Antifungal Activity of Essential Oils Extracted From Clove, Cumin and Cinnamon Against Blue Mold Disease on Citrus Fruit

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Abstract

Essential oils obtained from Cumin seeds, Clove buds and Cinnamon bark was checked for their antifungal potentials against *Penicillium italicum*, causal agent of blue mold disease in citrus fruit. Selected essential oils were checked in different concentrations of 3, 6, 12, 24 and 48µl/ml for their ability to inhibit the mycelial growth of the test fungi. The *in vitro* study revealed that the essential oils of cumin and clove have the potential to inhibit mycelial growth of test fungi completely at concentrations of 12 and 48µl/ml, respectively. Essential oil of cinnamon, however failed to completely inhibit the mycelial growth even at maximum used concentration of 48µl/ml. *In vivo* assays also supported these results. Clove and cumin oils showed complete fungal inhibition at concentration of 24 and 48µl/ml, respectively when applied on citrus fruits. Whereas, cinnamon essential oil could not stop fungal infection even at its highest tested concentration. The study was extended to chemical identification of tested essential oils through GC-MS.

Keywords: essential oils, citrus, Penicillium itallicum, antifungal activity

1. Introduction

Fungi cause significant losses in almost all perishables due to post harvest rots. One of these fungi is *Penicillium italicum* Whemer (blue mold) that results in a universal post-harvest disease of almost all kinds of citrus fruit[1]. Chemicals imazalil, sodium ortho-phenyl phenate, and thiabendazole, have been widely used to control this problem [2]. However increase in public concern regarding contamination of perishables with fungicidal residues and proliferation of resistance in the pathogenic population [3] has forced the community to search for environment safe strategies. In the past few years, there has been a huge increase in the search of natural substances such as essential oils and plant extracts as potential antifungal agents [4]. The use of essential oils to control post-harvest fruit diseases have been deeply investigated and is well documented since the volatile compounds may have better applicability as fumigants for control [5]. In the present work, it has been therefore thought desirable to discover the antifungal potencies of essential oils extracted from three different plants against *P. italicum*, a dominant mycotoxin producing fungi during citrus storage.

2. Methodology

2.1. Test Fungi

The strain of *Penicillium italicum* used in this study was isolated from the decaying citrus fruit (variety: Mandarin). The fungal culture was maintained on Malt Extract Agar (MEA) medium at 4±1°C. A 7-14 days old culture of the isolate was used as the source of inoculum and for the preparation of spore suspension for various studies. The spores were removed from the surface of the culture, suspended in 5 ml of sterile distilled water containing 0.05% (v/v) Tween 80 and its concentration was adjusted to 106sp/ml using a haemocytometer for further *in vivo* studies.

2.2. Extraction of Essential Oils

Seeds of cumin (Cuminum cyminum L.), clove buds [Syzygium aromaticum (L.) Merrill & Perry], and cinnamon bark (Cinnamomum verum J. Presl) were imperiled to hydrodistillation in a modified Clevenger apparatus for 3-4 hours. Isolated EOs were stored in glass after dehydrating with anhydrous sodium sulphate and were kept in the refrigerator at 4±1oC before use. Different concentrations of plant essential oils (Eos) were prepared by adding 3, 6, 12, 24 and 48 µl of pure Eos in 1 ml of 0.05% (v/v) Tween 80 in case of in vivo and 0.5% (v/v) Tween 80 in case of in vitro experiments.

2.3. In Vitro Antifungal Assay by Agar Dilution Method

Prepared concentrations of EOs were mixed with sterile molten MEA medium. Thirty milliliters of media containing different concentrations of EOs and 0.5% Tween 80 was poured into each petri plate which was then inoculated with test fungi and incubated for 7 days at 25±1°C.

2.4. In Vitro Volatile Assay to Check Antifungal Activity

In this method the MEA plates were first inoculated with test fungi and then pure extracted EOs in quantities of 3, 6, 12, 24, and 48 µl were applied on the surface of the sterilized filter paper, which was placed in the lid of the petri plate. After inoculation the plates were incubated in inverted position for seven days at 25±1°C. The zone of inhibition was measured in two directions at right angles to each other. The percentage of mycelial growth inhibition by each essential oil concentration was calculated from the mean colony diameter (cm) on medium without essential oil amendment (control) and from the mean colony diameter (cm) on each essential oil amended plate (zone of growth).

2.5. In vivo antifungal assay

Fruits were sterilized with 6% sodium hypochlorite solution followed by immersion in sterile distilled water for two minutes and surface sterilization in 70% ethanol for another two minutes. Fruit was wounded (2-wounds per fruit) at the equatorial side with a sterile stainless steel scalpel where each wound was about 4 mm long and 2 mm deep. 15 µl of spore suspension was inoculated into each wound using a micropipette under aseptic conditions. Two hours later, each wound was inoculated with a pre-determined concentration from each plant essential oil. Con-

trol fruit was subjected to the same treatments except that sterile distilled water was used instead of essential oil. The treated fruits were labeled, placed in sterilized petri plates and incubated at 23±2 °C for two weeks to assess decay and fungal growth symptoms on daily basis.

2.6. Chemical identification of essential oils by GC-MS

Qualitative analysis of the tested essential oils was undertaken by gas chromatography-mass spectroscopy (GC-MS) using a Hewlett-Packard mass detector (model 7890) coupled with mass spectrometer selective detector 5975. Analysis was carried out using a column HP5 mass-selective detector (MSD) (30 m x 0.25 mm; 0.25 μm film thickness), the operating conditions were as follows: Helium was the carrier gas at a flow rate of 1 ml/min. diluted samples (1:100 v/v, in methanol) of 1 μl were injected manually at temperature 250°C. NIST (National Institute of Standards and Technologies) Mass Spectra Library was also used as a reference.

3. Results and Discussion

3.1. In vitro antifungal efficacy of essential oil

In vitro antifungal activity of selected oils were checked through two methods i.e., dilution method and the volatile method (Table 1). A little variation was observed in results of both the assays. In dilution method cumin oil gave best control on the mycelial growth of P. italicum as its lowest concentration of 3μl/ml gave 96% inhibition. This increased with increase in oil concentration and 12μl/ml completely inhibited the fungal growth. The results of cumin oil was followed by clove oil, whose lowest tested concentration i.e., 3μl/ml showed 84% mycelial growth inhibition. However, the complete inhibition of fungal growth was recorded at maximum tested concentration i.e., 48 μl/ml. Cinnamon gave the lowest effect among the three tested EOs.

In volatile method clove oil gave best control of P. italicum instead of cumin that showed highest inhibition of mycelial growth in dilution method perhaps because of high volatility of phenols that are abundantly present in clove essential oil. Clove oil in 3, 6 and 12μl/ml concentrations gave 97% control on tested fungi. Increase in oil concentration of clove oil gave similar results as were recorded when the same oil was checked through dilution method. Complete inhibition of mycelial growth was observed at maximum tested concentration. In case of cumin the lowest tested concentration of 3μl/ml could only inhibit *P. italicum* up to 32% in contrast to its significant control of 96% recorded in dilution method. Concentration increased the inhibition percentage up to 98-99%. Romagnoli et al.,[6] reported a strong antifungal activity against dermatophytes and phytopathogens including fungi and yeast. They also found cumin aldehyde, pinenes, and *p*-cymene, and a fraction of oxygenate compounds such as alcohol and epoxides as the most active ingredients of cumin essential oil. Cinnamon oil also depicted better control on tested fungi in volatile method when compared to the dilution method perhaps because it is also rich in volatile phenols live clove essential oil. However, still the inhibition effect of cinnamon essential oil failed to match with the controlling capacity of other two tested oils.

Essential Oils	Concentrations (µl/ml)	Average colony diameter of *P. italicum* (cm)		Inhibition of mycelial growth of *P. italicum* (%)	
		Dilution Method	Volatile Method	Dilution Method	Volatile Method
Control	0	6.666±1.7a	5.66±0.83a	0.0±0.0g	0.0±0.0g
Cumin	3	0.266±0.03de	3.83±0.09bc	96±8.3a	32.33±7.1e
	6	0.1±0.07e	1.76±0.03e	98.49±13a	68.9±9.4b
	12	0.0±0.0g	0.066±0.02g	100±0a	98.83±13a
	24	0.0±0.0g	0.1±0.07f	100±0a	98.23±7.5a
	48	0.0±0.0g	0.066±0.03g	100±0a	98.83±3.9a
Clove	3	1.066±0.05c	0.166±0.08f	84±6.2b	97.06±19a
	6	0.733±0.09d	0.166±0.05f	89±11b	97.06±3.7a
	12	0.333±0.04d	0.166±0.09f	95±8.6ab	97.06±6.7a
	24	0.066±0.03f	0.1±0.06f	99±14a	98.23±9.1a
	48	0.0±0.0g	0.0±0.0h	100±0a	100±0.0a
Cinnamon	3	6.666±.74a	4.83±0.59b	0.0±0.0g	14.66±1.5f
	6	6.333±1.04a	3.33±0.37cd	4.995±0.03f	41.16±9.5cd
	12	6.166±0.07ab	3.5±0.83c	7.5±0.14e	38.16±6.2d
	24	5.833±0.43b	3.66±0.51bc	12.496±0.37d	35.33±6.8de
	48	4.5±0.21bc	3.166±0.09d	32.49±2.9c	44.06±5.7c

* Values are means (n=3). Mean values followed by different letters within the column are significantly different according to Duncan Multiple Range Test (P<0.05).

Table 1. In vitro antifungal activity of plant essential oils, used at various concentrations, on mycelial growth of Penicillium italicum*

3.2. In Vivo Antifungal Efficacy of selected Essential Oils

The results of in vivo antifungal efficacy indicated that all the three EOs had a good inhibitory effect on mycelial growth of P. italicum when tested on the surface of the citrus fruit. Yahyazadeh et al.,[7] revealed that essential oil can result in loss of pigmentation in fungal conidia as they became hyaline that may affect virulence of the pathogen; hence a decrease in the incidence of the infection.

Figure 1A, shows that clove essential oil exhibited most pronounced antifungal potentials against P. italicum as it completely inhibited the mycelial growth at concentration of 24µl/ml after 15 days of incubation. At concentration of 3µl/ml the inhibition of P. italicum was 62% and growth started at 4th day of inoculation. Increase in concentration to 6 and 12µl/ml increased fungal inhibition up to 79 and 93% respectively, and also the fungal growth on citrus fruit was delayed till 7th and 11th day.

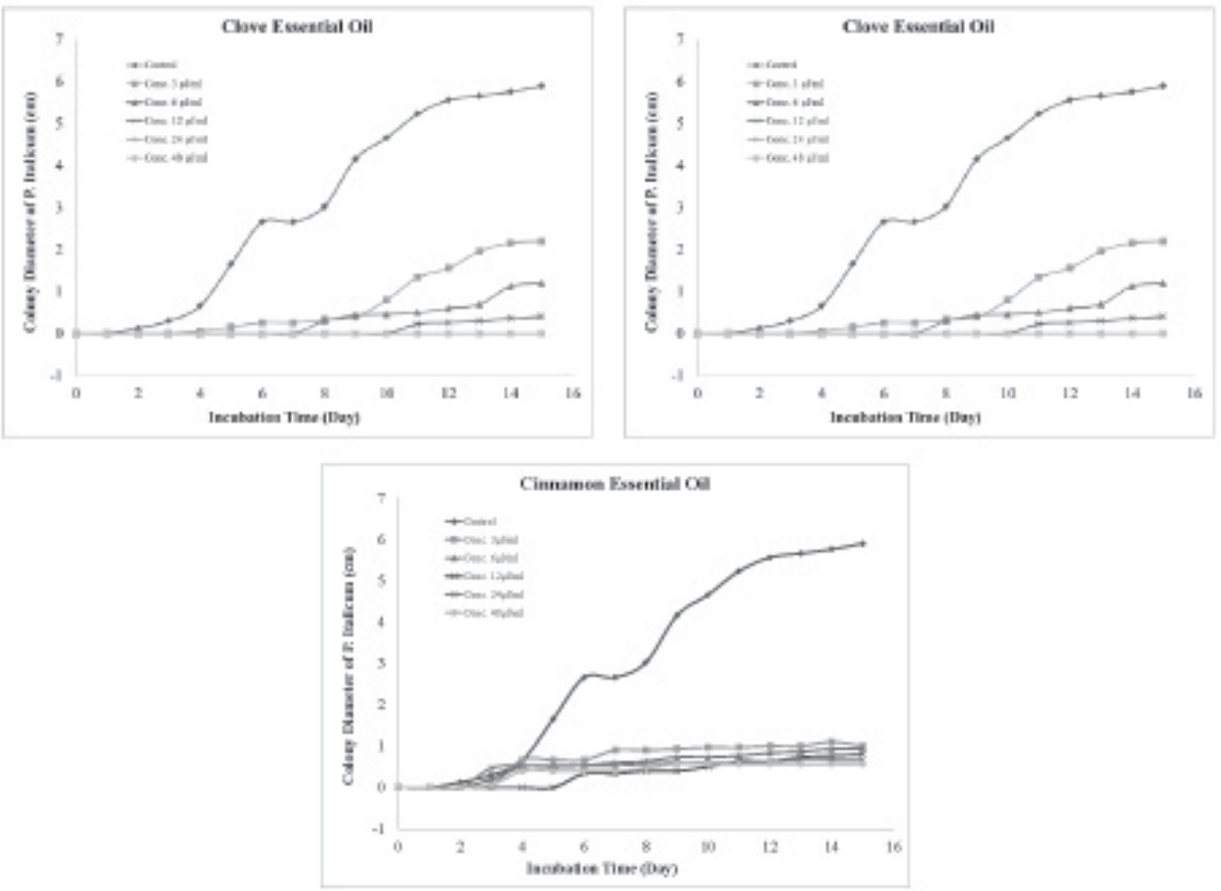

Fig 1. *In vivo* antifungal activity of **A:** Clove; **B:** Cumin**; C:** Cinnamon essential oils, used at various concentrations, on mycelial growth of *Penicillium italicum*

Cumin oil showed complete inhibitory effect at concentration of 48µl/ml while cinnamon oil did not completely inhibit fungal progression even at its highest concentration when applied on citrus fruit. The minimal inhibitory effect of cumin and cinnamon essential oils at lowest tested concentration (3µl/ml) was 88.27 and 83.05 % respectively. Whereas, no significant difference was recorded in inhibitory effects caused by both the cumin and cinnamon essential oils used in higher concentrations of 6 – 48µl/ml.

The data presented in Fig. 1B indicated that cumin oil in concentrations of 3 and 6µl/ml resulted in the inhibition of 88.71 and 89.27% respectively and growth started from the 5th day of the inoculation. However the concentrations of 12 and 24µl/ml delayed the mycelial growth in citrus fruit till 6th and 7th day. While the data presented in Fig. 1C shows that the mycelial development of tested fungus was started from 3rd day of inoculation at all tested cinnamon oil concentrations (3-48µl/ml) except the concentration of 24µl/ml that delayed the growth up to 5 days. These results depicted that the efficacy of clove essential oil during in vivo assays followed by that of cumin, whereas cinnamon oil showed least inhibitory effect.

In general, the results obtained from GC-MS analysis of the essential oils used were in accordance to the previous literature. Clove oil shows the presence of eugenol, alpha-terpineol, Isoeugenol and beta-terpinene as its major components. Eugenol has been reported by different

workers to be the most effective component of the clove and cinnamon EOs against various pathogens[8]. Vazquez et al.,[9] reported complete inhibition of P. citrinum by 2000 ppm of eugenol in a liquid medium.

The major components found in cinnamon oil were eugenol and cinnamaldehyde, whereas cumin oil revealed the presence of gamma-terpinen, cuminaldehyde and 4-carvomenthenol. Singh and Upadhayay[10] showed antifungal activity of Cuminaldehyde against Aspergillus flavus and Aspergillus niger. In a recent study Romagnoli et al.,[6] found cumin aldehyde, pinenes, and p-cymene, and a fraction of oxygenate compounds such as alcohol and epoxides as the most active ingredients of cumin essential oil.

4. References

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