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Essential Oils in Vapour and Direct Contact Treatments for Controlling Mold Growth on Cheese

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ABSTRACT

Background: Natural preservatives are of immense importance to meet the rising demand for food with either little or no synthetic preservatives. This study has evaluated the inhibitory effect of cinnamaldehyde, citral and eugenol essential oil (EO) against mold growth on solid media and cheese in the vapour phase and contact treatments.

Methods: The effect of EO at concentrations ranging from 0.06 to 0.25 µl/ml on radial growth of molds was examined. Further, the antifungal properties of vapors of EOs formulated into edible films at 1 to 5% were investigated using the microatmosphere method.

Result: The application of EOs directly on the surface of cheese at 0.03 mg/cm^2 caused the irreversible inhibition of molds as compared to the control. Both *A. flavus* and *A. niger* were sensitive to EO in contact treatments, but there was no significant difference between different EOs after 6 days. According to the results of vapour phase diffusion test cinnamaldehyde significantly suppressed the growth of *A. flavus* and *A. niger* on the solid medium and cheese surface at 25°C for 7 days storage. These findings suggest that constituents of EOs could be used as a possible substitute for antifungal chemicals to control mold growth on cheese.

Key words: Cheese, Essential oils, Molds, Vapour.

INTRODUCTION

Processed food products containing chemical or synthetic preservatives are highly rejected by modern-day consumers who are very health conscious. Nevertheless, the preservation of quality and safety of the foods throughout their shelf life is necessary. This has led the researchers to look for alternative antimicrobials available from different sources (Chen *et al*., 2014). In the last decades, considerable effort has been on essential oils derived from different plant sources to exploit them for food preservation applications. Plant-derived antimicrobials are 'eco-friendly' and harmless to consumers and can be a useful alternative without compromising the foods' shelf life and safety (Balaguer *et al*., 2013; Ouedrhiri *et al*., 2017; de Campos *et al*., 2022).

Essential oils (EOs) are terpenic mixtures extracted from various parts of plants by steam distillation or other methods. They cause the death of microorganisms by disturbing cell membrane integrity, loss of cellular constituents due to increased permeability and also impair major enzyme activity. EOs are gaining popularity because of being natural due to their origin and broad spectrum of activity against spoilage and pathogenic microorganisms (Keshavarzi *et al*., 2020). It is now a well-known fact that the biological activity of EO is because of some of their constituents present in major quantities. Recently, the major constituents are of special interest to industrial markets because of other potent biological activities. Besides being responsible for the antimicrobial activity of the whole EO as such, some of the major individual constituents of EOs are generally regarded as safe as designated by US-FDA (Liang *et al*., 2015). Furthermore, individual constituents of EOs could be standardized for applications in real foods.

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The antifungal activities are studied by numerous researchers by broth dilution and agar dilution methods. However for the control of mold growth application of EOs on the surface or vapor contact is ideal because that does not allow migration of EO into the product at concentrations higher than the sensory tolerance limits (Feng *et al*., 2011; Torrijos *et al*., 2022). Thus studies on EOs in direct contact on the surface of the product and vapour contact have been reported more rarely (Ju *et al*., 2018). It is noteworthy that molds are useful as starters in some cheese varieties however in many cheese varieties molds are considered spoilage causes (Jeong *et al*., 2014). Mainly, contamination of cheese by *Aspergillus flavus, Aspergillus niger* and *Penicillium* sp could cause spoilage (Kuorwel *et al*., 2014; Casquete *et al*., 2017).

In this sense, cinnamaldehyde, citral and eugenol the main constituents of cinnamon, lemon and clove,

respectively demonstrated antibacterial properties taken up in this study. Therefore this study aimed to determine the antifungal effect of constituents of EOs cinnamaldehyde, eugenol and citral against molds on the surface of laboratory solid media and cheese.

MATERIALS AND METHODS

Essential oils

The EOs used in this study were cinnamaldehyde (99%), eugenol (99%) and citral (99%). They were purchased from Sigma Aldrich Pvt Ltd, Bangalore, India.

Mold strains

The molds used in this study *R. oryzae* NCDC 52, *A. niger* NCDC 315, *A. flavus* NCDC 267 and *A. niger* NCDC 268 were obtained from the National Collection of Dairy Cultures, NDRI, Karnal, India. They were maintained on slants of YEPD agar (pH 4.0: Himedia Laboratories, Mumbai, India).

Preparation of conidial suspension

Conidia were harvested from 7 days old mold grown on Petri plates by adding sterile distilled water containing 0.05% Tween 80 and spores were gently scraped from the mycelia with a sterile inoculation loop to facilitate the release of conidia. The conidial counts were determined using a hemocytometer and subsequent dilutions were made to obtain approximately 10^6 conidia/ml.

Antifungal direct contact bioassay

The effect of the EO constituents on mycelial growth was tested using the poison food plate technique by measuring the radial growth of the fungal colony (Feng *et al*., 2011). The desired concentration of EO constituent was mixed with 20 ml of YPD medium containing 0.1% Tween 80 and then poured into the Petri plates (9.0 cm in diameter). After solidification, Whatman No. 4 paper discs of 6 mm diameter were impregnated with 10 µl containing 10^6 spores per ml and placed at the center of the plate. Essential oil-free medium with conidia and nystatin was used as a positive and negative control, respectively. The plates were incubated at 25°C for 5-7 days until the growth in control plates reaches the edges of the plates. Growth inhibition of each fungal strain was calculated as the percentage of inhibition of radial growth relative to the control. The plates were used in triplicate for each treatment. The relative growth inhibition of treatment compared to control was calculated by percentage, using the following formula:

$$\% \text{ Inhibition of growth} = \frac{\text{Diameter of radial growth of control - Treatment}}{\text{Diameter of radial growth of control}} \times 100$$

The effect of EO on mold spore growth on the paneer surface was assayed by the following method described by Gandomi *et al.* (2009). Briefly, freshly prepared paneer using a sterile knife top 5mm layer was removed and the paneer was then cut and trimmed to fit into a sterile Petri plate. The plates containing slices without a lid were exposed to UV radiation in a laminar hood for 1 hour. Using a cotton swab surface was treated with EO 0.03 mg/cm^2. Then a 10 µl of fungal spore suspension containing approximately 10^5 spores/ml was spot inoculated at the center. The lids were placed back and the gap between the lid and base was sealed with Parafilm® and incubated at 25°C for 7 days. The change in diameter of mycelium was measured in centimeters on a daily basis using a caliper.

Antifungal vapour contact bioassay

The invert plate technique was followed based on the method of Goñi *et al.* (2009). A 20 ml of sterilized YEPD agar was allowed to solidify in a Petri plate. A 0.1 ml of spore suspension (10^6 conidia/ml) was spread uniformly on the surface of the agar and left to dry. Plates were kept in an inverted position and a pre-sterilized disc (Whatman No. 1, Ø=6 mm) was placed in the center of the inner side of the lid and EO (0, 1, 2 and 4 µl) was added to the disc. The plates were then sealed with Parafilm® and incubated at 25°C for 7 days. The control consisting of a disc without EO is included as a control. The diameter zone of inhibition formed above the disc was measured.

Antimold activity of active WPI film on cheese

Edible film casting

Whey protein isolate films were cast on the inner side of the Petri plate by following the method described by Shakeri *et al.* (2011). The film-forming solution (10 ml) added with different levels of EO at 1%, 2%, 4%, 6% and 8% (v/v) concentrations was cast onto a 90 mm Petri dish and kept for drying in an oven at 35°C for 36 h. Further, the film-coated lids were used for evaluation.

Further, 15 ml YEPD agar was allowed to solidify in the base of the Petri dish. The solidified agar surface was inoculated at three points with 10 µl of fungal spore suspension containing approximately 10^5 spores/ml. The lids previously cast with the desired concentration of EO in a film-forming solution were placed. The gap between the lids was sealed with Parafilm® to prevent the loss of EO vapours. After incubation at 25°C for 5 days, the diameter of the colony was recorded in centimeters.

Antifungal activity testing

The modified microatmosphere method described by Balaguer *et al.* (2013) was followed. The commercial processed cheese was procured from the local market. Each slice of cheese was aseptically trimmed to fit in the base of the Petri plate. Three point inoculations were made on the surface of the cheese with 10 µl of fungal spore suspension with approximately 10^5 conidia/ml. Cheese samples without conidial suspension inoculation were used as a control. Active packaging experiments included the lids casted with antimicrobial film in situ and samples with closed empty lids served as control. The gap between the lids was sealed with Parafilm® to prevent the loss of EO vapors. After

incubation at 25°C for 5 days, the diameter of the colony was recorded in centimeters. The results were converted into percentage inhibition by comparing them to the control.

Statistical analysis

All the experiments were performed in triplicates and statistical analyses of the data were performed with SPSS statistical software.

RESULTS AND DISCUSSION

Effects of EO on molds by contact assay

Several individual compounds present in whole EOs can vary widely in antimicrobial effectiveness and compatibility with the sensory properties of food products. The influence of different processing conditions on antimicrobial activities is mostly unknown. Therefore evaluation of purified individual components for antimicrobial efficacy could provide candidate antimicrobial agents for incorporation into food products.

As summarized in Table 1 the EO in direct contact assay has effectively inhibited radial fungal growth of molds *A. niger*, *A. flavus* and *R. oryzae* on a solid growth medium. Amongst the EO tested cinnamaldehyde has shown the greatest diameter of zone of inhibition against molds. The cinnamaldehyde at 0.125 µl/ml caused 100% inhibition of mycelial growth *A. niger* NCDC 315, *A. niger* NCDC 268 and R. *oryzae* NCDC 52 and 66.5% inhibition of *A. niger* NCDC 267 was observed. Citral at 0.125 µl/ml inhibited the growth of *A. niger* NCDC 315, *A. flavus* NCDC 267, *A. niger* NCDC 268 and *R. oryzae* NCDC 52 by 55.7%, 100%, 58.2% and 62.5%, respectively. The total inhibition of these fungi required eugenol at a concertation of more than 0.25 µl/ml. The results have shown that the diameter of the zone of inhibition was proportional to the concentration. The control without EO showed no inhibition. The inhibition of sporulation of mold spores by EO has been previously reported (Feng *et al*., 2011; Ouedrhiri *et al*., 2017; Keshavarzi *et al*., 2020).

We also evaluated the potential application of cinnamaldehyde, eugenol and citral on paneer surfaces as a food model and results are presented in Fig 1. EOs have showed an inhibitory effect against radial growth (diameter) of *A. niger* NCDC 315 and *A. flavus* NCDC 267. In the control group as well as EOs at a concentration 0.03 mg/cm^2 group paneer growth of molds was visible after day 1 of the study. However, a greater reduction of colony diameter was observed in EO treated samples compared to the control. This demonstrates that the surface application of cinnamaldehyde at 0.03 mg/cm^2 has more inhibitory activity

Table 1: Effect of essential oil on radial growth of molds.

EO	Concentration (µl/ml)	*A. niger* NCDC 315		*A. flavus* NCDC 267		*A. niger* NCDC 268		*R. oryzae* NCDC 52	
		Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)	Colony diameter (cm)	Inhibition (%)
Control	0	8.5±0.05	0	5.08±0.45	0	7.55±0.9	0	8.5±0.05	0
Cinnamaldehyde	0.0625	4.7±2.0	44.7	4.5 ±0.2	11.8	6.1±0.45	18.6	7.5±0.05	12.3
	0.125	NG	100	1.8 ±0.24	66.5	NG	100	NG	100
	0.25	NG	100	NG	100	NG	100	NG	100
Eugenol	0.0625	6.9±0.52	19.2	4.13±0.18	18.1	6.5±0.28	13.9	7.5±0.5	13.9
	0.125	4.6±0.05	46.4	3.02±0.04	43.4	4.5±0.05	41.8	5.5±0.1	41.8
	0.25	2.5±0.08	70.1	NG	75.7	1.8±0.05	75.7	1.7±0.13	75.7
Citral	0.0625	6±0.2	29.8	4.9±0.2	67.6	6.2±0.68	17.2	5.6±0.2	34.7
	0.125	3.8±0.2	55.7	NG	100	3.2±.24	58.2	3.2±0.2	62.5
	0.25	NG	100	NG	100	NG	100	1±0.01	88.4

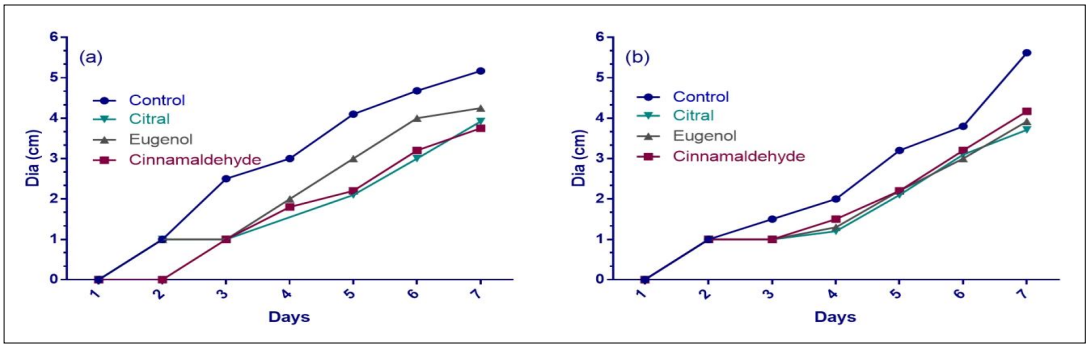


Fig 1: Effect of essential oil on the radial growth growth of *A. flavus* NCDC 267 (a) and *A. niger* NCDC 315 (b) on Indian cheese (paneer) during storage at 25°C.

on *A. niger* NCDC 315 and *A. flavus* NCDC 267 than citral and eugenol. The observed inhibition of *A. niger* and *A. flavus* by EO applied on the cheese surface is consistent with the work of Gandomi *et al*. (2009). Application of thyme and spice EOs by rubbing on cheese surface increased the lag period for *P. camemberti* and *P. roqueforti* has been reported (Wendorff and Wee, 1997; Makhal *et al*., 2014; Mohajeri *et al*., 2018). *A. flavus* NCDC 267 was more sensitive to cinnamaldehyde and citral treated samples than eugenol. No significant difference in the inhibition of mold growth between eugenol and citral was observed. This may be because of several intrinsic factors of foods that could lead to the trapping of hydrophobic EOs thereby making them unavailable for interactions with microorganisms. Therefore many authors warrant for evaluation of the effectiveness of antimicrobials in food systems (Feng *et al*., 2011).

Effects of EO on molds by vapour-phase

Keeping the future applications of films in food products, it's likely that packaging film is not necessary to come in contact with the surface of the food to exert antifungal activities, hence, the microatmosphere method was adopted (Balaguer *et al*., 2013). The antifungal activity of cinnamaldehyde, eugenol and citral EOs through the vapour phase against different mold species was assessed by the presence or absence of a zone of inhibition. The diameter of the inhibition zone is given in Fig 2. All the EOs showed consistently strong antifungal activity which generally decreased in the following order cinnamaldehye, citral and eugenol. *A. flavus* NCDC 267 was more susceptible to EO whereas *R. oryzae* NCDC 52 was the least.

EOs being volatile their concentration is increased in the head-space of the food system by migrating from the film and thereby come in contact with microorganisms on the surface of food (Çakmak *et al*., 2020). The antimicrobial films were prepared by coating WPI with EOs. The WPI being edible has the advantage of longer protection to the food products and also releases antimicrobial agents slowly onto the surface of the packaged food product. The antifungal films were initially tested on a solid medium before being used in the experiments involving the cheese using the microatmosphere method. The diameter of the zone of inhibitions mold growth resulting from films is presented in Table 2. The control film containing no EO in the casted film displayed no appreciable inhibition of *A. niger* NCDC 315 and *A. flavus* NCDC 267 as indicated by a large area of circular growth on the surface of the solid medium and on the cheese surface (Table 2). EO in WPI based film systems at tested concentrations caused the inhibition of circular growth of molds. The percentage of inhibition calculated from the diameter of mold mycelium growth, the film containing cinnamaldehyde has shown more effectiveness

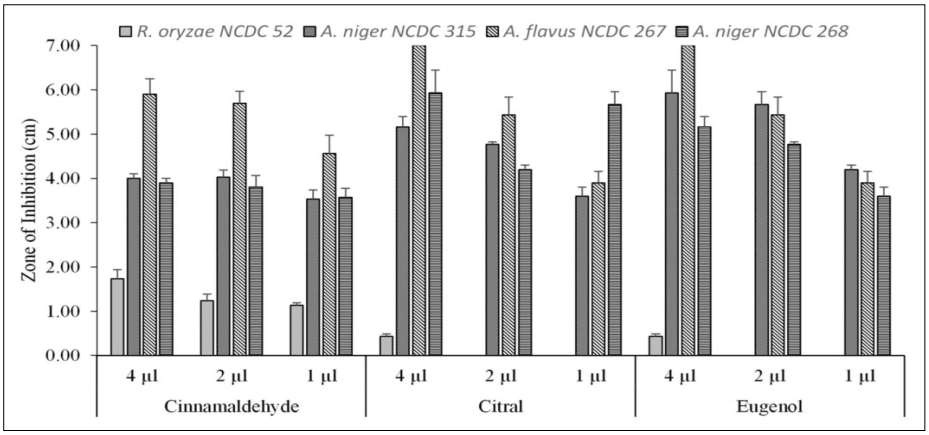


Fig 2: Inhibitory effect of essential oils in vapour phase on the growth of molds.

Table 2: Effect of essential oils in WPI based films against molds after 7 days storage at 25°C (presented as diameter of colony in centimeter).

Essential oil (%)		On YEPD agar		On cheese	
		A. niger NCDC	*A. flavus* NCDC	*A. niger* NCDC	*A. flavus* NCDC
		315	267	315	267
Citral	0	4.23±0.22	4.10±0.25	4.63±0.34	4.44±0.35
	1	NG	0.78±0.24	3.08±0.33	3.11±0.20
	3	NG	NG	1.73±0.09	1.26±0.35
	5	NG	NG	1.00±0.09	1.22±0.23
Cinnamaldehyde	1	NG	NG	0.70±0.09	0.70±0.10
	3	NG	NG	0.70±0.09	0.70±0.10
	5	NG	NG	0.70±0.09	0.70±0.10

NG=No growth.

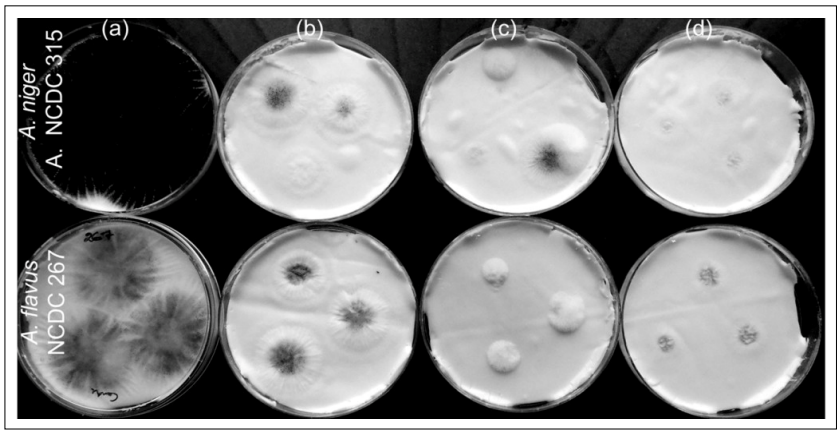


Fig 3: Radial growth of mold in cheese exposed to edible films containing citral essential oil a) 0% b) 1% c) 2% and d) 3% after 7 days storage at 25°C.

than eugenol containing film. The photograph in Fig 3 illustrates typical inhibition of molds *A. niger* NCDC 315 and *A. flavus* NCDC 267. In WPI based film system cinnamaldehyde showed strong activity and eugenol showed weak activity. Studies on the fungal growth in cheeses and other foodstuffs (Çakmak *et al*., 2020; Srisa and Harnkarnsujarit, 2020) have demonstrated that EOs in the antimicrobial films present antifungal activity. The stronger antifungal effect of cinnamaldehyde on the cheese surface observed in this study is consistent with the results of Jeong *et al.* (2014) on the inhibitory effect of cinnamon EO on cheese against *Penicillium* sp. Balaguer *et al*. (2013) found that gliadin films incorporated with 3% cinnamaldehyde was effective in inhibition of *P. expansum* and *A. niger* on sliced bread and cheese spread. The low-molecular weight and highly lipophilic components pass easily through cell membranes and disrupt the fungal cell organization. It has been shown that EOs are lipophilic compounds involved in membrane disruption leading to the death of molds (OuYang *et al*., 2019).

CONCLUSION

The present study highlighted the application of antifungal properties of EOs by different treatments for fungal growth control on the cheese. This study also provides a useful approach to developing antifungal packaging using active constituents of EOs through vapour phase. This is of paramount importance from the point of view of food preservation without detrimental effects on sensorial properties.

Conflict of interest

All authors declare that they have no conflict of interest.

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