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# Efficacy of the ethanolic extract of *Cuminum cyminum* and *Zingiber officiniale* on protein profiles of allergenic *Alternaria alternata* isolates

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**Abstract**

*Alternaria alternata* (*A. alternata*) is considered a fungal pathogen. The aims of the study were to determine the effect of the ethanolic extract from *Cuminum cyminum* (*C. cyminum*) and *Zingiber officiniale* (*Z. officiniale*) on growth inhibition and protein profile of *A. alternata* pathogens. Antifungal activity of the ethanolic extracts of *C. cyminum* and *Z. officiniale* against *A. alternata* isolates were performed with the pour plate technique. Also, characterization of protein profiles of *A. alternata* isolates was performed before and after treatment with *C. cyminum* and *Z. officiniale* extracts using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Both herbal extracts had anti-*A. alternata* activity to varying extents, indicating 28.80% for *C. cyminum* and 38.46% for *Z. officiniale* at concentration of 2000 mg at day 3. *C. cyminum* reduced the total protein (10.85%) and biomass weight (8.87%) of fungal isolates on day 7, whereas these reductions were 16.36% and 20.39% of total protein and biomass weight for *Z. officiniale,* respectively. The results from SDS-PAGE revealed that the extracts from *C. cyminum* and *Z. officiniale* reduced total protein bands from 44 to 24 for *C. cyminum* (45.5% reduction) and from 44 to 29 for *Z. officiniale* (34.1% reduction). Interestingly, *C. cyminum* and *Z. officiniale* deleted 25 and 30 kDa allergenic bands of *A. alternata* isolates. The predominantly observed decrease in protein fractions of *A. alternata* after treatment with the ethanolic extracts should be considered as an important response of *A. alternata* to the herbal components at their sub-inhibitory concentrations.



## Introduction

*Alternaria alternata* (*A. alternata*) is one of the most common allergenic fungi in the worldwide and has been clinically associated to respiratory allergies such as asthma, allergic rhinosinusitis and hypersensitivity pneumonitis (Pulimood *et al*., 2007; Chowdhary *et al*., 2012; Singh and Denning, 2012). Although there is a wide range of clinical manifestations, *A. alternata* is rarely found to be a cause of invasive infections in animals. This fungal specie is mainly related to the induction of immunoglobulin E (IgE)-mediated respiratory diseases. *A. alternata* spores are considered one of the most abundant and potent sources of airborne sensitizer allergens. The most intense exposure to *A. alternata* allergens is likely to occur outdoors; however, *A. alternata* can colonize in indoor environments and thereby increase exposure levels (Salo *et al*., 2005).

Because *A. alternata* has been increasingly recognized as a powerful respiratory allergic disease inducer (Gabriel *et al*., 2016), it has been proposed that at least some *A. alternata* allergens might play a role in the underlying mechanisms of allergic reaction severity. Therefore, the full definition and understanding of the *A. alternata* allergen repertoire

seem to be crucial in finding an explanation for why sensitization to *A. alternata* is a risk factor for respiratory allergies. The study of the allergenic protein components of this fungus may also provide valuable clues for understanding the interesting recent finding that *A. alternata* activates the innate immune system and enhances lung inflammation induced by unrelated allergens such as grass pollen (Kim *et al*., 2014). Nevertheless, compared with other allergenic sources that are commonly found in the environment, *A. alternata* is still a neglected and underestimated source of allergens (Crameri *et al*., 2014). Considering the above-mentioned facts, there is a clear need to identify the complete array of allergenic components from *A. alternata* and reduce the role of these proteins in the development of respiratory allergies. The herbal extracts have antimicrobial activities and are currently categorized as GRAS (Generally Recognized as Safe). The studies on the effect of herbal essential oils and extracts on protein profiles of allergenic fungi are scanty. So, the present study was conducted to study the effect of the ethanolic extract from *Cuminum cyminum* (*C. cyminum*) and *Zingiber officiniale* (*Z. officiniale*) on protein profile of allergenic *A. alternata* isolates obtained from the air of Ahvaz city, Iran.

## Materials and Methods

*A. alternata* strains: *A. alternata* strains (Aa1-Aa5), isolated from the air of Ahvaz city, and were cultured on Sabouraud dextrose agar (SDA) (Merck Co., Germany) at 27°C for 7 days.

Plant materials: The aerial parts of *C. cyminum* and *Z. officiniale* plants were purchased from Ahvaz city, Khozestan province (South of Iran) in spring 2017. The plants were identified at the Herbarium of Pharmacognosy Department, Faculty of Medicine, Jundishapur University of Medical Sciences, Ahvaz, Iran.

Extraction procedure: The air-dried plants were ground into fine powders. The plant powders were extracted with 90% ethanol employing maceration method (Salehi Surmaghi, 2008). Subsequently, the extracts were filtered through Whatman paper (No.1), and the solvents were distilled at 40°C. The remaining extracts were finally dried in an oven at 40°C for 3 hours to ensure the removal of any residual solvent and kept at 4°C until use.

Antifungal assay: Antifungal activity of the ethanolic extracts of *C. cyminum* and *Z. officiniale* against *A. alternata* isolates were performed with the pour plate method as described by Askarne *et al*. (2012). Briefly, the agar plates were prepared using SDA (15 ml per Petri dish) amended with various concentrations of extracts; 40, 60, 100, 200, 400, 800, 1600, and 2000 mg/plate. After inoculating the mycelia of fungus (1 × 1 $cm^2$ segment) onto the center of agar, the dishes were incubated at 27°C for 3 and 7 days. The plates containing ethanol without extracts were cultured as controls. Then, the inhibition percent (IP) was calculated as follows:

$$IP = (C-T) / C \times 100$$

Where "C" was colony diameter of control sets and "T" was colony diameter measured in treatment sets.

Preparation of cytoplasmic extract of *A. alternata*

1. Mass cultivation: Each fungal isolate was transferred to erlens containing 100 ml of Sabouraud dextrose broth (SDB) and 1 ml of *C. cyminum* extract (400 mg/ml), and also SDB containing 1 ml of *Z. officiniale* extract (400 mg/ml), and agitated in the shaker (100 g) at 27°C for 7-10 days. Fungal mycelia and spores were separated from the broth media by centrifugation at 3000 g for 15 minutes. The pellets were washed three times with 10 mM Phosphate buffered saline (PBS), pH 7.5 by centrifugation at 3000 g for 15 minutes.

2. Cell fractionation and crude extract preparation: The fungal elements were disrupted using homogenizer set. After cell disruption, the crude extracts were separated from other cell components by centrifugation at 13200 g for 30 minutes. The extracts were sterilized using a filter (0.2 µ, Sartorious Co., USA) and stored at – 20° C until required.

3. Determination of protein: Protein concentrations of obtained samples of the cytoplasmic extracts were determined by Bradford method (1976).

4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was performed using the Laemmli method (1970). The fungal extracts were dissolved in a sample buffer (Novex Co., USA) and boiled for 5 minutes. Aliquots of the sample (volume = 10 µl, which equals 60 µg of the protein) and standard markers (3 ± 185 kDa) (Novex Co., USA) were applied to a Novex precast NuBis-Tris gel (4 ± 12%) for the separation of fungal antigenic proteins. Electrophoresis was performed with a Biometra Minigel-Twin for 40 minutes at a constant voltage of 200 watt. The gel was fixed and stained with silver nitrate.

Statistical analysis: The data analysis was undertaken with a SPSS (version no. 12, USA) program using the Student's t-test statistical method for analysis of significance. A *p*-value less than 0.05 was regarded as significant

## Results and Discussion

The inhibition percent (IP) values of herbal extracts were determined 3 and 7 days after culturing (Table 1). All the tested extracts in our study showed anti-*A. alternata* activity to varying extents. The incorporation of increased concentrations of both herbal extracts to the media led to progressive and significant reduction in growth for all *A. alternata* isolates. The highest inhibitory percentages of *C. cyminum* were found to be 28.80% and 24.56% at days 3 and 7, respectively. The inhibition of the fungus by the extract of *C. cyminum* is in line with the study carried out by Ramognoli *et al*. (2010). These authors showed a dose-dependent inhibition of *C. cyminum* that reached 81.4% at the dose of 20 µl against *A. alternata*. In a study performed by Kedia *et al*. (2014), *C. cyminum* showed a fungicidal effect against *A. alternata* that reached 100% mycelial inhibition at the dose of 0.6 µl/ml concentration. In addition, Pawar and Thaker (2007) showed the inhibitory zone of *Alternaria* by *C. cyminum* at value of 28.5 mm. Previous studies proved that inhibitory effect of *C. cyminum* is associated with a group of terpenes such as a-pinene and 1,8 cineole (Davidson and Naidu, 2000; Hammer *et al*., 2003).

**Table 1.** The inhibition percent (IP) of the ethanolic extracts from *Cuminum cyminum* and *Zingiber officiniale.*

| Fungus | Plant extract | 100 (mg/plate) | 200 (mg/plate) | 400 (mg/plate) | 800 (mg/plate) | 1600 (mg/plate) | 2000 (mg/plate) | Growth time |
|---|---|---|---|---|---|---|---|---|
| *Alternaria alternata* | *C. cyminum* | 20.51% | 25.64% | 27.70% | 28% | 28.80% | 28.80% | 3 days |
| | | 12.10% | 14.03% | 15.78% | 17.50% | 22.80% | 24.56% | 7 days |
| | *Z. officiniale* | 28.20% | 30.77% | 33.33% | 35.89% | 38.46% | 38.46% | 3 days |
| | | 12.03% | 17.54% | 21.05% | 28.07% | 31.57% | 33.33% | 7 days |

In the present study, the highest inhibitory percentages were found to be 38.46% and 33.33% for *Z. officiniale* extract at concentration of 2000 mg at days 3 and 7, respectively. The inhibitory effect of *Z. officiniale* against *A. alternata* isolates was higher than that of *C. cyminum*. Results on the effectiveness of *Z. officiniale* is in line with findings by Fawzi *et al*. (2009), who showed that plant extracts, in particular *Z. officiniale*, had strong antifungal activity with high inhibition on growth of *A. alternata*. In another study by Mudyiwa *et al*. (2016), *Z. officiniale* had the potent antifungal activity on *Alternaria* isolates with mycelial diameter mean of (2.4 cm) at 50%, (2.1 cm) at 75% and (1.2 cm) at 100%. Pawar and Thaker (2018) also showed the inhibitory zone of *Alternaria* by *Z. officiniale* at value of 12.6 mm. The strong inhibition potential of *Z. officiniale* is attributed to the fact that it contains over 400 different compounds, a mixture of both volatile and non-volatile chemical constituents such as zingerone, shogaols and gingerols, sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene) and a small monoterpenoid fraction (β-phelladrene, cineol, and citral (Grzanna *et al*., 2005).

Table 2 represented the results of biomass measurements and total protein quantification for *A. alternata* isolates grown in SDB before and after exposed to *C. cyminum* and *Z. officiniale* ethanolic extracts. *C. cyminum* reduced the total protein (10.85%) and biomass weight (8.87%) of fungal isolates on day 7 in the cultures containing 400 mg/ml of *C. cyminum*, whereas these reductions were 16.36% and 20.39% of total protein and biomass weight for *Z. officiniale*, respectively. According to the results, the effect of *Z. officiniale* on protein synthesis of *A. alternata* isolates was higher than that of *C. cyminum*. To the best our knowledge, there was no report concerning the effect of *C. cyminum* and *Z. officiniale* on total protein and mycelia weight of *A. alternata* and other species of *Alternaria*. In a study by Hadizadeh *et al*. (2009), the inhibitory effect of nettle, thyme, eucalyptus and Rue oils against *A. alternata* was proved. Nettle oil at 1500 ppm showed a potent and completely inhibitory effect on the radial growth of *A. alternata* and biomass weight reduction. The oil of thyme exhibited a moderate to high

**Table 2.** The effect of the ethanolic extracts of *Cuminum cyminum* and *Zingiber officiniale* on total protein and mass.

| Fungal isolate | *C. cyminum* (400 mg/ml) | | *Z. officiniale* (400 mg/ml) | | Control | |
|---|---|---|---|---|---|---|
| | Total protein (g/ml) | Biomass weight (g) | Total protein (g/ml) | Biomass weight (g) | Total protein (g/ml) | Biomass weight (g) |
| Aa1 | 1.5 | 2.36 | 1.25 | 1.77 | 1.63 | 2.35 |
| Aa2 | 1.02 | 2.12 | 1.01 | 2.14 | 1.2 | 2.56 |
| Aa3 | 1 | 2.22 | 0.97 | 1.42 | 0.95 | 2.41 |
| Aa4 | 1.17 | 2.69 | 1.1 | 2.24 | 1.12 | 2.96 |
| Aa5 | 0.98 | 1.92 | 1.03 | 2.31 | 1.46 | 2.13 |
| Total | 5.67 | 11.31 | 5.36 | 9.88 | 6.36 | 12.41 |
| Mean | 1.134 | 2.262 | 1.072 | 1.976 | 1.272 | 2.482 |

antifungal activity against the pathogen tested, ranging from 68.5-74.8% at 1500 and 2000 ppm, respectively. Low effects of Rue and eucalyptus oils were observed against *A. alternata in vitro* contact assay with fungal mycelial growth inhibition percentage from 7.3-30.6% and 8.7-20.7%, respectively. The ability of herbal components to reduce biomass of filamentous fungi, in particular *Alternaria* sp., has recently been described by Silva *et al*. (2009). The authors suggested polyphenols as the active components in the herbal extracts, since these components could form complexes with proteins, inactivating enzymes essential for fungal growth (Haslam, 1996). In a study conducted by Khosravi *et al*. (2011), a marked depletion of cytoplasmic proteins of *Aspergillus flavus* treated with *C. cyminum* oil accompanied by lysis and disruption of membranes of major organelles such as nuclei and mitochondria. This effect proved that the herbal components passed not only through the cell wall but also through the plasma membrane and then interacted with membranous structures of the cytoplasmic organelles and protein biosynthesis.

Our results from the SDS-PAGE stained with silver nitrate indicated that the cytoplasmic extracts from *A. alternata* isolates had different 24 protein bands, which ranged from 17 to 83.5 kDa (Fig. 1 and Table 3). Most bands were concentrated between 19 and 38 kDa in all of the species. Among these fungal isolates, "Aa2" isolate had the maximum number of protein bands (12 bands) ranging from 25 to 84 kDa and "Aa3" isolate had the minimum protein bands (5 bands) ranging from 36 to 73 kDa. In a study conducted by Sabokbar *et al*. (2014), a total of 9 protein bands ranging from 22 kDa to 178 kDa were detected in *A. alternata*. Identification of a protein in a complex antigenic extract on the sole basis of its molecular weight is a difficult process and sometimes the same antigen may have small differences in molecular weight in different studies. In addition, the differences observed in molecular weight may be related to differences in the calculation of the molecular weight or to the presence of several antigens with the same molecular weight, the species used as the source of antigenic proteins and sometimes even the methods of detection. Furthermore, a major problem in purifying proteins from *Alternaria* is the presence of large amounts of pigmented material which hampers chromatography. The identification and purification of *A. alternata* allergens is important in the standardization of allergenic extracts used in the diagnosis of allergic diseases as well as developing ways to improve treatment of these common diseases. In our study, the ethanolic extracts from *C. cyminum* and *Z. officiniale* reduced total protein bands from 44 to 24 for *C. cyminum* (45.5% reduction) and from 44 to 29 for *Z. officiniale* (34.1% reduction) in SDS-PAGE stained with silver nitrate. To the best our knowledge, there was no previous report concerning the effect of *C. cyminum* and *Z. officiniale* on protein profile of *A. alternata*. Previously, the investigators demonstrated a broad spectrum of changes in protein profiles of *C. albicans* induced by thyme, tea tree, clove and peppermint oils (Rajkowska *et al*., 2016). Scanning and transmission electron microscopy revealed significant morphological alterations with cellular deformity of fungal cells in the presence of herbal essential oils (Stringaro *et al*., 2014). The decrease in the number of protein fractions in whole cell proteins profiles of *A. alternata* can be explained by the loss of cell membrane integrity and leakage of intracellular compounds induced by herbal components oils, as

Rajkowska *et al*. (2014) have shown previously. Cytotoxic and genotoxic activity were already recognized for herbal components (Chaieb *et al*., 2007; Bakkali *et al*., 2008), but an explanation of mechanisms of action of *C. cyminum* and *Z. officiniale* on *A. alternata* proteome requires further studies. More than 9 allergens have been described in *A. alternata* extracts, although only 2 of them are major allergens such as Alt a1 (30 kDa) and Alt a2 (25 kDa) (Gabriel *et al*., 2016). Alt a1, which is the most relevant allergen in *A. alternata* extracts, reacts with serum IgE in 82 to 100% of *A. alternata*-sensitized patients (De Vouge *et al*., 1996). Alt a2 is recognized by IgE antibodies of 16 (61%) of 26 individuals allergic to *A. alternata*. Minor *A. alternata* allergens, such as Alt a11 (the highly conserved fungal allergen enolase), Alt a3, Alt a4, Alt a6, Alt a7, Alt a10, Alt a12 and nuclear transport factor 2, have been also reported (Simon-Nobbe *et al*., 2000). In this study, *C. cyminum* deleted 25 and 30 kDa allergenic bands of "Aa1", "Aa2" and "Aa5" isolates whereas *Z. officiniale* deleted 25 and 30 kDa allergenic bands of "Aa1" and "Aa5" isolates (Table 3).

From our findings it can be concluded that plant extracts from *C. cyminum* and *Z. officiniale* can be used for inhibiting of allergenic *A. alternata* since they have antifungal properties. Also, it has been demonstrated that these plant extracts can effectively reduce the fungal biomass and protein synthesis of *A. alternata*. Both herbal extracts deleted 25 and 30 kDa protein bands as the most allergenic bands *of A. alternata*. It can be concluded that plant extracts affected protein patterns of *A. alternata* isolates.

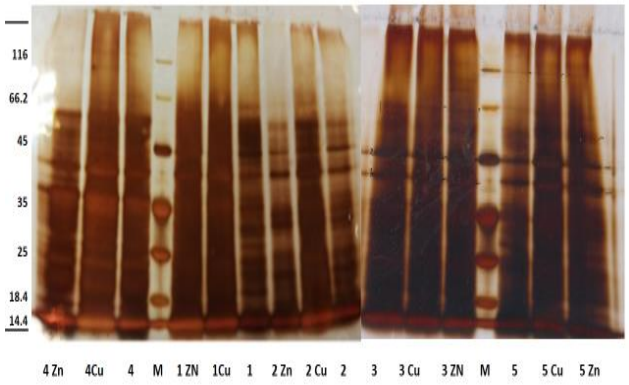


**Fig. 1.** Protein pattern of cytoplasmic extracts of *Alternaria alternata* isolates in gradient SDS-PAGE (silver nitrate staining).

**Table 3.** Protein profiles of *Alternaria alternata* control isolates and isolates treated with *Cuminum cyminum* and *Zingiber officiniale* ethanolic extracts in SDS-PAGE technique.

| Fungal isolate | Control (protein bands) | Total bands | *C. cyminum* treatment (protein bands) | Total bands | *Z. officiniale* treatment (protein bands) | Total bands |
|---|---|---|---|---|---|---|
| Aa1 | 19, 22, 25, 27, 28.5, 30, 35, 38, 45, 53, 76.5 | 11 | 35, 45 | 2 | 36, 45 | 2 |
| Aa2 | 25, 27, 30, 34, 38, 42, 45, 48.5, 57, 62.5, 69, 84 | 12 | 45, 48.5, 57, 62.5, 84 | 6 | 22, 25, 30, 32, 34, 45, 54, 66, 77.5 | 9 |
| Aa3 | 25, 36, 45, 52, 73 | 5 | 27, 38, 45, 52 | 4 | 51, 64, 73 | 3 |
| Aa4 | 19, 22, 34, 35, 45, 64 | 6 | 19, 22, 35, 45, 66 | 5 | 19, 22, 35, 38, 45, 47.5, 69 | 7 |
| Aa5 | 17, 19, 22, 25, 27, 30, 34, 45, 51, 62.5 | 10 | 17, 22, 42, 45, 50, 61.5, 69.5 | 7 | 17, 21, 25, 42, 45, 50, 60, 71 | 8 |
| Total | | 44 | | 24 | | 29 |

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## Conflict of Interest

None declared.

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