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Epicarp of *Citrus sinensis* (Osbeck): A Potential Source as A Fungitoxic and Insecticidal Fumigant for the Management of Storage Fungi and Pests

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Abstract: The essential oil extracted from the epicarp of *Citrus sinensis* was used as a potential source for the management of storage fungi and pests. The essential oil exhibited absolute fungitoxicity as fumigant against the storage fungal pathogens viz., *Alternaria alternata*, *Aspergillusniger*, *A. fumigates*, *A. ochraceous*, *Cladosporium cladosporiodes*, *Penicillium chrysogenum*, *P. citrinum*, *P. italicum*, *P. oxalicum*, *Rhizopus arrhizus* and *R. nigricans* in the 7.0 l/l air to 10.0 l/l air, range of concentration.. The oil also exhibited absolute insecticidal activity as fumigant against stored pests viz., *Sitophilus oryzae*, *Trogoderma granarium* and *Tribolium castaneum* at LD_{50} 15.0 l/l, 12.8 l/l and 10.9 l/l air concentration of oil respectively. The activity of the oil did not changed even at exposure to 100℃ temperature or autoclaving the oil. The oil also retained its activity after 24 months of storage. GC – MS studies of the oil revealed the presence of 10 chemical constituents. Limonene was found to be the major component (84.2 %). *Citrus sinensis* natural volatile could therefore be a safer fumigant than those currently used, to control storage fungi and pests.

Key words: *Citrus sinensis*, fungitoxic, insecticidal, fumigant, GC – MS.

Introduction

Storage fungi and pests are the major cause of production losses in stored commodities. Storage fungi are generally present as mycelia below the pericarp, or as dormant spores on the surface of seeds. They cause spoilage of stored foods through discoloration, loss of viability, heating and mustiness, biochemical changes leading to quality loss and production of toxins[1]. The postharvest losses and quality deterioration caused by storage pests are major problem throughout the world[2]. The intensification of food production has led to several problems in the postharvest phase including the major concern of pest infestation during storage. This is further aggravated by the increased attention paid to maintenance of buffer stocks to provide food security for a country. Pest problems have increased side by side with the increase in the quantity of stockpiled food and the longer duration of storage. Such pest problems are more acute in the tropics than in temperate zones because the environment in the former is more conducive to the growth and development of pests.

To control storage fungi, fungicide application is the usual practice. However, using synthetic chemicals to control these pathogens can cause carcinogenicity, teratogenicity, high and acute residual toxicity and other side effects on humans[3,4]. The development of resistance is also becoming a significant problem within the populations of the pathogen. due to the application of the synthetic fungicides[5,6].

Like fungicides synthetic insecticides have been successfully used to protect stored grains from insect infestations but their indiscriminate and massive use have created serious problems such as hazards to the environment including human health and non-target organisms[7], residues in food grains[8], environmental pollution[9,10], and development of resistant strains[11,12]. It would be highly desirable to find safe alternatives to synthetic insecticides to protect stored grains and grain products from insect infestations.

The negative consumer perception of chem ical preservatives drives attention towards natural alternatives. Particular interest is focused on the potential application of plant essential oils. Exytracts from plants have recently been of great interest. Their possible use as natural additives emerged from a growing tendency to replace synthetic antimicrobial agents with natural

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ones. Phyto-compounds are expected to be far more advantageous than synthetic pesticides due to their sheer magnitude of complexity, diversity and novelty of chemicals, reactions and phenomenon[13]. They are also bio-degradable in nature, non-pollutant and posses no residual or phytotoxic properties[14,15,16]. The general antifungal activity of essential oils is well documented[17,18]. Some of the essential oils have been reported to inhibit postharvest fungi under *in vitro* conditions[13,19,20,21,22]. In recent years, some pesticidal plants, e. g. *Azadirachta indica*, *Chrysanthemum cinerariaefolium* and *Carum carvi* have been receiving global attention and their secondary metabolites have been formulated as botanical pesticides in plant protection[23].

There are very few reports on the antifungal activity of the *Citrus sinensis* essential oil against different microbial species[22,24,25,26]. Effects of the citrus oils and some spices on the growth and aflatoxin production by *Aspergillus parasiticus* was reported by Karapinar[27]. Ernestina et al. [28] also reported fungicidal activity of citrus oil against the causal agent of the anthracnose disease on tropical fruits. An added advantage of some of the essential oils is their bioactivity in the vapour phase, a characteristic which makes them attractive as possible fumigants for stored product protection[16]. The findings thus indicate the possibility of exploiting *Citrus sinensis* essential oil as an effective inhibitor against the storage fungi and pests.

Recently, attention has been drawn to the possible use of plant products or plant derived compounds as promising alternatives to synthetic insecticides in controlling insect pests of stored products[29,30,31,32]. The effectiveness of many plant derivatives for use against stored grains pests has been reviewed by Golob and Webley [33] and Jacobson[34,35].

No reports have appeared are using *Citrussinensis* as fumigants against storage fungi and stored pests. The present findings thus, indicate the possibility of exploiting *Citrus sinensis* essential oil as a fumigant against storage-contaminating fungi and pests.

Materials and Methods

Extraction of Essential Oil

Fresh epicarp of *Citrus sinensis* (L.) Osbeck (Musambi) was collected from various juice shops of Lucknow, India during the months of May to October. The essential oil was extracted from collected material by hydro-distillation for 5h using a Clevenger-type apparatus[36]. A clear, light yellow, oily layer was obtained on the top of the aqueous distillate which was separated from the latter and dried with anhydrous sodium sulphate. The extracted essential oil was stored at 4℃ in air-tight sealed glass vials, covered with aluminum foil until further analysis.

GC – MS Analysis of Essential Oil

The GC – MS of essential oil was analysed on a Shimadzu QP – 2000 instrument at 70 eV and 250℃. GC Column: ULBON HR – 1 equivalent to OV – 1, utilizing a fused silica capillary – 0. 25mm 50M with film thickness – 0. 25. The GC – MS was operated at an initial temperature of 60℃ for 5 minutes and then heated at the rate of 5℃ per minute to 250℃. Carrier gas (helium) flow was 2 ml per minute. The identification of components was based on comparison of their mass spectra fragmentation patterns with those of Mass Spectrometry Data Centre, the Royal Society of Chemistry. U. K. (Eight Peak Index of Mass Spectra, 3rd Ed. 1983) and with those reported in the literature[37].

Fungal Species

Strains of organisms used were: *Alternariaalternata* MPPLU 01 (Aa), *Aspergillus niger* MPPLU 05 (An), *A. fumigates* MPPLU 07 (Af), *A. ochraceous* MPPLU 09 (Ao), *Cladosporium cladosporioides* MPPLU 14 (Cc), *Penicillium chrysogenum* MPPLU 27 (Pch), *P. citrinum* MPPLU 31 (Pci), *P. italicum* MPPLU 29 (Pit), *P. oxalicum* MPPLU 33 (Pox), *Rhizopus arrhizus* MPPLU 43 (Ra) and *R. nigricans* MPPLU 45 (Rn) from the collection of Mycology and Plant Pathology Division, Botany Department, University of Lucknow. The cultures of the phytopathogenic organisms were maintained on Potato Dextrose Agar (PDA) at 4℃.

Stored Pests

Stored pests viz. Rice weevil *Sitophilus oryzae* L. (Coleoptera: *Curculionidae*), Khapra beetle *Trogoderma granarium* Everts (Coleoptera: Dermestidae) and Red flour beetle *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) were collected from private store houses of Lucknow district,. Mass cultures were maintained in earthen pots (2 kg) and/or large plastic containers (1. 5 kg) and sub – cultures in beakers (500 g) or small plastic containers (100 g) with the wheat as food medium. The beetles were allowed to grow in a natural environment as occurs in a traditional storehouse and were checked at regular intervals. A huge

number of beetles were thus reared to set a continuous supply of newly formed adults. The wheat seeds were thoroughly washed with tap water to remove dusts and other insect contamination present in the materials and carefully dried under sun-light, having 13% – 14% moisture content. Then the foods were kept in an incubator for about 24 hours at 60℃ to disinfest them. The sterile foods were then preserved in airtight glass jars (1000 ml) in order to impede further infestation.

Volatile Activity Assay of Essential Oil against Storage Fungi

Tests for the volatile activity of oil were carried out by inverted Petri dishes method in 90mm Petri dishes (Borosil) containing 20 ml of solidified PDA. A 5 mm diameter disc of the test fungus, cut from the periphery of an actively growing culture, was placed on the agar in each Petri dish and the dishes were kept in an inverted position. Sterilized cotton swab was placed on the upper lid of each of the inverted dishes. Different concentrations of oil were pipetted on cotton swab and were sealed by parafilm to check the release of volatile oil. For each corresponding control an equal amount of water was pipetted onto the sterilized cotton swab. The inverted Petri dishes were incubated at 25 ± 1℃ for 7 days. Fungitoxicity was expressed in terms of percentage of mycelia growth inhibition and calculated according to the formula of Pandey et al.[38] (1982).

Percentage of mycelial growth inhibition = [dc – dt/dc 100], where dc = Average diameter of fungal colony in control and dt = Average diameter of fungal colony in treatment.

Volatile Activity Assay of Essential Oil against Storage Pests

Glass vials (5.5 cm long by 2 cm in diameter), capped with polypropylene stoppers were used for the bioassay. Pests were transferred to the vials in groups of 15 adults along with the food material. The vials were covered with fine nylon cloth secured with adhesive tape. The vials containing the insects were then turned upside down over the vials containing the oil such that the oil vapours saturated the atmosphere of the containers containing the pests. The control consisted of a similar setup but without essential oil. This procedure was replicated three times. The vials were placed at room temperature with a photoperiod of 14 h light and 10 h dark. Mortality was determined after 24 h of treatment. The LD_{50} and LD_{95} values were calculated by Probit analysis[39]. Control mortality was accounted by Abbott's[40] formula.

Effect of Temperature and Autoclaving on Fungitoxicity and Insecticidal Activity of Oil

Experiments were performed to determine the thermostable or thermolabile nature of the oil. Different glass vials containing five ml oil each were subjected to different temperature treatments for three hours in incubators previously adjusted to 40, 60, 80 and 100℃. Antifungal activity of oil was also tested after autoclaving it at 121℃ for 15 min. The glass vials were then allowed to cool down to room temperature and the fungitoxicity of the treated oil from each set was tested at its MIC against the test fungus using the volatile activity assay. Insecticidal activity of oil was determined in a same manner.

Effect of Storage or Self-life on Fungitoxicity and Insecticidal Activity of oil

Experiments were undertaken to ascertain the duration for which the oil can be stored without loosing its fungitoxicity. Five ml of essential oil was stored in an air tight glass vial at room temperatures (20℃ to38℃ ± 2℃). The fungitoxicity of the stored oil at its MIC was tested at regular intervals of 2 months using the volatile activity assay. The insecticidal activity of oil was determined in a similar manner.

Results and Discussion

Extract from epicarp (waste product) of *C. sinensis* by hydro-distillation yielded 1.8 % essential oil. GC – MS analysis of the oil led to identification of 10 components. The main components of the *C. sinensis* epicarp essential oil studied and their percentages are presented in Table 1, the major constituents were Limonene (84.2 %), Linalol (4.4%) and Myrcene (4.1%).

The activity of the oil was tested against storage fungi by the volatile activity assay. In this assay the oil shows better activity[22]. The *Citrus sinensis* essential oil exhibited absolute fungitoxicity as fumigant against the storage fungal pathogens viz., *Alternaria alternata*, *Aspergillusniger*, *A. fumigates*, *A. ochraceous*, *Cladosporium cladosporiodes*, *Penicillium chrysogenum*, *P. citrinum*, *P. italicum*, *P. oxalicum*, *Rhizopus arrhizus* and *R. nigricans* in the 7.0 L/L air to 10.0 L/L air, range of concentration. (Table, 2), which is less than many of essential oils tested reviously by different workers[38,41,42,43]. The findings of our studies indicate that the essential oil of *C. sinensis* is an effective fungitoxicant a-

gainst storage fungi.

The oil also exhibited absolute insecticidal activity as fumigant against stored pests viz., *Sitophilus oryzae*, *Trogoderma granarium* and *Tribolium castaneum* at LD_{50} 15.0 L/L, 12.8 L/L and 10.9 L/L air concentration of oil respectively (Table 5). A large number of essential oils extracted from various spice and herbaceous plants have already been screened for toxicity as potential fumigants. Toxicity of a number of monoterpenes has been evaluated against various stored-product insects. Coats et al. [44] found that exposure of *S. oryzae* for 24 h to linalool and *d*-limonene had an LC_{50} of 14 and 19 L/L air whereas the LC_{50}'s for myrcene and α-terpineol were >100 μL/L. Citrus sinensis essential oil showed potent toxicity to all the stored pests tested. The primary component of the essential oil was limonene which was found to be the principal toxic constituent. Therefore this oil may show a promise as an alternative to fumigants currently used to control storage-grain insect pests.

The thermostability of the oil was tested by the volatile activity assay at MIC against storage fungi. It was found that at temperature ranging from 40 – 100℃ and even after autoclaving the oil, at 121℃ for 15 min, its activity was not altered (Table, 3). The thermostability of the oil was also tested against stored pests and it was found that the oil was effective even after receiving treatments of temperature (data not shown). The efficacy of essential oil was determined in terms of percent inhibition of mycelial growth of storage fungi after various storage periods using the volatile activity assay at MIC. It was observed that the oil retained its fungitoxicity even after 24 months of storage of (Table, 4). Similarly the oil did not alter its activity against all the stored pests tested (data not shown).

Table 1. Components of *Citrus sinensis* (L.) Osbeck epicarp essential oil identified by GC – MS

Peak No.	Components	Retention time (scan)	Percentage in total oil
1	α – pinene	12.36(192)	0.9
2	β – pinene	13.70(232)	0.6
3	Myrcene	14.60(259)	4.1
4	Limonene	16.60(319)	84.2
5	Linalol	18.60(379)	4.4
6	Citral	19.96(420)	0.5
7	α – Terpineol	21.10(454)	0.8
8	Terpinolene	21.56(468)	1.3
9	Citronellal	22.93(509)	1.9
10	Geraniol	23.86 (537)	1.3

Table 2. Effect of different concentrations of *Citrus sinensis* oil on per cent radial growth inhibition of different storage fungi at 25 1℃ using volatile activity assay (VA).

Conc. of oil (μL/L air)	Percent radial growth inhibition										
	Aa	An	Af	Ao	Cc	Pch	Pci	Pit	Pox	Ra	Rn
1.0	12.3	12.9	8.9	10.7	9.6	11.3	15.8	13.6	16.7	9.4	12.6
2.0	20.1	26.5	19.6	23.6	15.6	18.9	23.1	25.3	28.9	18.7	19.7
3.0	35.6	41.2	31.3	34.9	31.3	30.6	33.9	36.5	39.6	29.7	30.4
4.0	59.3	59.6	45.8	51.0	43.6	49.8	53.8	58.9	55.6	43.2	39.8
5.0	73.5	80.1	66.9	63.7	54.6	71.5	70.4	73.1	71.3	57.5	53.7
6.0	97.8	96.3	81.5	77.9	83.6	85.9	81.3	88.7	84.6	69.3	67.2
7.0	100	100	95.6	86.9	100	100	96.7	100	97.7	74.8	76.4
8.0	100	100	100	98.6	100	100	100	100	100	88.6	90.6
9.0	100	100	100	100	100	100	100	100	100	97.8	98.2

Conc. of oil (μL/L air)	Percent radial growth inhibition										
	Aa	An	Af	Ao	Cc	Pch	Pci	Pit	Pox	Ra	Rn
10.0	100	100	100	100	100	100	100	100	100	100	100
11.0	100	100	100	100	100	100	100	100	100	100	100

Table 3. Effect of different temperature treatments and autoclaving on fungitoxicity of *Citrus sinensis* oil (at MIC) against different storage fungi incubated at 25 1℃ using volatile activity assay (VA).

Temperature (℃)	Percent inhibition of mycelial growth										
	Aa	An	Af	Ao	Cc	Pch	Pci	Pit	Pox	Ra	Rn
40	100	100	100	100	100	100	100	100	100	100	100
50	100	100	100	100	100	100	100	100	100	100	100
60	100	100	100	100	100	100	100	100	100	100	100
70	100	100	100	100	100	100	100	100	100	100	100
80	100	100	100	100	100	100	100	100	100	100	100
90	100	100	100	100	100	100	100	100	100	100	100
100	100	100	100	100	100	100	100	100	100	100	100
autoclaving (121℃for 15 min)	100	100	100	100	100	100	100	100	100	100	100

Table 4. Effect of different storage periods on fungitoxicity of *Citrus sinensis* oil at MIC against different storage fungi incubated at 25 ± 1℃ using volatile activity assay.

Storage period (months)	Percent inhibition of mycelial growth										
	Aa	An	Af	Ao	Cc	Pch	Pci	Pit	Pox	Ra	Rn
6	100	100	100	100	100	100	100	100	100	100	100
8	100	100	100	100	100	100	100	100	100	100	100
10	100	100	100	100	100	100	100	100	100	100	100
12	100	100	100	100	100	100	100	100	100	100	100
14	100	100	100	100	100	100	100	100	100	100	100
16	100	100	100	100	100	100	100	100	100	100	100
18	100	100	100	100	100	100	100	100	100	100	100
20	100	100	100	100	100	100	100	100	100	100	100
22	100	100	100	100	100	100	100	100	100	100	100
24	100	100	100	100	100	100	100	100	100	100	100

Table 5. Fumigant toxicity of *Citrus sinensis* essential oil against the three stored pests.

Stored pest	LD_{50} (95% FL[b]) (μL/L air)	LD_{95} (95% FL) (μL/L air)	Slope Chi – Square (χ^2)
Sitophilus oryzae 15.0(10.5 – 19.6)	26.4 (16.9 – 35.9)	1.26	0.22
*Trogoderma granarium*12.8(9.4 – 16.2)	21.8(18.3 – 25.3)	0.98	2.11
*Tribolium castaneum*10.9(8.6 – 13.2)	17.7(12.2 – 23.2)	1.830.78	

[b]FL indicates fiducial limits.

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