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Full Length Research Paper

In vitro antifungal activity of allicin alone and in combination with two medications against six dermatophytic fungi

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Dermatophytes are fungi capable of invading keratinized tissues of humans and animals, causing dermatomycosis. Azole antifungal drugs are often used in the treatment of dermatomycosis. Because of increased use of these medications, azoles are known to cause drug resistance; hence this study investigated an alternative anti-dermatophyte which is plant-based, and biodegradable natural product. Allicin is a pure bioactive compound derived from garlic, which is known worldwide for its antifungal activities. This study evaluated the *in vitro* efficacy of pure allicin alone against six dermatophyte isolates and the MIC50 and MIC90 ranged from 0.098 – 25.0 µg/ml. Results of this study showed that the order of efficacy based on the MICs values was fluconazole > allicin > ketoconazole at 28ºC for both 7 and 10 days incubation. On the other side, most of tested drug combinations demonstrated synergistic or additive interaction for all isolates for both 7 and 10 days incubation at 28ºC. In conclusion allicin alone showed very good potential as an antifungal compound against mycoses-causing dermatophytes, performing better than the synthetic drug fluconazole, and almost the same as ketoconazole, furthermore allicin in combination with ketoconazole or with fluconazole frequently showed synergistic or additive interaction against dermatomycosis.

Key words: Allicin, antifungal drugs, dermatophytes, MIC (minimal inhibitory concentration).

INTRODUCTION

Dermatomycosis is amongst the most prevalent infectious disease which has increased in recent years that caused by dermatophytes. Dermatophytic fungi are able to invade keratinized tissues (skin, hair, and nail) of humans and animals (Barros et al., 2007). The azoles group of antifungal drugs such as ketoconazole and fluconazole have been used for the treatment of various fungal infections especially dermatomycosis. This groups are synthetic drugs and although effective, but, because of increased use of these medications, an incidence of drug resistance to all agents in the azoles group has been reported (Al-Mohsen and Hughes, 1998; Odds et al., 2003; Pyan and Shin, 2006). Furthermore, they are known generally to cause side effects (Al-Mohsen and Hughes, 1998), hence this study investigated the use of a plant-based, biodegradable natural product as an alternative. The data showed that the extracts of garlic (*Allium sativum)*, were the most potent plant material tested against fungal and bacterial pathogens. Garlic includes organosulphur groups, so consists in antimicrobial features (Woods-Panzaru et al., 2009). The antimicrobial effect of garlic related to interaction of sulphur compounds (such as allicin) with sulphur (thiol-containing enzymes) groups of microbial enzymes (such as trypsin and other proteases), causing to an inhibition of microbial growth (Shadkchan, 2004; Wilson and Demmig, 2007).Allicin is a pure, bioactive and the most powerful medicinal compound isolated from garlic. It is manufactured via an enzymatic response from freshly crushed garlic (Gardner et al., 2007). Alliinase, the

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enzyme that is stocked in isolated section in garlic, is mixed with a compound named alliin in fresh garlic and makes allicin. Allicin has been reported to show antibacterial properties (Cai et al., 2007) and antifungal activities (Shadkchan, 2004; Pyan and Shin, 2006; Gardner et al., 2007; Woods-Panzaru et al., 2009). Ajoene is the ingredient from allicin that has antifungal action against *Aspergillus niger* and *Candida albicance* (Yoshida et al., 1987). Currently, pure grade allicin is available commercially. Many organisms can be inhibited with garlic, and some of them were inhibited much more strongly by allicin compared to antibiotics (Wilson and Demmig, 2007). Pyan and Shin (2006) compared the activity of allicin with the activities of essential oils from *Allium* plants and demonstrated weak performance of the oil fractions in comparison with allicin against *Trichophyton* spp. Currently a standardized *in vitro* protocol for susceptibility testing is available from the National Committee for Clinical Laboratory Standards (NCCLS) established in 2002. Barros et al. (2007) have reported that the susceptibility tests to antifungal agents were suitable and reliable. This method is based on the calculation of the MIC, with more calculations of FICI (fractional inhibitory concentration index) for drug combinations. Pyun and Shin (2006) used broth microdilution method to investigate the activity of allicin alone and the combined effects of *Allium* oils with ketoconazole against *Trichophyton* spp.

In the present study, *in vitro* antifungal activity of allicin alone and in combination with ketoconazole and fluconazole against six dermatophytic fungi was investigated. This study sought to evaluate the microdilution susceptibility testing for the determination of MICs and FICIs of allicin and two antifungal medications, as a potential alternative treatment of dermatomycosis using a plant-based product.

MATERIALS AND METHODS

Study design

Six isolates of dermatophytic fungi were examined for their interactions with allicin, ketoconazole and fluconazole respectively; following the protocol outlined NCCLS susceptibility testing guidelines for filamentous fungi M38-A (National Committee for Clinical Laboratory Standards, 2002). Besides; it was assessed two incubation periods up to 7 and10 days.

Isolates

A total of 5 isolates were used, namely *Trichophyton rubrum* (6443), *Trichophyton mentagrophytes* (1233), *Trichophyton verrucosum* (5213), *Microsporum canis* (1437), and *Epidermophyton flocossum* (883). All isolates were kept in sterile saline (0.85%) v/v NaCl at 4ºC until required for bioassays. *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.

Media

The standard RPMI 1640 medium according to NCCLS guidelines was prepared by adding 10.4 g of powdered RPMI 1640 (Sigma) medium in 1000 ml distilled water. Then, it was buffered with 0.165 mol/L 3-[N-morpholino] propane sulfonic acid (MOPS) at 34.54 g per liter and use magnetic stirrer until it was dissolved. It was adjusted to PH 7.0 at 25ºC and sterilized by Millipore filter (0.22 µm) and stored at 4ºC until use.

Antifungal compounds and dilutions

Three antifungal drugs were used namely, allicin (Alexis–Biochemicals Co, San Diego, USA) was dissolved in 10 mg/ml in methanol / water / formic acid (60:40:0.1), then stored at -20 to -70℃. Ketoconazole and fluconazole (Sigma chemicals Co, USA), were dissolved in 100% dimethyl sulfoxide (DMSO) at 1.5 mg/ml and 5 mg/ml respectively, in accordance with the protocol of NCCLS M38-A, and prepared as a stock solution of 1000 µg/ml. The stock was 2x diluted in RPMI 1640 when the antifungal compound was assayed singly and 4x when it was used in combination with another compound to give the same final strength needed for the test (Santos et al., 2006). A series of twofold dilutions were prepared at 100 times higher than the highest desired test final concentration (intermediate concentration). The DMSO solutions were diluted 1:50 in RPMI 1640 medium to produce twice the final intensity needed for the test (final concentration). The stock solutions were stored at -70℃ until use. The concentration of allicin, ketoconazole, and fluconazole drug dillution ranged from 0.05 to 25.0 µg/ml, 0.03 to 16.0 µg/ml, and 0.03 to 64.0 µg/ml respectively.

Inoculum preparation

All isolates were removed from sterile saline (0.85%) v/v NaCl solution to potato dextrose agar (Difco Laboratories, Detroit, Michigan) and incubated at 28ºC for 7 days to induce conidia formation. The seven-day-old colonies were covered with about 5 ml of sterile saline (0.85%) v/v NaCl solution, and the culture surface was gently probed with the tip of a Pasteur pipette to dislodge the spores. The resulting mixture of conidial and hyphal fragments was filtrated with a Whatman's filter no. 40 (pore size: 8 µm), which filtered the hyphal fragments but allowed the passage of microconidia into the collecting flask below as recommended by Santos and Hamdan (2005); Santos et al. (2006); Barros et al. (2007). The densities of the conidial suspensions in cuvettes were adjusted with a UV spectrophotometer at a wavelength of 520 nm that ranged from 0.15 to 0.17 (70% to 72% transmittance). The inoculum concentrations ranged from 2- 4 × 10 µ conidia/ ml. The inoculum quantifications were prepared by counting microconidia using a hematocytometer and by plating 0.01 ml of each inoculum suspensions on sabouraud dextrose agar (Difco Laboratories, Detroit, Michigan). The plates were kept at 28ºC and were observed daily for the presence of colony from units (CFU) and counted as soon as growth becomes visible. These suspensions were diluted 1:50 in RPMI 1640 medium, which corresponded to 2-4x10□ the density required in about 2- 4×10□ conidia/ ml. The test inoculums were prepared in enough volume to inoculate directly each well with 100 µl of the corresponding diluted inoculum suspensions.

Test procedure

U-bottomed 96 well microdilution plates (Brand 781660, Wertheim, Germany) were used; according to the NCCLS protocol to deter mine the *in vitro* susceptibility of fungal organisms to allicin, ketoco-

nazole and fluconazole, and to evaluate the *in vitro* activity and trend of interaction of allicin in combination with ketoconazole or fluconazole.

Antifungal susceptibility testing: 100 µl of the corresponding fungal inoculum suspension prepared earlier and 100 µl of the twofold drug concentration was pippeted into each microdilution well. Each test plate contained two drug-free controls, one of them with only RPMI 1640 medium (negative control) and another with100
µl of RPMI 1640 with 100 µl of the inoculum suspension
(positive control).

***In vitro* drug interactions:** Two drug interactions were examined. The first combination of allicin with ketoconazole and the second was allicin with fluconazole on all of 6 isolates. Preparation of inoculum and the medium were similar to the susceptibility testing. Santos et al. (2006) used of one microplate to examine each kind of inoculum of each strain for each pair of drug combinations based on the method of Gupta et al. (2003).

Incubation time and temperature

All microdilution trays were incubated at 28º C without agitation and were read visually after 7 days to observe the growth colonies as recommended by Santos and Hamdan, (2005), Santos et al. (2006), and Barros et al. (2007). The plates were then read with a spectrophoto-meter (Optizen 14/2v, Mecasys Co., Ltd, Korea) set at a wavelength of 520 nm that ranged from 0.15 to 0.17 (70 to 72% transmittance) after 7 and 10 days of incubation. The MIC 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 et al., 2001; Cai et al., 2007). Spectrophotometric readings were done by measuring the OD at this wavelength, after agitation of the plates. The raw OD readings were converted into measurements of growth as percentages of control readings, as suggested by Pelletier et al. (2002), and Swinne et al. (2005).

Evaluation of the MIC and FICI

The minimal inhibitory concentration (MICs) is the lowest concentration of an antifungal agent that considerably prevents visible growth of an organism on an agar or broth dilution susceptibility test. MIC results were recorded in micrograms per milliliter. For drug combinations, endpoint determination was achieved visually as the lowest concentration showing noticeable growth inhibition for each pair of drug concentration. To determine the endpoint in microdilution procedure, all growths in the microdilution wells were compared to the growth control. For ketoconazole and fluconazole, the MIC was determined as the lowest concentration of the antifungal agent showed about 80% growth inhibition when compared to growth control results (Ghannoum et al., 2004; Santos and Hamdan, 2005; Barros et al., 2007). To compare the actions of the tested drugs, the MIC of all drugs were acquired, including readings of the MIC50 that is the MIC at which 50% of the isolates were inhibited; correspondingly, the MIC90 is the reading at which 90% of the isolates were inhibited (Santos and Hamdan, 2005; Santos et al., 2006).

The fractional inhibitory concentration index (FICI) can be classified as synergistic, additive, 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 et al., 2002; Ozawa et al., 2005). Determination of all the MICs and FICIs were performed in triplicate.

RESULTS

The results of the study showed that the MICs of allicin was obtained at 0.098 – 25.0, ketoconazole 0.0625 – 4.0, and fluconazole 0.5 – 64.0 µg/ml, so the order of efficacy was as follow: fluconazole > allicin > ketoconazole. As for the response of dermatophytes to allicin,the MICs attained for *T.mentagrophytes* > *T. rubrum* > *T. verrucosum* > *Microsporum canis*, and *Epidermophyton flocossum*. The same tendency was observed for ketoconazole and fluconazole at 28ºC at both 7 and 10 days incubation (Tables 1 and 2). As a result of the study, FICI of allicin with ketoconazole or with fluconazole was found to be different 0.375– 3.0. Besides, the results also showed that FICIs for combination of allicin/ketoconazole were lower than the FICIs for combination of allicin/fluconazole at 28ºC for 7 and 10 days after the incubation. Furthermore, the results revealed that about 54% of the treated samples with combination of allicin/ ketoconazole and allicin/fluconazole after 7 days incubation at 28ºC had synergistic or additive properties. Minority of the treated samples displayed indifferent reaction (Table 1). Moreover, about 33.5% of the treated samples with drug combinations after 10 days incubation at 28ºC had synergistic or additive properties. Most of the treated samples showed indifferent reaction (Table 2).

DISCUSSION

Dermatophytes are a group of fungi able to invade keratinized tissues of human and animals, causing dermatomycosis (Barros et al., 2007). Dermatophytosis is one of the popular and prevalent infectious diseases worldwide (Santos and Hamdan, 2005; Barros et al., 2007). The azole antifungal drugs such as imidazole (e.g. ketoconazole) and triazole (e.g. fluconazole) are commonly used in the treatment of dermatomycosis. These antifungal agents are effective, but with increasing usage of these medications, an incidence of drug resistance to the azoles group has been increased. In addition, they generally have toxic effects such as itching, allergic rash, hepatotoxicity (for ketoconazole) and gastrointestinal complaints, elevated liver enzyme (for fluconazole) (Al-Mohsen and Hughes, 1998), as a consequence the use of the natural safe products are necessary..Therefore we investigate the use of a plant-based, biodegradable natural product as an alternative namely allicin. Allicin is a pure, bioactive and the most powerful medicinal compound isolated from garlic and based on the results of several studies, allicin has different biological properties such as antimicrobial and antifungal activities. These studies showed that it could be used as the treatment of fungal infections (Shadkchan, 2004; Cai et al., 2007). This

Table1. Effects of allicin, ketoconazole, and fluconazole alone and in combination on dermatophytes at 28ºC at 7 days incubation.

Dermatophyte species	Allicin		Ketoconazole		Fluconazole		A + K [1]				A + F [2]			
	MIC 50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50[3]	FICI	MIC90	FICI	MIC50	FICI	MIC90	FICI
T.rubrum	0.78	6.25	0.5	2.0	2.0	8.0	0.39/0.125	0.75	3.125/1.0	1.0	0.39/1.0	1.0	3.125/8.0	1.5
T.rubrum (ATCC)	0.78	6.25	0.5	2.0	2.0	8.0	0.39/0.125	0.75	3.125/1.0	1.0	0.78/1.0	1.5	3.125/8.0	1.5
T.mentagrophytes	1.56	25.0	1.0	4.0	4.0	64.0	0.78/1.0	1.5	12.5/4.0	1.5	1.56/4.0	2.0	25.0/64.0	2.0
T.verrucosum	0.39	1.56	0.25	1.0	1.0	4.0	0.098/0.0313	0.375	0.39/0.25	0.5	0.098/0.25	0.5	0.195/1.0	0.375
M. canis	0.098	0.195	0.0625	0.125	0.5	2.0	0.049/0.0313	1.0	0.049/0.0625	0.75	0.098/0.25	1.5	0.39/2.0	3.0
E.floccosum	0.098	0.195	0.0625	0.125	0.5	2.0	0.049/0.0313	1.0	0.049/0.0313	0.5	0.098/0.25	1.5	0.39/2.0	3.0

[1]A+ K:Allicin + Ketoconazole. [3] MIC_{50} is the MIC at which 50% of the isolates were inhibited (µg/ml). Fractional inhibitory concentration index.
[2]A+ F: Allicin + Fluconazole. MIC_{90} is the MIC at which 90% of the isolates were inhibited (µg/ml).

Table 2. Effects of allicin, ketoconazole, and fluconazole alone and in combination on dermatophytes at 28ºC at 10 days incubation.

Dermatophyte species	Allicin		Ketoconazole		Fluconazole		A + K [1]				A + F [2]			
	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90	MIC50[3]	FICI	MIC90	FICI	MIC50	FICI	MIC90	FICI
T.rubrum	1.56	12.5	0.5	2.0	4.0	16.0	0.78/0.25	1.0	6.25/2.0	1.5	0.78/4.0	1.5	12.5/16.0	2.0
T.rubrum (ATCC)	1.56	12.5	0.5	2.0	4.0	16.0	0.78/0.25	1.0	6.25/2.0	1.5	1.56/4.0	2.0	12.5/16.0	2.0
T.mentagrophytes	6.25	25.0	1.0	4.0	16.0	64.0	6.25/1.0	2.0	25.0/8.0	3.0	6.25/8.0	1.5	25.0/64.0	2.0
T.verrucosum	0.78	6.25	0.25	1.0	2.0	8.0	0.195/0.125	0.75	1.56/0.25	0.5	0.39/0.5	0.75	0.78/4.0	0.625
M.canis	0.098	0.195	0.0625	0.125	1.0	4.0	0.049/0.0313	1.0	0.098/0.125	1.5	0.098/1.0	2.0	0.195/8.0	3.0
E.floccosum	0.098	0.195	0.0625	0.125	0.5	2.0	0.049/0.0313	1.0	0.098/0.25	2.5	0.098/1.0	3.0	0.195/4.0	3.0

[1]A+ K:Allicin + Ketoconazole. [3] MIC_{50} is the MIC at which 50% of the isolates were inhibited (µg/ml). Fractional inhibitory concentration index.
[2]A+ F: Allicin + Fluconazole. MIC_{90} is the MIC at which 90% of the isolates were inhibited (µg/ml).

study used allicin as an antifungal agent against dermatophytes species in comparison with two antifungal medications namely, ketoconazole and fluconazole. The MICs of the allicin, ketoconazole and fluconazole against the dermatophytes spp., assessed by broth microdilution assay were listed in Tables_1 and 2.

Yamada and Azuma (1977) proved that the MICs of allicin obtained at 28ºC for 5 days incubation are 0.78 – 6.25 µg/ml. Result of the present study showed that the MICs of allicin obtained at 28ºC for 7 days incubation are MIC_{50} with 0.098 – 1.56 µg/ml and MIC_{90} with 0.195 – 25.0 µg/ml. Also the MICs of ketoconazole attained at 28ºC for 7 days incubation are equal to MIC_{50} and MIC_{90} of ketoconazole with 0.0625 – 1.0 µg/ml and 0.125 – 4.0 µg/ml respectively. The results were in agreement with Santos and Hamdan study (2005) and Korting et al. (1995), but proved to be different from the study of Fernandez-Torres et al. (2003). Besides, the MICs of fluconazole obtained at 28ºC for 7 days incubation are MIC_{50} and MIC_{90} of fluconazole with 0.5 – 4.0 µg/ml and 2.0 – 64.0 µg/ml respectively which are in agreement with Ghannoum et al. (2004); Santos and Hamdan (2005); Santos et al. (2006); and Barros et al. (2007), but demonstrated to be different from the study of Korting et al. (1995). Finally, Santos and Hamdan (2005) demonstrated that ketoconazole is much more effective than fluconazole. Meanwhile, the results of this study revealed that the order of efficacy based on the MIC_{50} and MIC_{90} was flucanozole > allicin > ketoconazole at 28ºC

for 7 and 10 days incubation respectively. The MICs attained in this study could be presented as *T. mentagrophytes* > *T.rubrum* > *T. verrucosum* > *Microsporum canis*, and *Epidermophyton flocossum* for all tested medications separately at 28ºC for 7 and 10 days incubation respectively. They were in agreement with the studies by Odds et al. (2004) and Fernandez-Torres et al. (2003). As a concluding remark, allicin showed to be potentially very good as antifungal compound against dermato-phytosis, doing better than the synthetic medicine such as fluconazole and almost the same as ketoconazole.

This study revealed that the MICs for 10 days incubation increased from 1 to 2 dilutions for drugs tested separately in comparison with the MICs for 7 days incubation except for ketoconazole. The results are in agreement with an extant study by Santos and Hamdan (2005) who proved that an increased incubation time of 10 days compared to 7 days increases MICs from 1 to 2 dilutions with the same medium, 7 days incubation time compared to 4 days proved to generate similar results.

In this study, antifungal susceptibility tests were performed with allicin, ketoconazole and fluconazole. Isolates were resistant *in vitro* to fluconazole, with the MICs > 51.2 µg/ml, and resistant to ketoconazole, with the MICs > 0.8 µg/ml (Therese et al. 2006). The results of this study revealed that the isolates were all susceptible to ketoconazole in MIC50 except for *T. mentagrophytes*, but for MIC90 isolates were all resistant to ketoconazole except for *Microsporum canis*, and *Epidermophyton flocossum*. Besides, isolates were all susceptible to fluconazole except for *T. mentagrophytes.* The results of this study have been agreed with earlier articles by Korting et al. (1995); Kantarcıoglu and Yücel (2002); Ghannoum et al. (2004); Santos et al. (2006); Therese et al. (2006); Barros et al. (2007).

On the other hand, our results showed that the FICIs of allicin with ketoconazole or with fluconazole was found to be different 0.375– 3.0. Furthermore, the FICIs for combination of allicin and ketoconazole were also lower than the combination of allicin and fluconazole.

As in line with Pyun and Shin (2006) reported that ketoconazole combined with allicin against other isolates of *Trichophyton* spp. resulted in additive effects (FICI=0.53 up to 0.75). The results indicate that 54% of the tested medication combinations after 7 days incubation and about 33.5% of the treated samples with drug combinations after 10 days incubation at 28ºC represented synergistic or additive interaction (FICI ≤ 1.0) for all tested isolates. The perivious studies showed that utilizing from the combination of natural products with synthetic drugs decrease the side effects of these medicines and it is useful in the treatment of the patients (Pengelly, 2004). Consequently it could be concluded that allicin alone revealed to be potentially very good as antifungal compound against dermatomycosis, performing better than the synthetic drug (fluconazole) and almost the same as ketoconazole, so this antifungal agent probably appears to be suitable alternative for the treatment of dermatomycosis. Moreover, allicin in combination with ketoconazole or with fluconazole often showed synergistic or additive interaction against mycoses-causing dermatophytes. Since, FICI results are new study in this field, further studies must be conducted *in vitro* or *in vivo* and also tested other combinations as a result of insufficient data in the field.

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