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ORIGINAL ARTICLE

Fungitoxic Effect of Essential Oil of *Citrus sinensis* Test against Storage Fungi of Crude Drug Sample

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ABSTRACT

The peel of the Citrus sinensis fruits was subjected to hydrodistillation through clevenger's apparatus so as to collect the volatile antifungal fraction. The fungitoxic properties of the oil viz., minimum inhibitory concentration (MIC), nature of toxicity, effect of storage and effect of temperature were investigated. The MIC of the oil against the test fungi i.e. A. niger, A. flavus and P. citrinum was found to be 2200 ppm, 2100 ppm and 1300 ppm respectively. At these concentration, the oil exhibited both fungistatic and fungicidal nature in A. niger and A. flavus while it showed fungicidal nature at 1400 ppm for P. citrinum. Essential oil (Citrus sinensis) fumigation showed best result in control of natural infection of test fungi of crude drug sample. Thus, the oil may prove to be an ideal and indigenous effective fumigant and preservative for protection of crude drug sample.

Key words: *Extraction, Efficacy, Citrus sinensis, Test fungi*

INTRODUCTION

Crude drug sample becomes wasteful if it gets damaged before reaching the consumer. large quantities of crude drug sample are either damaged or lost due to various fungal infection during storage.In recent years plant parts and their products have proved their worth in providing less phytotoxic, more systematic, biodegradable and host metabolism stimulating fungicides (Sharma, 2000). In addiotion the volatile substances obtained from higher plants have proved their usefulness in controlling Post harvest fungal diseases and there, are considered to have bright future (Dubey, *et al.,* 1984)

MATERIALS AND METHODS

Essential oil extracted from the pericarp of the fruits of *Citrus sinensis* was tested for their antifungal activity against dominant contaminating fungus viz., *Aspergillus niger, Aspergillus flavus* and *Penicillium citrinum*. The essential oil was extracted by hydro-distillation for 6-8 hours in Clavenger's apparatus (Clavenger, 1928; Guenther, 1948). The volatile fraction thus obtained, exhibited two distinct fractions comprising an upper oily layer and a lower aqueous layer were obtained which were separated by carefully regulating the stopper of the apparatus A clear, light yellow coloured, oily layer was obtained on the top of the aqueous distillate which was separated from the latter and treated with anhydrous sodium sulphate to remove traces of moisture. The obtained essential oil was kept in air tight sealed glass vials and stored for further study.

Antifungal activity was tested by poisoned food technique (Grover and Moore, 1962). Measured amount (according to required concentration) of oils were added to melted cool Potato dextrose agar medium and mixed thoroughly before pouring, for homogenous distribution of oil in medium and plates were poured. Tween-80 (0.05%) mixed in medium, served as control. Treated and control plates were inoculated with 5 mm diameter disc of fungal culture (Table 1). The plates were incubated at 25±1ºC and percent inhibition was calculated as per formula of Panday, *et al.,* (1981). The Minimum inhibitory concentration (MIC) was determined as the minimum amount of oil needed for the inhibition of mycelial growth completely of test fungi.

Percent inhibition over control = $[(dc - dt)/dc] \times 100$

Where, dc = Growth (mm) of fungus in control, dt = Growth (mm) of fungus in treatment.

The fungitoxicity of a substance against a fungus may be temporary (fungistatic) allowing the latter to regain its vitality when the former is removed from its viscinity or the toxicity of the substance may be permanent (fungicidal) not allowing the fungus to regain its vitality when the former was removed from its viscinity (Garber and Houston, 1959). When there was full growth in control plates, the inhibited fungal discs of treatment sets were taken out, washed with sterilized water and re-inoculated, separately in petriplates containing the fresh medium. The plates were incubated similarly and the observation for the revival of growth of the re-inoculated fungal discs was recorded as presence (+) or absence (-) of the mycelial growth (Table 2). In the spore germination assay, nine concentrations of oil (100, 200, 300, 400, 500, 600, 700 and 800 ppm) and control were tested for spore germination of each test fungi (Table 3). Fungal spores obtained from seven day old culture of the fungi were taken and placed on glass slides containing different concentrations of oil and water in control in triplicate form. Slides containing the spores were incubated in a moist chamber at 25±1ºC for 24 hours. When incubation time over then each slide fixed in lactophenol-cotton blue and observed under the microscope for spore germination. About 200 spores were counted and the number of spores germinated scored to calculate the percentage of spore germination. As the oil exhibited acute toxicity against the test fungi, experiments were carried out to examine its *in vivo* efficacy in controlling fungal infestation. Fresh aonla fruits were purchased from local market of Lucknow. Requisite amount of the oil (0.1 ml), so as to obtain the MIC and hyper MIC dose was soaked in sterile cotton swab, which was wrapped in sterilized muslin cloth and placed at the bottom to allow complete coverage of stored samples by vapor of the oil. The untreated sample was prepared by using sterile cotton swab without the oil. Fruits were packed in pre-sterilized polythene bag and kept in incubator at 25±1ºC. The number of infected fruit was counted in control and treatment sets and results were expressed in percent infection. Each treatment had 3 replicates.

Table 1: Effect of different concentrations of *Citrus sinensis* (peel) oil on spore germination of *Aspergillus niger, Aspergillus flavus* and *Penicillium citrinum*

S.No.	Concentration of oil (ppm)	Percent radial growth inhibition		
		A. niger	***A. flavus***	***P.citrinum***
1	Control	00.00	00.00	00.00
2	100	32.40	30.20	62.50
3	200	47.00	45.00	75.00
4	300	65.90	62.00	89.20
5	400	78.00	74.80	100.00
6	500	86.50	85.00	100.00
7	600	93.60	91.20	100.00
8	700	100.00	97.50	100.00
9	800	100.00	100.00	100.00

Each value is mean of three replicates.

Table 2: Nature of fungitoxicity of *Citrus sinensis* oil against *Aspergillus niger, Aspergillus flavus* and *Penicillium citrinum*

S.No.	Conc. of oil (ppm)	Presence (+) and Absence (-) of mycelial growth *A. niger*	*A. flavus*	*P. citrinum*
1	400	T + R +	+ +	- -
2	500	T + R +	+ +	- -
3	600	T - R +	+ +	- -
4	700	T - R +	- +	- -
5	800	T - R -	- -	- -

T: Treated set.
R: Re-inoculated set.

Table 3: Effect of different concentration of *Citrus sinensis* (peel) oil on spore germination of *Aspergillus niger, Aspergillius flavus* and *Penicillium citrinum*

S.No.	Conc. of oil (ppm)	Spore germination (%)		
		A. niger	*A. flavus*	*P. citrinum*
1	Control	100.00	100.00	100.00
2	100	48.80	50.00	32.90
3	200	31.70	37.00	20.50
4	300	19.00	19.50	8.90
5	400	09.70	10.20	0.0
6	500	0.0	0.0	0.0
7	600	0.0	0.0	0.0
8	700	0.0	0.0	0.0
9	800	0.0	0.0	0.0

Each value is mean of three replicates.

RESULTS AND DISCUSSION

The results presented in Table 1 showed *in vitro* efficacy of *Citrus sinensis* peel-oil on the percent radial growth inhibition of test fungi i.e. *Aspergillus niger, Aspergillus flavus* and *Penicillium citrinum*. The results indicated that 100 ppm concentration did not show considerable reduction in growth of *A. niger, A. flavus* while 62.50% in *P. citrinum*. Above 300 ppm, considerable reduction in growth of all test fungi was recorded. MIC was 700 and 800 ppm for *A. niger* and *A. flavus* while MIC was lower as 400 ppm in case of *P. citrinum r*eported higher MIC values, more or less similar result is reported by Sharma and Triphathi (2005) while Singh, *et al.,* (1980); Panday, *et al.,* (1982); Zambonelli, *et al.,* (1996); Antonov, *et al.,* (1997); Beg and Ahmad (2002) and Nguefack, *et al.,* 2004., Sharker, *et al.,* (2009) Rahman, *et al.,* (2010).

It is evident from Table 2 that the oil showed fungicidal activity at 800 ppm for *A. niger* and *A. flavus* while 400 ppm (MIC) also showed fungicidal nature in case of *P. citrinum*. The oil retained its toxicity against test fungi after exposure upto the temperature of 100^0C. The fungi toxic nature of the oil is thermostable. The oil retained its toxicity against the test fungi even after 360 days.

Results of spore germination assay are presented in Table 3 and its corresponding. Results indicated that 100 ppm concentration showed 48.80%, 50.00% and 32.90% spore germination in case of *A. niger, A. flavus* and *P. citrinum* respectively. Spore germination was decreased with the concentration of oil increased. Above 300- ppm, spore germination was greatly reduced and at 500 ppm concentration of oil, there was complete inhibition of spore germination in *A. niger* and *A. flavus*. However, spore germination of *P. citrinum* was completely inhibited at 400 ppm concentration. It was also observed that those spores which germinated in presence of low concentration of oil, produced small germ tubes as compared to control.

From *in vitro* studies, it was evident that 800-ppm concentration of oil was fungicidal for all the test fungi. Hence, this concentration (and above) was used for *in vivo* studies. Essential oil showed best control on natural infection of aonla, only 14.4% fruits were infected at 800 ppm and none of the fruits were infected at 1200 ppm while 100% fruits were infected in control set. Results are presented in and it's corresponding.

In the present study, control and treated samples were analyzed after 2, 4 and 6 months for quality analysis. Dilution method for counting spore load/gm sample was used and similar method was also used by Neergaard and Saad (1962); Christensen, (1957) and Lutomski and Kedzia (1980). Less than 100 spores were found in hot water and essential oil treated fruits while control fruits contained approximately 106 spores/gm sample. According to FIP requirements, pharmaceutical preparations should not contain more than 100 spores per gm, so it is clear that hot water and essential oil treatment are best method for safe preservation of herbal crude drug samples.

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