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Inhibitory Effects of Aqueous Onion and Garlic Extracts on Growth and Keratinase Activity in *Trichophyton mentagrophytes*

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ABSTRACT

The effect of onion and garlic extracts on fungal growth and keratinolytic activity was studied in *Trichophyton mentagrophytes* as one of the major etiologic agents of human and animal dermatophytosis in Iran and other parts of the world. In order to find out the best keratinase producer for further steps, culture conditions for 30 strains of *T. mentagrophytes* isolated from human dermatophytosis were optimized on specific solid and liquid media. All of the isolates produced the enzyme on both selective culture media. The maximum keratinolytic activity at submerged cultivation was reported for cultures of *T. mentagrophytes* isolate No. 1 grown for a 12-day period at 32ºC. Extracellular keratinase activity was in the range of 0.28 to 2.18 u/mg protein in different isolates at predetermined optimal conditions. The growth of *T. mentagrophytes* isolate No. 1 was inhibited in the presence of various concentrations of onion and garlic extracts. This inhibition reached to a maximum of 100% for both extracts at 10% v/v concentrations. Keratinase synthesis was also inhibited by two extracts as a dose-dependent manner with maximums about 58.54 and 71.36 percent at 5% concentrations, accordingly. In contrast to the fungal growth, keratinolytic activity was inhibited more by garlic as compared with onion extract. This is the first report on keratinase inhibition by these two natural compounds. Since fungal growth and keratinolytic activity are important factors in pathogenesis of the dermatophytes, their inhibition by onion and garlic indicate that these substances may have potential values for treatment of human and animal dermatophytosis. *Iran. Biomed. J. 7 (3): 113-118, 2003*

Keywords: *Trichophyton mentagrophytes*, Keratinase, Onion, Garlic, Dermatophytosis

INTRODUCTION

Filamentous fungi can synthesize a diverse range of hydrolytic enzymes as proteases, carbohydrases and lipases [1]. Dermatophytes are a specialized group of fungi able to cause zoonotic superficial infections as a consequence of invading keratinized tissues of skin, hair and nails. This ability is related to the action of extracellular enzymes named keratinases [1-3]. Keratinases are the key enzymes in fungal invasion of skin and have been mostly studied in dermatophyte species belonging to the *Trichophyton* and *Microsporum* genera, some pathogenic yeasts as *Candida albicans* and also some other fungi and bacteria [4-10]. The ability of the dermatophytes and other keratinolytic microorganisms to invade skin and subsequent dissemination through the stratum corneum is governed partly by their proteolytic enzymes specially keratinases [1, 5-10]. Keratinolytic enzymes are also involved in microbial bioconversion of keratinous wastes and therefore they are potential targets for biotechnological researches. *T. mentagrophytes* is a well-known producer of keratinases and this ability is probably essential for invasion of host tissues [4, 5, 7, 11, 12]. This fungus is unique among the dermatophytes and some other zoonotic organisms for its high prevalence and distribution and also for several pathogenic varieties. As fungi are

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eukaryotic organisms similar to mammalians, known antifungal drugs have a wide range of side effects on human and animals besides the high cost and limit routes of administration [3]. Indeed, drug resistance problems have already occurred for dermatophytes after several applications. Thus, many research programs have been conducted to find out new natural and synthetic compounds with antifungal properties and minimum side effects [1-3].

Discovery of antimicrobial activities of onion and garlic has a long history and it is reported on different microorganisms e.g. fungi, bacteria and viruses [13-21]. Also, these two naturally occurring phytochemicals has some other important applications in cancer chemoprevention, inhibition of aflatoxin synthesis, mycotoxin-induced toxicity and free radical formation, and prevention of getting an important human viral disease known as hepatitis A [22-26]. Since the role of fungal growth and keratinolytic activity in virulence and pathogenesis of the dermatophytes has been established, this work was conducted to evaluate the effects of aqueous onion and garlic extracts on these two important parameters in *T. mentagrophytes* as a major etiologic agent of dermatophytosis. In the present study, 30 isolates of *T. mentagrophytes* obtained from patients with dermatophytosis have been studied. This is the first report on inhibition of fungal keratinase as an important virulence factor by aqueous onion and garlic extracts.

MATERIALS AND METHODS

Organisms. Thirty strains of *T. mentagrophytes*, isolated from patients with dermatophytosis during routine diagnostic works, were used in this research.

These strains were identified based on colony and microscopic morphology, urease test, hair perforation test, and ability to pigment production on corn meal agar (CMA) plus 2% dextrose [27].

Screening of keratinolytic activity on agar plates. The isolates were screened for keratinase production based on the method of Wawrzkiewicz *et al.* [28] using solid mineral medium. For preparation of the medium, standard keratin powder as a keratin source was added to the sterile agar medium at a final concentration of 0.06%. This medium consists of Bacto agar (15 g), $MgSO_4.7H_2O$ (0.5 g), KH_2PO_4 (0.1 g), $FeSO_4.\ 7H_2O$ (0.01 g), $ZnSO_4.7H_2O$ (0.005 g), NaH_2PO_4 (3.86 g), Na_2HPO_4 (3.97 g), cycloheximide (0.5 g) and chloramphenicol (0.05 g) in one liter of distilled water. The agar plates were inoculated with 20 µl of fungal suspensions (prepared by gently rubbing of slants in the presence of 0.01% Tween 80) contain 5×10^5 cells/ml. Keratinolytic activity of the isolates was detected as a clear zone around the colony after incubation at 25°C for 6 days. The diameter of the clear zone was measured to quantify enzyme activity.

Preparation of aqueous onion and garlic extracts. Freshly prepared Hamedan white onion and garlic were thoroughly washed with distilled water. One hundred gram of each onion and garlic were separately homogenized with 250 ml of 10 mM phosphate buffer, pH 7.0 by a Heidolph DIAX 600 homogenizer. The homogenates were squeezed through three layers of cheesecloth to remove larger particles and then centrifuged at 100,000 ×g at 4°C for 30 min. The obtained supernatants were sterilized by passing through 0.22 µm Millipore filters and then kept at -70°C before use for maximum 24 hours.

Submerged cultivation. Mineral liquid medium contained all ingredients of solid medium except agar was transferred to 250 ml flasks at 50 ml aliquots and sterilized at 121°C for 15 min. Different concentrations of aqueous onion and garlic extracts (0.62, 1.25, 2.50, 5.00 and 10.00% v/v in culture medium) were separately added to the culture media. The flasks were inoculated with *T. mentagrophytes* isolate No. 1 spore suspension as 10^6 cells/ml of the medium. The inoculated flasks were separately shaken in 120 rpm at different temperatures (25, 28, 32 and 36°C) and incubation times (6, 9, 12 and 15 days). Phosphate buffer without plant extracts was used as control. Each experiment was done as triplicate.

Keratinase activity assay. Keratinolytic activity of culture filtrates was measured spectrophotometrically according to the method of Takiuchi *et al.* [29] with some modifications (using keratin powder instead of guinea pig hair as a keratin source). Keratin powder (20 mg), 3.0 ml phosphate buffer (28 mM, pH 7.8) and 2.0 ml culture filtrate were incubated in a shaker water bath at 150 rpm at 37°C for 1 hour. After the addition of 10% trichloroacetic acid (TCA) and centrifugation at 10,000 ×g for 15 min, the optical absorption of the supernatant was measured at 280 nm wavelength using a double-beam UV/VIS 1601 Shimadzu spectrophotometer toward the blank. The

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blank was treated in the same way except for the addition of TCA which done before the initiation of enzyme reaction. The increase of 0.1 unit absorption is equal to one unit of enzyme activity. Protein content was measured according to the Bradford method [30]. Fungal dry weight was determined after the complete drying of a known amount of the wet mycelium at 80°C and considered as growth index.

RESULTS

Fungi. *T. mentagrophytes* isolates were separated from skin scales of dermatophytic patients after culturing of the specimens on Mycobiotic agar (Difco) plates. The isolates were identified based on the production of powdery or cottony white-cream colonies, microscopic features as spiral hyphae and grape-like globose microconidia, positive results in urease and hair perforation tests and finally inability to pigmentation on CMA plus 2% dextrose [1].

Agar plate screening. Screening of 30 *T. mentagrophytes* isolates for keratinase production on solid mineral medium showed that all of the examined isolates were able to produce extracellular keratinase at different levels. Keratinolytic activity was assessed based on the observation of a clear zone around the fungal colony on the plate (Fig. 1). The diameter of clear zone was in the range of 9.5 to 47.5 mm among the isolates. There was a significant difference in keratinolytic activity on solid medium among some isolates (P<0.05).

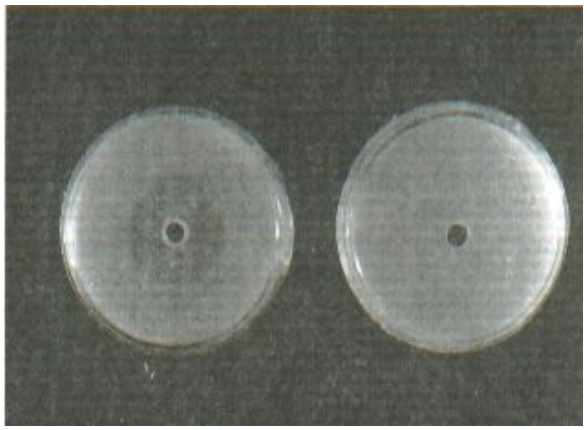

Fig. 1. Screening of keratinase-producing ability based on clear zone production around the fungal colony on solid mineral medium: keratinase-producing *Trichophyton mentagrophytes* isolate (left) and non-keratinolytic *Epidermophyton fluccosum* isolate (right).

Optimization of keratinolytic activity in submerged shaken cultures. In order to confirm the previous screening results, *T. mentagrophytes* isolate No. 1, the best producer of extracellular keratinase, was studied in mineral liquid medium.

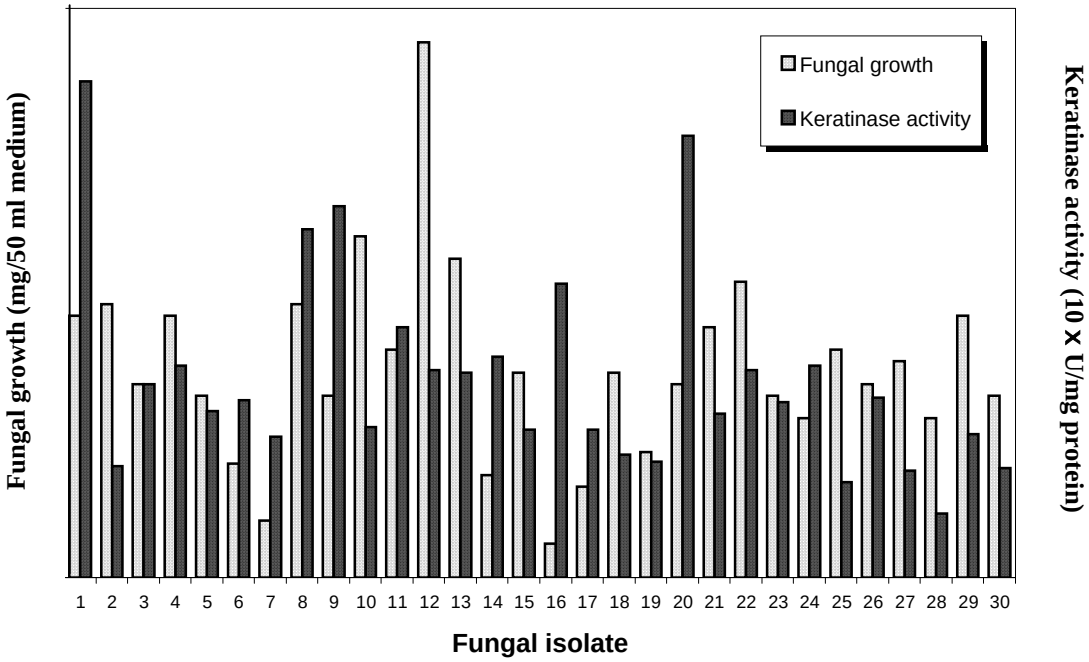

Fig. 2. Comparison of growth rate and extracellular keratinase activity in *Trichophyton mentagrophytes* isolates maintained in submerged cultures at 32°C for 12 days.

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Table 1. Effect of aqueous onion and garlic extracts on *Trichophyton mentagrophytes* (isolate No.1) growth and extracellular keratinase activity in submerged cultures[a].

	Concentration of extract (% , v/v)	Mycelia dry weight (mg)	Growth inhibition (%)	Keratinase activity (% of control)
Onion	0.00	10.00 ± 0.53	0.00	100.00[b]
	0.62	4.10 ± 0.43	59.00	73.11
	1.25	2.50 ± 0.22	75.00	63.32
	2.50	2.35 ± 0.29	76.50	61.31
	5.00	2.30 ± 0.25	77.00	42.46
	10.00	0.00	100.00	ND
Garlic	0.00	10.00 ± 0.53	0.00	100.00[b]
	0.62	6.50 ± 0.70	35.00	41.46
	1.25	5.00 ± 0.38	50.00	34.17
	2.50	4.50 ± 0.31	55.00	31.41
	5.00	4.30 ± 0.32	57.00	28.64
	10.00	0.00	100.00	ND

[a]The results are the means of 2 experiments with triplicate each; [b]100% refers to 3.98 unit/mg protein keratinase activity; ND, not determined.

The fungus was cultured on this medium and keratinase activity was measured after incubation in different culture conditions as described in materials and methods section (data not shown in details). The maximum keratinolytic activity was obtained for 12 days cultures maintained at 32°C.To compare the results in solid and liquid media, all 30 isolates were also monitored for growth rate and keratinase producing ability in determined conditions at submerged cultures.

The fungal growth rate was between 1.5 to 23.5 mg dry weight of 50 ml cultures among the isolates (Fig. 2). Extracellular keratinase specific activity was measured as 0.28 to 2.18 u/mg protein among the isolates (Fig. 2). Total keratinase activity of the isolates was ranged between 0.65 to 4.02 u/50 ml culture media. There was a significant difference in both fungal growth and keratinolytic activity among some of the *T. mentagrophytes* isolates (P<0.05).

Effects of aqueous onion and garlic extracts on fungal growth and keratinase production. The effect of aqueous onion and garlic extracts on fungal growth and keratinase production by *T. mentagrophytes* isolate No. 1 cultured in the presence of different concentrations of both extracts was studied. The results showed that fungal growth was significantly inhibited by both onion and garlic extracts in all concentrations as compared with controls (Table 1). Minimum inhibition for onion and garlic extracts was measured as 59% and 35% at 0.62% v/v concentrations, respectively. The growth was completely inhibited in the presence of maximum concentrations of both extracts (10 %, v/v). It is interesting to note that the keratinase activity was also inhibited by both extracts in a dose-dependent manner (Table 1). Aqueous onion and garlic extracts at the lowest concentrations (0.62 %, v/v) caused approximately 26% and 58% inhibition in enzyme activity by the fungus. Keratinase activity was profoundly inhibited in fungus fed with higher concentrations of both extracts. The maximum inhibition of keratinolytic activity of 5% (v/v) concentration of onion and garlic extracts was 57.54% and 71.36%, respectively (Table 1). These inhibitions were significant as compared with the controls (P<0.05).

DISCUSSION

Dermatophytes are a closely-related group of mycelial fungi that are classified in three major genera: *Microsporum*, *Trichophyton* and *Epidermophyton.* These fungi produce different types of proteolytic enzymes specially keratinases that have key roles in fungal invasion and pathogenesis in human and animal dermatophytosis [1, 6-8]. The proteolytic activity has also been confirmed in some saprophytic fungi, yeasts and even bacteria [5, 7, 9, 10, 31-34].

The long history of the medicinal applications of onion and garlic is well documented [13]. The antibacterial, antifungal, antiviral and anti-carcinogenic properties of these compounds are widely known [14-26]. In present study, the inhibitory effects of aqueous onion and garlic extracts on growth and keratinolytic activity of a selected high keratinase producer isolate of *T. mentagrophytes* were established. Thirty *T. mentagrophytes* isolates were first screened for the selection of best keratinase producer for further

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analysis using solid mineral medium. The obtained results were suitably correlated with results from other workers on keratinolytic activity of different saprobes and dermatophytes [11, 12, 28, 31, 35, 36]. The keratinolytic activity of *T. mentagrophytes* isolates was further confirmed by testing the isolates in submerged cultures at determined optimal conditions. There was an inter-strain variation in fungal keratinase expression in *T. mentagrophytes* isolates similar to the screening results. These results were correlated with other results of keratinolytic activity in some dermatophytes and also a hyphomycete *Scopulariopsis brevicaulis* [12, 29, 34, 36, 37]. Our results showed that there was no significant correlation between fungal growth and keratinolytic activity in *T. mentagrophytes.* We also could not find an obvious relationship between keratinase production in solid and liquid media. It is postulated that variation in keratinolytic activity for each *T. mentagrophytes* isolate between solid and liquid media can be resulted from different culture ingredients and conditions. Also, inter-strain variation in keratinase activity may be related to the ability of the isolates in production of different types and kinds of keratinases.

After preliminary studies on optimization of keratinolytic activity in *T. mentagrophytes*, the effect of onion and garlic extracts on growth pattern and keratinase synthesis by this fungus was subjected. The growth was completely inhibited for both extracts at the highest concentrations of 10%, v/v. Interestingly, the inhibitory effects of onion extract on *T. mentagrophytes* growth was higher than garlic extract at all concentrations tested. However, there were no significant differences in this regard ($P>0.05$). Several researchers have also reported the antifungal properties of onion and garlic against dermatophytes and other fungi [14-19, 23, 24]. It is interesting to note that the keratinolytic activity of *T. mentagrophytes* was also inhibited by both onion and garlic extracts. However, this inhibition was only significant for the garlic extract at all used concentrations as compared with the controls ($P<0.05$). Unlike growth, keratinase production is more inhibited (up to 1.5-2.0 folds) by garlic as compared with onion at all concentrations (Table 1). Our recent works on fungal ultrastructure indicate that aqueous onion extract disrupts hypha cell wall and causes massive necrosis and disarrangement in some cellular compartments specially nucleus and mitochondria in *T. mentagrophytes* and *T. rubrum* (data not shown). Thus, changes in hyphal structure may be responsible for inhibitory effects of onion extract on growth of these two important dermatophytes. Further results are needed for confirming this hypothesis and also finding actual mechanism(s) of onion and garlic extracts mediated keratinase inhibition.

The data show that onion and garlic extracts can be used as potential candidates for preparation of anti-dermatophytic drug formulations and thus may be useful in the treatment of different kinds of dermatophytosis in human and animals. On the other hand, since the keratinolytic enzymes have an important role in microbial bioconversion of keratinous wastes, two high keratinase producer isolates of *T. mentagrophytes* (No. 1 and No. 20) identified in this study and can be used as rich sources of the enzyme for biotechnological researches.

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