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IN VITRO ASSESMENT OF FUNGITOXICITY OF CINNAMON BARK OIL AGAINST ASPERGILLUS SPP.

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ABSTRACT

The fungitoxic properties of vapours of essential oil extracted from Cinnamon (*Cinnamomum zeylanicum* Breyn) bark have been investigated against five species of *Aspergillus viz. A. flavus* Link*, A. fumigatus* Fresenius*, A. nidulans* (Eidam) Wingate*, A. niger* van Tiegham and *A. terreus* Thom, causing biodeterioration of paper manuscripts in Gorakhpur. The fungitoxicity was determined *in vitro* as minimum inhibitory concentration (MIC), minimum lethal concentration (MLC) and inoculum density sustained at MIC and higher doses (fungicidal or fungistatic nature). It is concluded that this oil is effective against all the five selected species of *Aspergillus* and can be recommended for further *in vivo* investigations against *Aspergillus* spp. It is also suggested that the oil should be tested *in vitro* and *in vivo* against species of *Aspergillus* causing diseases in birds, animals and humans too, in order to explore the possibility of their use as a chemotherapeutic agent.

KEYWORDS: **Fungitoxicity, Cinnamon bark, Essential oil, *Aspergillus*.**

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INTRODUCTION

Aspergillus Micheli species are highly aerobic and are found in almost all oxygen-rich environments, where they commonly grow as molds on the surface of a substrate. These species are common contaminants of starchy foods and grow in or on many plants and trees. In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. Species of *Aspergillus* are common saprobes, responsible for natural degradation of a variety of organic matter and deterioration in storage of a number of commodities, including grains, vegetables, fruits, paper, textiles and leather etc.[1-6]. In India, damage to cultural properties by fungal biodeterioration is enormous. Paper manuscripts and paintings are damaged by fungi, including *Aspergillus* and other fungal genera[7]. Species of *Aspergillus* are important medically and commercially also. More than 60 *Aspergillus* species are medically relevant pathogens[8]. Occasionally, some species of this genus are opportunistic pathogens in the respiratory tracts of birds and animals, including man and cause serious diseases[9]. Aspergillosis is the group of diseases caused by *Aspergillus* spp. In humans, the major forms of disease are allergic broncho-pulmonary aspergillosis, acute invasive aspergillosis, disseminated invasive aspergillosis and aspergilloma, a "fungus ball" that can form within cavities such as the lungs. The inappropriate use of synthetic fungicides cause adverse effects on ecosystems and a possible carcinogenic risk[10-13]. These synthetic fungicides are mostly non-biodegradable, heavily pollute the environment, adversely affect the non-target organisms and deface and destroy the cultural objects[14]. Moreover, the fungi develop resistance against these fungicides, which in turn become ineffective[15]. Therefore, there is an urgent need to develop new management system to reduce the dependence on synthetic fungicides. Recent trends favour the use of alternative substances derived from natural plant extracts to control these fungi. Volatile essential oils of plant origin have shown antifungal activity against a wide range of fungi[16-17]. These natural substances do not deface and destroy the objects, including cultural properties, are biodegradable, eco-friendly, cause no pollution and non-toxic. In recent years, volatile constituents of various higher plants, *i.e*. , many essential oils and their constituent terpenoids, have shown potent fungitoxic activity in their vapours against *Aspergillus* spp. and other fungi. Use of such volatiles for protection of stored foods against fungal infestation and also for controlling fungal diseases of crops has been suggested[18-24]. A perusal of literature proves that of all these plants and their parts, cinnamon bark oil is a potent fungitoxicant against an array of fungi, including *Aspergillus* spp.[25-31]. Therefore, the present investigation has been done with an aim to investigate the fungitoxic properties of vapours of essential oils extracted from Cinnamon (*Cinnamomum zeylanicum* Breyn) bark against five species of *Aspergillus viz. A. flavus, A. fumigatus, A. nidulans, A. niger and A. terreus,* causing biodeterioration of paper manuscripts in Gorakhpur.

MATERIALS AND METHODS

(i) Fungal Strains Used as Test Fungi

Five strains of *Aspergillus viz. A. flavus, A. fumigatus, A. nidulans, A. niger and A. terreus* isolated from deteriorated pages of Webster's New International Dictionary of the English Language, 1934[5] were used as test fungi. These fungi were examined by Direct Observation and were isolated by direct lifting with inoculation needle and by Standard Blotter Method[32] and Agar Plate Method (Czapek Dox Agar[33] and Streptomycin Rose Bengal Agar[34]. The fungi obtained in mixed culture were purified by streaking on PDA Medium.

Figure 1
WEBSTER'S DICTIONARY,1934 infested by fungi
(Source of the 5 strains of test fungus – Aspergillus)

Figure 2
Deteriorated inner cover of the Dictionary

Figure 3
Back cover of the same showing fungal colonies

(ii) Plant Material
Cinnamon (*Cinnamomum zeylanicum* Breyn) dried bark was obtained from local market of Gorakhpur.

(iii) Extraction of Essential Oil
Essential oil of the Cinnamon bark was obtained by hydrodistillation in Clevenger's apparatus.

Figure 4
Clevenger's Apparatus used for extraction of essential oil

(iv) Assessment of Antifungal Activity of Essential Oil

Antifungal activity of vapours of extracted essential oils was assessed by the inverted Petri plate technique[35].

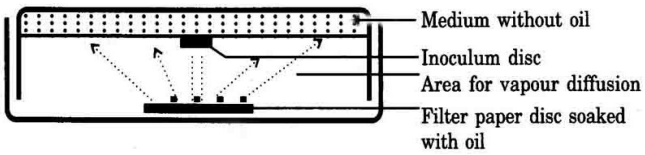

Figure 5
Inverted Petri plate Technique

A 5 mm. diameter inoculum disc of the test fungus, cut from the periphery of the mycelial colony of a seven day old culture, was inoculated on 10 ml. Czapek Dox Agar medium in an 80 mm. diameter Petri dish. The dish was then inverted , and the requisite amount of oil in 0.5 ml. acetone, soaked on a 25 mm. diameter sterile filter paper disc, was placed in the dish on its lid. Sterile distilled water, taken in place of oil in 0.5 ml. acetone, was used as control. Every experiment was repeated ten times and the average of results was recorded. The dishes were incubated at 25° ± 1 °C, and on the 7th day, fungitoxicity was recorded as per cent inhibition of mycelial growth, calculated by the formula:

$$\% \text{ Mycelial Inhibition} = \frac{Gc - Gt}{Gc} \times 100$$

Where, Gc = Colony diameter of the control set,
Gt = Colony diameter of the treatment set.

The dose of vapuors of essential oil was expressed as ppm (parts per million), *i.e.*, parts (volume) of oil per million parts of aerial volume inside the Petri dish available for diffusion of oil vapour, arbitrarily assuming that the given volume of oil volatilizes to produce an equal volume of vapour[35].The Corning glass Petri dish (80 mm. diameter) used in this study had an average inner volume of 60 ± 2 ml., of which 10 ml. was occupied by the medium and 50 ml. medium-free aerial space was available for diffusion of oil vapour. The ppm dose of oil was calculated by progression as the amount of oil (µl) used per litre of medium-free aerial space available for diffusion of oil vapour.

(v) Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of essential oil vapour was determined by observing per cent inhibition of mycelial growth of the test fungus by progressively lower doses of oil, in the range of 100 – 10 ppm. The minimum dose required for 100% inhibition (fungistatic/fungicidal) was recorded as the MIC. The fungistatic/fungicidal nature of fungitoxicity was observed at the MIC and higher doses for determining the minimum lethal concentration (MLC), which was recorded as the minimum dose required for fungicidal action[36].

(vi) Nature of Fungitoxicity

For determining the nature of fungitoxicity of essential oil vapour, the treatment and control sets were prepared at MIC. After 7 days of incubation, the mycelial discs were removed from the Petri plates and re-inoculated on the fresh medium. The presence/absence of mycelia growth in the re-inoculated discs proved the fungistatic/fungicidal nature of the toxicity of vapours, respectively[36] .

(vii) Inoculum Density Sustained

Inoculum density sustained by vapours of oil at MIC and hyper MIC doses was determined by increasing the number of inoculums discs in each assay dish of the treatment set in arithmetic progression of 2, up to a maximum of 24 discs[34].

OBSERVATIONS

Table – 1

MIC* and nature of fungitoxicity of Cinnamomum zeylanicum Breyn bark oil vapours against five strains of Aspergillus

Concentration of Oil (ppm)	Per cent Mycelial Inhibition					Nature of Fungitoxicity** (at MIC)
	A. flavus Link	***A. fumigatus*** Fresenius	***A. nidulans*** (Eidam) Wingate	***A. niger*** van Tiegham	***A. terreus*** Thom	
10	84.8	82.4	84.6	80.0	90.2	+
20	100	100	100	100	100	-
50	100	100	100	100	100	-
100	100	100	100	100	100	-

* = *Minimum Inhibitory Concentration (fungicidal/fungistatic)*

** + = *Fungistatic Nature (presence of mycelial growth in re-inoculated discs)*

\- = *Fungicidal Nature (absence of mycelial growth in re-inoculated discs)*

Table – 2

Inoculum density sustained (Number of inoculum discs of 5 mm. diameter inhibited)

Test Fungi	Inoculum Density Sustained	
	At MIC dose (20 ppm)	At Hyper MIC dose (5 x MIC dose = 5 x 20 = 100 ppm)
A. flavus Link	2	22
A. fumigatus Fresenius	2	24
A. nidulans (Eidam) Wingate	1	22
A. niger van Tiegham	1	20
A. terreus Thom	2	24

RESULTS AND DISCUSSION

Data of Table – 1

reveal that minimum inhibitory concentration (MIC) of *Cinnamomum zeylanicum* bark oil vapours is 20 ppm dose, at which the oil shows fungicidal nature against all the five strains of *Aspergillus viz. A. flavus, A. fumigatus, A. nidulans, A. niger* and *A. terreus,* causing biodeterioration of paper manuscripts in Gorakhpur. At 10 ppm dose also, it is effective, but is fungistatic in nature and mycelial growth is present in re-inoculated discs. The nature of fungitoxicity reveals that at the same 20 ppm dose, the mycelial growth is absent in re-inoculated discs. Therefore, minimum lethal concentration (MLC) of the oil is also 20 ppm. Consequently, MIC and MLC, both values are 20 ppm against all the aforesaid five species of *Aspergillus*.

Data of Table – 2

reveal that vapours of cinnamon bark oil can inhibit not more than two inoculum discs of the all five species of the test fungus *Aspergillus* at MIC dose. However, at hyper MIC dose (5 x MIC), these vapours retain fungitoxicity for appreciably higher inoculums density and a maximum of 24 inoculum discs of 5 mm. diameter are inhibited.

CONCLUSION

It is therefore, concluded that vapour of the oil of *Cinnamomum zeylanicum* Breyn bark is effectively toxic at very low dose (20 ppm.) against all the five species of the selected test fungus – *Aspergillus* causing biodeterioration of paper manuscripts in Gorakhpur. Also, it can inhibit high inoculums density at hyper MIC dose. Therefore, it is recommended for further detailed study under *in vivo* conditions to protect our cultural heritage in paper and textiles damaged by *Aspergillus* spp. and other cellulolytic fungi. It is also suggested that the oil should be tried *in vitro* and *in vivo* against species of *Aspergillus* causing diseases in birds, animals and humans too, in order to explore the possibility of their use as a chemotherapeutic agent.

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