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THE USE OF CINNAMON AQUEOUS AND ETHANOLIC EXTRACTS TO CONTROL SOME GROWTH CRITERIA OF THE OPPORTUNISTIC MOLD *ASPERGILLUS FUMIGATUS* I_2

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Abstract

The aim of the present study is to assess the effect of aqueous and ethanol cinnamon extract on some growth criteria of the opportunistic mold *Aspergillus fumigatus* I_2. Ethanol extract or hot water extract of cinnamon were prepared and mixed with *Aspergillus fumigatus* I_2 spore suspension and calculations were performed for count and percentage of the germinated spores. Various concentrations of ethanol cinnamon extract were performed to test their effect on fungal linear growth on Czapek Dox's agar medium and dry weight, protein and polysaccharide content in Czapek Dox's liquid medium. The results showed that Cinnamon ethanol extract was effective as it completely inhibited the germination of spores for 48 hours while the aqueous one has a fungistatic effect which reduced with an increase in the incubation period. There was significant effect of cinnamon ethanol extracts (different concentration) on both *A. fumigatus* I_2 linear growth and dry weight that reduced, notably when concentration increases. The presence of the cinnamon extract in *A. fumigatus* I_2 growth medium is found to decrease the mycelium polysaccharide and protein contents. GC-Mass spectrometry also showed the presence of cinnamaldehyde in the cinnamon ethanol and aqueous extracts alongside benzoic acid, benzaldehyde, and volatile oil. The test revealed an increasing concentration of the active ingredients with the ethanol extract highlighting its substantial antifungal activities.

Key words: Antifungal activity, Benzaldehyde, Benzoic acid, Benzoic acid, Cinnamaldehyde.

Introduction

The presence of the microbial pathogens in the food leads to food spoilage and the occurrence of foodborne diseases posing a detrimental effect on human health (Liu *et al*., 2017). The infection caused by these pathogens has accounted for the increase in the morbidity and mortality rate across the world. The World Health Organization (WHO) reported that 55 million of the people lost their lives as a result of these infectious diseases in 2011 (Nabavi *et al*., 2015a). Correa-Royero *et al*., (2010) stated that plants are generally used in traditional medicine as antimicrobial agents and their extracts play an important role in development of new antifungal compounds.

Various studies have reflected upon *A. fumigatus* as a saprotrophic fungus that disseminates spores (conidia) into the environment, continuously (Fang & Latgé, 2018; Akoumianaki *et al*., 2016). It is labelled as difficult to treat pathogen given its fungicide resistance and adaptability to the various conditions (Moussaid *et al*., 2019). It affects the patients through inhalation, air filtration, direct inoculation through surgical tools, and mechanical ventilation. Traditionally, the use of plants as antimicrobial agents as well as their essential extract has been well accounted in literature. It is because these are claimed to constitute of the antifungal (Bacha *et al*., 2016) as well as antibacterial activities (Bukhari *et al*., 2021).

One of the earlier studies by Marasini *et al*., (2015) used traditional plants to evaluate their antibacterial activity. The results depicted that these plants were good source of antibacterial to combat bacterial infections, displaying a potent antibacterial activity. Whereas, Bansod & Rai (2008) screened the activity of medicinal plants against *A. fumigatus* and *A. niger* to show the maximum activity of mixed oils. The results demonstrated that mycotic infections can be cured by using plant oils as they act as pharmaceutical product and preservative. Similarly, previous studies have shown that extract, essential oil, and spices are effective and showed antifungal activities against the *Aspergillus fumigatus and A. niger* (Jaime *et al*., 2019; Nabavi *et al*., 2015b). Bansod & Rai (2008) demonstrated that tikhadi oil showed the maximum antimycotic activity followed by lemon grass, nigri oil, and cinnamon (*Cinnamomum zeylanicum*). However, a recent study by Mostafa *et al*., (2018) has exhibited the effective antimicrobial activity of the cinnamon with variable efficiency against the tested bacterial strains. Khan & Ahmed (2011) evaluated the effectiveness of essential oils and their major components on the growth, virulence factors, and hyphal ultrastructure of *Trichophyton rubrum* and *Aspergillus fumigatus*. Researches have exhibited that cinnamon bark holds rich ingredients such as cinnamaldehyde that interfere with the biological procedures involving electron transfer and reaction with components containing nitrogen (Fang & Latgé, 2018; Bacha *et al*., 2016).

At present, there are only a few antifungal drugs such as amphotericin B, azoles, and the candins demonstrate partial effectiveness whereas the resistance pertaining to the *Aspergillus* strains is still emerging (Fang & Latgé, 2018). The resistance towards fungal drug, drug interaction, increase in the drug toxicity, and inadequate bioavailability of conventional antifungal drugs promote the use of natural products and develop effective antifungal agents. However, the dearth of information related to the use of cinnamon and its antifungal activities for the multidrug-resistant *A. fumigatus* is observed in Saudi Arabia. Therefore, the study aims to assess the

effect of aqueous and ethanol cinnamon extract on some growth criteria of the opportunistic mold *Aspergillus fumigatus*. The results of the study are assumed to assists in the devising of an alternative for chemical fungicides treatment against the opportunistic *A. fumigatus* I_2.

Materials and Methods

Plant samples and microorganisms: The study included the samples of the cinnamon sticks that were collected from the local market of Al-Madinah Al-Munawarah. While, *Aspergillus fumigatus* I_2 and Czapek Dox Agar (HIMEDIA) were collected from the Microbiological Laboratory of the Biology Department, Taibah University. *Aspergillus fumigatus* I_2 was confirmed by Raper & Fennell (1965) and El-Shafie (1996) to belong to genus Aspergillus. Czapek Dox Agar was used as culture medium to grow *Aspergillus fumigatus* I_2.

Plant extract preparation: Initially, the cinnamon sticks were thoroughly washed in fresh water then completely dried at room temperature for 24 hours, ground to a fine powder and sterilized by passing through a 0.4-micron filter. They had standardized particle size. For hot aqueous extract, the 20gm powder was soaked into 100 ml sterile distilled water (20 percent). For collecting the hot aqueous extract, the solution left in a water bath at 60°C for 30 minutes. The suspended mixture was centrifuged at 5000 rpm for ten minutes, following its sterilization through Stericup filter (Sharaf & Abo Ellil, 2004).

The ethanol extract was prepared using the Varghese (1976) method. Twenty gram of plant powder was mixed in 100ml of the ethanol, left overnight and later filtered through Whatman filter paper No.1, which gave a concentration of 20 percent (w/v) (200 mg/ml). It was then centrifuged at 5000 rpm for ten minutes and the supernatant fluid was evaporated using rotary evaporator. The concentrated extracts were dissolved in dimethyl sulfoxide (10 percent) and were stored at -20°C (Hoque *et al*., 2008).

Cinnamon extracts effect on spore germination: The present study adopted similar method as performed by Sharma & Tripathi (2008) for performing the spore germination. A mixture of either aqueous extract or ethanol extract (0.1 ml) for cinnamon was mixed with 0.1ml *Aspergillus fumigatus* I_2 spore suspension (about 10^6 spores/ml). The mixture was placed in the Vantigian slides central cavity. The glass cover was spread on the slide, which was later incubated for 24 hours or 48 hours or 72 hours at 25 to 28ºC. After the incubation period ends, the lactophenol-cotton blue stain was fixed for each slide, which was observed for germination of spore under a microscope. The count and percentage of the germinated spores were computed. Distilled water or dimethyl sulfoxide was used for exerting control. The replicate slides (3) and microscopic fields (10) were examined. The cinnamon ethanol extract was selected for its prominent inhibitory activity on germination of *A. fumigatus* I_2 spores as well as its comparison with the aqueous extract.

Determination of the *Aspergillus fumigatus* I_2 growth criteria using cinnamon ethanol extract: For linear growth determination, ethanol cinnamon was prepared using various concentrations (20 mg/ml, 40 mg/ml, 80 mg/ml, 120 mg/ml, 160 mg/ml, 180 mg/ml and 200 mg/ml). These were placed in a flask which contains Czapek Dox's agar medium in a melted and cooled form, and which was sterilized for fifteen minutes at 121°C. Following sterilization, the medium was left to solidify on the sterile Petri dishes at room temperature. The fungal disc was used for the central inoculation of the solidified medium where sterile cork borer (0.5 cm) was used to cut the edge of the *Aspergillus fumigatus* I_2 (five days old). The Petri dish was incubated at 28℃ for seven days, which were measured for linear growth for every two days. The triplicates approach was used for the treatment where the linear growth mean diameter was calculated after the fungal disc had been subtracted (Natheer, 2010).

In dry weighing, the cinnamon ethanol extract with different concentration (mg/l) (20, 40, 80, 120, 160, 180 and 200) was introduced in the flask which holds the Czapek Dox's medium in a liquid form which was then sterilized for 15 minutes at 121℃. After its cooling, the fungal disc was inoculated in every flask which was derived from the five day-old culture edge. The incubation time was 7 days at 28℃. The harvesting of the mycelia was performed, after incubation, which was filtered through Whatman filter paper (grade 6) and later washed. At 70°C, the drying of the mycelia occurred which was left overnight until completely dried, where mean and constant weight was calculated. The triplicate approach was adopted for treatments and where control was exerted without plant extract.

Polysaccharide content: For calculating the content of polysaccharide, the ethanol cinnamon (200 mg/ml) was introduced in the Czapek Dox's liquid medium flask (50ml) which was sterilized then incubated with the fungal disc taken from the five-day culture edge. The incubation of the flask was held for seven days at 28°C. The mycelia were gathered, washed, plotted, and dried to be used for the estimation of the polysaccharide content through Nelson solution. The control was conducted in Czapek Dox's liquid medium, which had no extract of plants.

Protein content: The impact of cinnamon ethanol extract on the *Aspergillus fumigatus* I_2 protein content was calculated. The fungal biomass used for polysaccharides estimations, helped in protein estimation through adaptation of the Bradford (1976) method in Giza Agricultural Research Center, Egypt.

Cinnamon extracts phytochemical analysis: Gas-chromatography/mass spectrometry was used to conduct phytochemical analysis for both cinnamon aqueous and ethanolic extracts (Gerhardt *et al*., 1981; Stalikas, 2007). Clevenger's apparatus was used for the evaluation of the total volatile oil in cinnamon extracts using a hydro distillation of oils and oleoresins (Singh *et al*., 2007).

Data analysis

The collected data were statistically analyzed using IBM SPSS Statistical Software. The study performed ANOVA (Analysis of variance) to determine the significant differences in the mean ($p \leq 0.05$).

Results

The impact of ethanol extract of cinnamon on spore germination of the *A. fumigatus* I_2 after being incubated for one (24 hours) to two days (48 hours) where no germination for the spores observed (Table 1). It was observed that incubations following 72 hours (3days), accounted for the spore's recovery following which germination started (7 percent). Whereas, the germination was found to be directly proportional to the increase of the incubation period for the aqueous cinnamon extract. The germination for the aqueous extract was low after 1 day (24 hours) with 2 percent which increased to 20 percent after 48 hours (2 days) and 31 percent after 72 hours (3 days).

The linear growth of *A. fumigatus* I_2 in presence of different concentration for cinnamon ethanol extract is tested (Table 2). The linear growth increase with the increase of the incubation period for the seventh day of growth, though, it was substantially lower as compared to control values, from 20-80 mg/ml. The extract of cinnamon for the range 120 to 200 mg/ml had a linear growth complete inhibition following the fourth day of incubation. After this, incubation for 7 days was associated with decreased linear growth which was 12mm at 120 mg/ml, 7mm at 160 mg/ml, 5mm at 180 mg/ml and 2mm at 200 mg/ml, in contrast to the 90mm attained with control.

The impact of cinnamon extract on the *Aspergillus fumigatus* I_2 dry weight is exhibited in Table 3 after it was incubated for seven days at 28 °C. The dry weight results are consistent with the findings of the linear growth. The *A. fumigatus* I_2 mycelia dry weight reduces as an effect of the increase in extract concentration (Table 3). The increase in concentration of the ethanol extract led to the concomitant decrease in gaining the dry weight which reached its optimal at extract concentration for 200mg/ml when the dry weight was 0.02 g/50ml in comparison to the attained weight of the control (2.06 g/50ml).

Table 4 shows the influence of the cinnamon ethanol extract on the *A. fumigatus* I_2 polysaccharide and protein. It shows that the total mycelia polysaccharide was impacted because of ethanol extract presence (200mg/ml) in the *A. fumigatus* I_2 growth medium. The values 178.2 mg/g was observed for polysaccharide whereas for the control (or untreated), it was 398.9 mg/g. Moreover, the protein content was 235.8 mg/g for treated sample while it was 300.0 mg/g for untreated (Table 4).

Cinnamon extracts (aqueous and ethanol) were evaluated using GC-Mass spectrometry to determine the presence of an active substance. Table 5 shows that cinnamon, cinnamaldehyde, benzoic acid, cinnamic acid, eugenol and benzaldehyde are the active ethanol extract components. The highest among the found components are cinnamaldehyde (58.681%), benzoic acid (5.480%), and benzaldehyde (2.228%). Whereas, the low amount was observed for cinnamic acid, eugenol, and cinnamon, i.e., 1.183%, 0.833%, 0.937%, respectively. However, anisol was not detected in the ethanol extract.

For the cinnamon aqueous solution extract, the presence of some components was recorded through the concentration level, which was generally low, excluding cinnamic acid (4.642%). The aqueous solution lacks two components, i.e., eugenol and cinnamon, while anisol was observed (4.149%). Both extracts ethanol and aqueous extract constitute of volatile oil, i.e., 2.15% and 1.95%, respectively.

Table 1. Effect of cinnamon extracts (10% conc) on spore germination of *A. fumigatus* I_2 after different incubation periods.

Spore germination (%)	Control		Cinnamon	
	DMSO	Water	Aqueous	Ethanol
1 day (24h)	50	53	2	0.0
2 days (48h)	73	76	20	0.0
3 days (72h)	97	100	31	7

Table 2. Effect of cinnamon ethanol extract (different concentration) on linear growth (mm) of *A. fumigatus* I_2 after incubation at 28°C.

Ethanol cinnamon extract (mg/ml)	Linear growth (mm)		
	Incubation days		
	2	4	7
Control (DMSO)	38	48	90
20	17	22	34
40	11	14	25
80	9	12	17
120	-	-	12
160	-	-	7
180	-	-	5
200	-	-	2

- Represents no growth

L.S.D. at 5 percent: 1.2

*The mean diameter of linear growth was calculated after inoculums disc subtraction

Table 3. Effect of cinnamon ethanol extract (different concentration) on the dry weight of *A. fumigatus* I_2.

Cinnamon ethanol extract (mg/ml)	Increase in dry weight (from initial mass/ inoculum)
Control (untreated fungus)	2.06
20	1.10
40	0.91
80	0.63
120	0.27
160	0.15
180	0.06
200	0.02

L.S.D. at 5 percent: 0.035

Table 4. Cinnamon ethanol extract effect on the *A. fumigatus* I_2 polysaccharide and protein content.

A. fumigatus biomass	Total polysaccharide (mg/g)	Total protein (mg/g)
Treated	178.2	235.8
Control (untreated)	398.9	300.0

Table 5. Cinnamon extracts active ingredients and volatile oil.

Active substances	Relative concentration (%)	
	Ethanol	Aqueous
Cinnamon	0.937	0.0
Cinnamic acid	1.183	4.642
Cinnamaldehyde	58.681	48.226
Benzaldehyde	2.228	2.011
Benzoic acid	5.480	4.149
Eugenol	0.833	0.0
Volatile oil	2.15	1.95
Anisol	0.0	4.149

Discussion

The present study assessed the impact of cinnamon extract (aqueous and ethanol) on the growth criteria of the *A. fumigatus* I_2. The findings of the study provide promising results by showing remarkable antimicrobial activity by the ethanol extract. These results are found consistent with the recent study of Zhang *et al*., (2016), who demonstrated the effectiveness of the cinnamon essential oil on membrane integrity and membrane permeability causing cell damage. Various other researches have also highlighted the antifungal activities of the cinnamon extracts (Correa-Royero *et al*., 2010; Sibi *et al*., 2013). For instance, Correa-Royero *et al*., (2010) showed that cinnamon oil extracts improved formulations, stimulated toxicity, and determined optimal concentrations for clinical applications of drugs. Similarly, Sibi *et al*., (2013) narrated that methanolic extracts increase rate for fungal killing, decrease the therapy time, mitigate the drug resistance, expand the spectrum activities, and decline the level of drug toxicity.

The present study also revealed that cinnamon aqueous or ethanol extracts significantly inhibit *A. fumigatus* I_2 spore germination at a variable time interval where the ethanol one was more effective. Likely, Sana & Ghori (2012) and Husain *et al*., (2108) reported the ethanol extract of cinnamon more active as antimicrobial compound than the aqueous one. A fungistatic effect was exerted by aqueous extract of cinnamon on the spore germination, which was decreased as the time interval increased. López Meneses *et al*., (2017) and Sharma & Tripathi (2008) has also reported the *Cinnamon Zeylanicium* suppressive effect on the fungi spore germination, which was found to be dependent upon dose. The use of essential oil, notably *Cinnamomum zeylanicum* was found to inhibit the mold growth. This highlights the strong inhibitory effect of *Cinnamomum zeylanicum* on the growth and spore germination of the pathogenic *Aspergillus* species, which accounts for damaging and changing cell morphology (Correa-Royero *et al*., 2010). Like the present study, Salma *et al*., (2019) confirmed the inhibitory activity of Cinnamon bark ethanol extract against *Staphylococcus aureus* and *E. coli*, more on the former species.

The current study also assessed the different cinnamon ethanol extract concentration to evaluate their direct effect on the *Aspergillus fumigatus* I_2 linear growth. The findings revealed that as the concentration increases, the fungistatic effect on linear growth decreased at all intervals, i.e., 2 days, 4 days, and 7 days. The highest and complete fungicidal effect was observed at the 120 mg/ml to 200 mg/ml following four days incubation. However, the increase in the incubation period accounted for 7 days of growth reported a linear growth of 2mm as compared to 90mm for the controlled values. Similarly, significant inhibition was observed for the cinnamon ethanol-dependent on the concentration, where the maximum inhibition was reached at 200mg/ml when the quantity of dry weight is 0.02 g/50ml in contrast to the control 2.06 g/50ml. These are consistent with the study of Hashim *et al*., (2008), stating that high concentrations of *Cinnamomum zeylanicum* inhibit the growth of *Aspergillus flavus* (100%) mycelium. Pundir & Jain (2010) related the fungistatic or fungicidal action of cinnamon to cytoplasm granulation, cell membrane rupture and cell enzymes suppression.

The present study also revealed the impact of cinnamon ethanol extract on *A. fumigatus* I_2 polysaccharide and protein content. The treatment with the cinnamon ethanol extract showed a reduction in the protein (235.8 mg/g) and polysaccharide component (178.2 mg/g). This is corroborated by Khan & Ahmed (2011) study which examined the *Aspergillus fumigatus* and *Trichophyton rubrum* cellular membrane morphological changes along with endomembranous structures after being treated with cinnamon oil using electron microscopy.

In the present study, the cinnamon extract (ethanol and aqueous) was also assessed for its active component using GC-MS detection. The presence of cinnamaldehyde was found to be highest in cinnamon (ethanol and aqueous extract), though its higher concentration was found in the ethanol extract. Moreover, in the present study benzoic acid, cinnamic acid, and volatile oil were detected in ethanol and aqueous extract, more in the former extract. The findings show that cinnamon bark antimicrobial component was more soluble in ethanol as compared to aqueous extract. Also, ethanol extracts constituted of cinnamon and eugenol, whereas aqueous extract only had anisol. These findings are corroborated by the previous findings of Mukhtar & Ghori (2012).

The cinnamaldehyde in cinnamon was described as the strongest highly electronegative antifungal compound that hinders electron transfer processes and interferes with synthesis of protein and nucleic acids so inhibit the microbial growth (Gupta *et al*., 2008) and also inhibits the amino acid decarboxylase activity (Wendakoon & sakaguchi ,1995).

The findings of the present study suggest that cinnamon extract serves as promising antifungal agents which can help overcome toxicity, examine optimal concentration as well as clinical applications. It also suggests that future studies can examine its use by comparing with the currently used drugs or alongside it.

Conclusion

The study assessed the effect of aqueous and ethanol cinnamon extract for their antifungal activity for fighting against the *Aspergillus fumigatus* I_2 (an opportunistic fungus). The tests carried out in the study reveal promising results where the remarkable antimicrobial activity was found by the ethanol extract. Due to cinnamon ethanol extract strong antifungal activity, the fungus growth criteria were found to decline inclusive of the spore germination, polysaccharides, and protein content. The results of the study concluded that cinnamon extract could be used as a form of green remedy, in comparison to chemical drugs. Moreover, these offer the added benefit of being eco-friendly and cheap as compared to chemical drugs that may impose hazardous effect.

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