0	2.0	0.25	0.375	0.375	0.5
<i>T. rubrum</i> (10)	0.0312	0.25	8.0	1.0	2.0	4.0	8.0	1.0	1.0	1.5	1.0
Growth control (+°C)	100% Growth			100% Growth		100% Growth		100% Growth		100% Growth	
Negative control (-°C)	0% Growth			0% Growth		0% Growth		0% Growth		0% Growth	

¹G+K:Garlic extract + ketoconazole.²G +F: Garlic extract + fluconazole. ³MIC₅₀ and ⁴ MIC₉₀ are MICS at which 50% and 90% of the growth of a microorganism was inhibited, respectively.⁵MFC is the lowest concentration with no visible growth. ⁶Fractional inhibitory concentration index. The values (MICS and MFC) are reported for garlic extract (mg/ml) and the MICS for ketoconazole and fluconazole (µg/ml).

Table 2. Antifungal activities of garlic extract, ketoconazole, and fluconazole alone and in combination on *Trichophyton rubrum* at 28°C at 10 days incubation.

Dermatophyte isolates	Garlic extract			Ketoconazole		Fluconazole		G + K ¹		G + F ²	
	MIC ₅₀ ³	MIC ₉₀ ⁴	MFC ⁵	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	FICI ₅₀ ⁶	FICI ₉₀	FICI ₅₀	FICI ₉₀
<i>T. rubrum</i> (1)	0.0312	0.125	4.0	2.0	4.0	4.0	8.0	0.5	1.0	1.0	1.0
<i>T. rubrum</i> (2)	0.0312	0.125	2.0	1.0	4.0	2.0	8.0	0.375	0.5	0.5	0.375
<i>T. rubrum</i> (3)	0.125	0.5	8.0	4.0	8.0	8.0	32.0	1.0	2.0	1.0	1.5
<i>T. rubrum</i> (4)	0.0312	0.0625	4.0	0.25	0.5	2.0	4.0	0.5	0.75	1.0	1.0
<i>T. rubrum</i> (5)	0.0625	0.25	8.0	2.0	4.0	4.0	8.0	1.0	1.0	1.0	1.5
<i>T. rubrum</i> (6)	0.125	0.5	8.0	1.0	2.0	8.0	32.0	1.0	1.5	1.0	2.0
<i>T. rubrum</i> (7)	0.0312	0.5	8.0	1.0	2.0	4.0	8.0	0.75	1.0	1.0	1.5
<i>T. rubrum</i> (8) (ATCC)	0.0312	0.5	8.0	1.0	2.0	4.0	16.0	0.75	1.0	1.5	2.0
<i>T. rubrum</i> (9)	0.0312	0.125	2.0	0.5	1.0	2.0	4.0	0.25	0.375	0.375	0.5
<i>T. rubrum</i> (10)	0.125	0.5	8.0	1.0	2.0	8.0	16.0	1.0	1.0	1.5	1.0
Growth control (+°C)	100% Growth			100% Growth		100% Growth		100% Growth		100%Growth	
Negative control (-°C)	0% Growth			0% Growth		0% Growth		0% Growth		0% Growth	

¹G+K:Garlic extract + ketoconazole.²G +F: Garlic extract + fluconazole. ³MIC₅₀ and ⁴ MIC₉₀ are MICS at which 50% and 90% of the growth of a microorganism was inhibited, respectively.⁵MFC is the lowest concentration with no visible growth. ⁶Fractional inhibitory concentration index. The values (MICS and MFC) are reported for garlic extract (mg/ml) and the MICS for ketoconazole and fluconazole (µg/ml).

Table 3. Antifungal activities of garlic extract, ketoconazole, and fluconazole alone and in combination on dermatophytes at 28°C at 7 days incubation.

Dermatophyte species	Garlic extract			Ketoconazole		Fluconazole		G + k ¹		G + F ²	
	MIC ₅₀ ³	MIC ₉₀ ⁴	MFC ⁵	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	FICI ₅₀ ⁶	FICI ₉₀	FICI ₅₀	FICI ₉₀
<i>T.rubrum</i>	0.0312	0.25	4.0	0.5	2.0	2.0	8.0	0.75	1.0	0.75	1.0
<i>T.rubrum</i> (ATCC)	0.0312	0.25	4.0	0.5	2.0	2.0	8.0	0.75	1.0	1.0	1.0
<i>T.mentagrophytes</i>	0.0625	0.5	8.0	1.0	4.0	4.0	64.0	1.0	1.0	1.5	2.0
<i>T.verrucosum</i>	0.0156	0.125	2.0	0.25	1.0	1.0	4.0	0.25	0.375	0.375	0.5
<i>M.canis</i>	0.0156	0.0625	1.0	0.0625	0.125	0.5	2.0	0.75	0.75	1.0	1.5
<i>E.floccosum</i>	0.0156	0.0625	1.0	0.0625	0.125	0.5	2.0	1.0	0.5	1.0	1.5

¹G+K: Garlic extract + ketoconazole. ²G + F: Garlic extract + fluconazole. ³MIC₅₀ and ⁴ MIC₉₀ are MICS at which 50% and 90% of the growth of a microorganism was inhibited, respectively. ⁵MFC is the lowest concentration with no visible growth. ⁶Fractional inhibitory concentration index. The values (MICS and MFC) are reported for garlic extract (mg/ml) and the MICS for ketoconazole and fluconazole (µg/ml).

Table 4. Antifungal activities of garlic extract, ketoconazole, and fluconazole alone and in combination on dermatophytes at 28°C at 10 days incubation.

Dermatophyte species	Garlic extract			Ketoconazole		Fluconazole		G + k ¹		G + F ²	
	MIC ₅₀ ³	MIC ₉₀ ⁴	MFC ⁵	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	FICI ₅₀ ⁶	FICI ₉₀	FICI ₅₀	FICI ₉₀
<i>T.rubrum</i>	0.0625	0.5	8.0	0.5	2.0	4.0	16.0	0.75	1.0	1.0	1.0
<i>T.rubrum</i> (ATCC)	0.0625	0.5	8.0	0.5	2.0	4.0	16.0	0.75	1.0	1.0	0.75
<i>T.mentagrophytes</i>	0.125	1.0	8.0	1.0	4.0	16.0	64.0	1.0	1.0	1.0	1.5
<i>T.verrucosum</i>	0.0312	0.25	4.0	0.25	1.0	2.0	8.0	0.375	0.375	0.375	0.5
<i>M.canis</i>	0.0156	0.125	2.0	0.0625	0.125	1.0	4.0	0.75	1.0	1.0	1.5
<i>E.floccosum</i>	0.0156	0.125	2.0	0.0625	0.125	0.5	2.0	1.0	1.5	1.5	2.0

¹G+K: Garlic extract + ketoconazole. ²G + F: Garlic extract + fluconazole. ³MIC₅₀ and ⁴ MIC₉₀ are MICS at which 50% and 90% of the growth of a microorganism was inhibited, respectively. ⁵MFC is the lowest concentration with no visible growth. ⁶Fractional inhibitory concentration index. The values (MICS and MFC) are reported for garlic extract (mg/ml) and the MICS for ketoconazole and fluconazole (µg/ml).

extract ranged 0.0156-0.125 mg/ml and the MIC₉₀ with 0.0625-1.0 mg/ml, but MFC value ranged from 1.0-8.0 mg/ml at 28 °C for 7 and 10 days incubation too. The results on FICI revealed that about 83.5% of the treated samples with combination of garlic extract/ketoconazole and garlic extract/fluconazole after 7 days incubation at 28 °C had synergistic or additive properties (Table 3). Furthermore, about 79% of the treated samples with drug combinations after 10 days incubation at 28 °C had synergistic or additive properties (Table 4).

DISCUSSION

The results of this study for ten isolates of *T.rubrum* and six other isolates showed that the MICs of aqueous garlic extract obtained at 28 °C for 7 days incubation

are 0.0156-0.5 mg/ml, and for 10 days incubation are 0.0312-1.0 mg/ml. The results were in agreement with Ghahfarokhi et al. (2006) who demonstrated that the MICs of aqueous garlic extract against *T.rubrum*, *T. mentagrophytes*, *M. canis*, and *E. floccosum* are 0.0039-0.0625 mg/ml.

The MICs and MFC attained in this study, for six other isolates of dermatophytes, could be presented as *T. mentagrophytes* > *T. rubrum* > *T. verrucosum* > *Microsporum canis*, and *Epidermophyton floccosum* for all tested drugs individually at 28 °C for 7 and 10 days incubation respectively. They were in agreement with the studies by Odds et al. (2004); Fernandez-Torres, Inza, Guarro, (2003). As a concluding remark, aqueous garlic extract showed very good potential as an antifungal compound against dermatophytosis, so this antifungal agent probably appears to be suitable alter-

native for the treatment of dermatomycosis.

In this study, fungicidal effect of aqueous garlic extract (MFC) was achieved to determine the minimum fungicidal concentration. The results of this study showed that at garlic extract concentrations less than 1.0 mg/ml, almost no significant fungicidal action was observed, but at concentrations with 2.0-8.0 mg/ml, no fungal growth was noted and no conidia survived. It could be concluded that garlic extract, at low concentrations is fungistatic against isolates of *T.rubrum* and other dermatophytes in this study, but at high concentrations it changed into fungicidal. The results were in agreement with earlier studies (Yamada and Azuma 1977; Yoshida et al. 1987; Davis et al. 1990) who showed that high concentrations of garlic extract have been shown to have both fungistatic and fungicidal activities *in vitro* and *in vivo*.

For synergistic effects or to increase efficacy on present dermatophytes infections, garlic extract can be added to reduce concentration of ketoconazole and fluconazole with better results. The present study as well as the previous investigations (Yin, Chang, Tsao, 2002; Pengelly, 2004) proved the ability of aqueous garlic extract to inhibit the fungal growth (e.g. dermatomycosis). These combinations can not only be used alone as antifungal drugs but also in combination with aqueous garlic extract with antifungal azoles which results in an increase in fungicidal effectiveness. The findings of the present study suggest that the application of these combinations may considerably inhibit the fungal pathogens. Moreover, it is possible to lower the dose of antifungal azoles in these combinations when using alone and this will reduce the use of chemical drugs hence, decrease its side effects and toxicity. Since, garlic extracts have broad spectrum fungicidal effects, the risk of fungal resistance is also reduced and helpful in the treatment of patients.

CONCLUSION

Aqueous garlic extract alone is potentially as good as antifungal compounds against dermatophytes, better than synthetic drug fluconazole and almost as good as ketoconazole. Therefore, it can be considered as alternative in the treatment of dermatomycosis. Combination of garlic extract with ketoconazole or fluconazole have synergistic or additive effect against dermatophytes. Combination of natural products and synthetic drugs may also decrease the side effects of synthetic antifungal agents. However, further research is required including "*in vivo*" studies, as well as using other combinations of natural compounds and synthetic drugs for effective treatment as there is a lack of data in this field.

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Conflict of Interests

The authors have declared that no conflict of interest exists.

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