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Full Length Research Paper

Assessment of broad spectrum control potential of *Eucalyptus citriodora* oil against post harvest spoilage of *Malus pumilo* L.

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***In vitro Eucalyptus citriodora* Hook. oil showed potent bioactivity against dominant post harvest fungal pathogens. The minimum bioactive concentrations with fungicidal action of the oil was found to be 1.0 µl ml^{-1} for *Alternaria alternata, Botrytis cinerea, Cladosporium cladosporioides, Colletotrichum capsici, Cyrtomium falcatum, Fusarium cerealis, Fusarium culmorum, Gloeosporium fructigenum, Penicillium digitatum, Penicillium expansum, Penicillium italicum, Penicillium implicatum, Penicillium minio-luteum,* 1.2 µl ml^{-1} for *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger*, *Aspergillus parasiticus, Curvularia lunata, Fusarium oxysporum, Fusarium udum, Penicillium variable, Helminthosporium oryzae, Helminthosporium maydis, Phoma violacea,* and 1.4 µl ml^{-1} for *Rhizopus nigricans*. The oil exhibited potency against heavy doses (30 mycelial disc, each of 5 mm in diameter) of inoculum at 2.0 µl ml^{-1} concentrations. The bioactivity of the oil was thermostable up to 100°C and lasted up to 72 months. The oil preparation did not exhibit any phytotoxic effect on the fruit skin (epicarp) of *Malus pumilo* up to 50 µl ml^{-1} concentrations. *In vivo* trials of the oil as a fungicidal spray on *M. pumilo* for checking the rotting of fruits, it showed that 30 µl ml^{-1} concentration controls 100% infection by pre-inoculation treatment, while in post-inoculation treatment, 40 µl ml^{-1} concentration of fungicidal spray were required for the 100% control of rotting. The fungicidal spray was found to be cost effective (INR 15/L) has long shelf life (72 months) and devoid of any adverse effects. Therefore, it can be used as a potential source of sustainable eco- friendly broad-spectrum herbal pesticide after successful completion of wide range trials.**

Key words: *Eucalyptus citriodora* Hook., fungicidal spray, fruit rot, herbal pesticide, *Malus pumilo.*

INTRODUCTION

Edible fruits are among the most important foods of mankind as they are nutritive and indispensable for the maintenance of health. They are also high-value commodities, offering good economic return even on small area of land. Based on policy directives of the planning commission of India, on the research priority area for enhanced fruit production was identified as reducing post harvest losses (Eckert and Sommer, 1967; Harvey, 1978). They identified weak post harvest management as major constraints and quoted 50% loss from harvesting, handling, storage and marketing of fruits according to FAO. India, being a geographically subtropical country with warm and humid climate, provides suitable environment for developing and spread of numerous plant pathogens. Harvested fruit and vegetables are attacked by microorganisms because of their high moisture content and rich nutrients (Simmonds, 1963).

Usually, synthetic pesticides are applied for the control of 'pest and disease' of the agricultural food commodities, as these are effective, dependable and economic. However, their indiscriminate use has resulted into several problems such as pest resistance to pesticides, resurgence of pests, toxic residues in food (causing health hazards to animals and human beings), water, air, soil and disruption of eco-system (Somasundaram et al.,

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1990). Natural products are an alternative to the use of these synthetic pesticides (Shahi et al., 2003). Keeping this view in mind, the present paper reports the bioactivity of the essential oil of *Eucalyptus citriodora* Hook. belonging to the family Myrtaceae also called lemon-scented gum, spotted gum, because of typical strong lemon like odour. It is grown naturally in Tamilnadu, Karnataka and Kerela, and is commercially cultivated in India. In the present investigation, the oil of *E. citriodora* were evaluated *in vitro* against dominant post harvest pathogenic fungi as well as control of rotting in apple.

MATERIALS AND METHODS

Maintenance of fungus culture

The test fungal pathogens, *Alternaria alternata* (Fr.) Keissler (MTCC 2724), *Aspergillus flavus* Link (MTCC 3396), *Aspergillus fumigatus* Fres (MTCC 2544), *Aspergillus niger* Van Tiegham (MTCC 1781), *Aspergillus parasiticus* Speare (MTCC 6768), *Botrytis cinerea* Pers. Ex. Fr. (MTCC 2104), *Cladosporium cladosporioides* (Fresenius) de Vries (MTCC 3478), *Colletotrichum capsici* (Syd) Butler and Bisby (MTCC 2071), *Cyrtomium falcatum* Went. (MTCC 2222), *Curvularia lunata* (Wakker) Boedijn (CBTC 2342), *Fusarium cerealis* (Cooke) Sacc (CBTC 2456), *Fusarium culmorum* (W.G Smith) Sacc (MTCC 2090), *Fusarium oxisporum* Schlecht.:Fr. (MTCC 2087), *Fusarium udum* (Butler) Snyder and Hansen (MTCC 2204), *Gloeosporium fructigenum* Berk (MTCC 2191), *Helminthosporium oryzae* Breda de Haan (CBTC 1256), *Helminthosporium maydis* Nisikado and Miyakel (CBTC 2314), *Penicillium digitatum* Sacc. (CBTC 1121), *Penicillium expansum* Link (MTCC 4485), *Penicillium italicum* Wehmer (CBTC 1029), *Penicillium implicatum* Biourge (CBTC 1034), *Penicillium minio-luteum* Dierckx (CBTC 1045), *Penicillium variabile* Sopp (CBTC 1046), *Phoma violacea* (Bertd) Eveleigh (CBTC 2051), *Rhizopus nigricans* Ehrenb (CBTC 2167) (Neergaard, 1977; Samson et al., 1995) were collected from Microbial type culture collection (MTCC), Chandigarh (India) and Collection of Bio-resource Type Culture (CBTC), Microbiology Department, CCS University, Meerut (India). All culture were maintained on potato dextrose agar medium (200 g scrubbed and diced potato in 1000 ml distilled water, 15 g agar, 20 g dextrose pH ± 5.6). A 7 day old culture of each fungus was used for bioactivity tests.

Isolation of active constituent(s)

The essential oil was extracted from the fresh leaves of *E. citriodora* Hook by hydro-distillation using Clevenger's apparatus (Clevenger, 1928). A clear light yellow green coloured oily layer was separated and dried with anhydrous sodium sulphate. The physiochemical properties of the oil were determined by the technique described by Langenau (1948).

In vitro studies

The minimum bioactive concentrations (MBCs) of the oil were determined following the poisoned food technique (PFT) of Grover and Moore (1962) with slight modification (Shahi et al., 1999). The requisite quantity of the oil was dissolved in 2 ml acetone and then added in 100 ml pre-sterilized potato dextrose agar (PDA) medium (pH- 5.6). In control sets, sterilized water (in place of the oil) and 2 ml acetone were used in the medium. Mycelial discs of 5 mm diameter, cut out from the periphery of 7-day old cultures of the test pathogens, were aseptically inoculated upside down on the agar surface of the medium. Inoculated Petri plates were incubated at 27 ± 1°C and the observations were recorded on the 7th day. Percentage of mycelial growth inhibition (MGI) was calculated as follows:

$$MGI\ (\%) = (dc\text{-}dt) \times 100 / dc$$

where, dc = mycelial growth diameter in control sets, dt = mycelial growth diameter in treatment sets.

The nature of antifungal activity, fungistatic (temporary inhibition) / fungicidal (permanent inhibition) of the oil was determined by the method of Garber and Houston (1959). The inhibited fungal discs (at minimum bioactive concentrations) were reinoculated up side down on plain PDA (potato dextrose agar) medium in Petri plate. Observations were recorded on 7^{th} day of incubation at 27±1°C. Fungal growth on 7^{th} day indicated fungistatic action of the oil, while absence of growth indicated fungicidal action of the oil. The effect of inoculum potentiality on bioactivity of the oil (2.0 µl ml^{-1}) was determined by the method of Shahi et al. (1999). Mycelial disc of 5 mm in diameter of seven day old cultures were inoculated in culture tube containing 2.0 µl ml^{-1} oil in liquid medium (Potato dextrose broth) separately. In controls, sterile water were used in place of oil and run simultaneously. The number of mycelial discs in the treatment as well as control sets were increased progressively up to 30 in multiple of five. Observations were recorded after the 7^{th} day of incubation. Absence of mycelial growth in treatment sets on the 7^{th} day exhibited the oil's potential against heavy doses of inoculum.

Effect of temperature and duration of toxicity during storage of the oil was evaluated according to Shahi et al. (1999). Five lots of oil were kept in small vials, each containing 5 ml of oil; these were exposed at 40, 60, 80 and 100°C in an incubator for 60 min. Residual activity was assayed by poisoned food technique of Grover and Moore (1962). Loss of toxicity of the oil was also determined by storing the oil at room temperature (30 ± 4°C) and withdrawn samples at intervals of 60 days up to 7 years and tested by poisoned food technique (Grover and Moore, 1962). All the experiments were repeated twice and each contained five replicates; the data presented mean values.

Phytotoxic investigation

Phytotoxic effect of the oil was carried out at different concentrations (ranging from 10 to 100 µl ml^{-1}) on fruits skin (epicarp) of *Malus pumilo*. Two sets of 50 samples (apples) were maintained one for the treatments and another for the controls. Each sample was first washed with distilled water followed by 70% ethyl alcohol and then allowed to dry. In treatment sets, 1 ml of the different concentrations of oil was sprayed to each sample separately. In controls, sterilized water was sprayed (in place of oil). The qualitative observations (morphological changes, such as colour, odour, weight, size, changes in epicarp and taste) have been recorded at the interval of 24 h up to 3 weeks.

In vivo investigation of the oil in the form of fungicidal spray

The study was designed to see the activity of the oil in the form of fungicidal spray applied on fruit skin for the control of fruit rot of *M. pumilo* by different methods. For *in vivo* study, both pre and post inoculation treatments (fungicidal spray) were applied to the fruits. In the pre inoculation treatment, two sets were prepared, treatments

Table 1. Physico-chemical properties of the oil of *Eucalyptus citriodora* oil.

Properties studied	Observations
Plant height (m)	25-40
Oil yield (%)	0.6
Colour	Light yellow
Specific gravity at 15°C	0.8640-0.8770
Refractive index at 20°C	1.4511-1.4570
Optical rotation	+3 to -3°
Saponification value	8.90 to 2.0
Ester value	12-60
Solubility in 70% alcohol	1.3 to 1.5 vols
Citronellol content (%)	65-85

as well as controls. In treatment set, fruits were sprayed in known concentrations (10 to 50 µl ml^{-1}) of oil preparation in vehicle. In controls, the fruits were sprayed with distilled water in vehicle. Thereafter, the fruits were injured using a sterilized needle, and the fungal inoculum of *P. expansum, B. cinerea, P. violacea* (5 mm diameter mycelial disc of each fungus) was placed over the injured areas. All inoculated fruits were incubated at 26 ± 1°C and the observations were recorded on the 7th day.

In post inoculation treatment, fruits were first wounded with a sterilized needle and fungal inoculum of *P. expansum, B. cinerea, P. violacea* (5 mm diameter mycelial disc of each fungus) was placed over the wounded areas. After 24 h of incubation, fruits were sprayed in different concentrations (10 to 50 µl ml^{-1}) of oil preparation. In controls, fruits were sprayed with distilled water in vehicle. Inoculated fruits were incubated at 26 ± 1°C and the observations were recorded on the 7th day. The data were average of 5 replicates and repeated twice. Percentages of inhibition (I) were calculated as follows.

$$I\ (\%) = (Ic-It) \times 100 / Ic$$

Where: Ic = average diameter of infected area in control set, It = average diameter of infected area in treatment sets.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance (P≥0.05) of the data obtained in all experiments. All results were determined to be within the 95% confidence level for reproducibility. The ANOVA was computed using the SPSS version 16.0 software package.

RESULTS

The leaves of *E. citriodora* on hydro-distillation yielded 0.6% essential oil. The physicochemical properties of the oil were shown in Table 1. The oil exhibited broad antifungal activity, the minimum bioactive concentrations with fungistatic action (temporary inhibition) of the oil was found to be 0 .4 µl ml^{-1} for *A. alternata*, 0.6 µl ml^{-1} for *A. niger*, *A. parasiticus, B. cinerea, C. cladosporioides, C. capsici, C. falcatum, F. cerealis, F. culmorum, G. fructigenum, P. expansum, P. digitatum, P. italicum, P. implicatum, P. minio-luteum,* 0.8 µl ml^{-1} for *A. flavus, A. fumigatus, C. lunata, F. oxysporum, F. udum, H. oryzae, H. maydis, P. variable* and 1.0 µl ml^{-1} for *R. nigricans* (Table 2). The minimum bioactive concentrations with fungicidal action (permanent inhibition) of the oil was found to be 1.0 µl ml^{-1} for *A. alternata, B. cinerea, C. cladosporioides, C. capsici, C. falcatum, F. cerealis, F. culmorum, G. fructigenum, P. digitatum, P. expansum, P. italicum, P. implicatum, P. minio-luteum,* 1.2 µl ml^{-1} for *A. flavus, A. fumigatus, A. niger*, *A. parasiticus, C. lunata, F. oxysporum, F. udum, P. variable, H. oryzae, H. maydis, P. violacea,* and 1.4 µl ml^{-1} for *R. nigricans* (Table 2).

The oil inhibited heavy doses (30 fungal mycelial disc, each of 5 mm in diameter) of inoculum at 2.0 µl ml^{-1} concentration. The bioactivity of the oil persists up to 100°C, and it did not expire even up to 72 months of storage.

The oil did not exhibit any phytotoxic effect up to 50 µl ml^{-1} level on fruit skin (Table 3). Formulation of the oil prepared at different concentrations (10 to 50 µl ml^{-1}) in the form of fungicidal spray. The fungicidal spray, when tested in vivo on *M. pumilo* for checking the rotting, it showed complete inhibition at 20 µl ml^{-1} concentration by pre inoculation treatment while in post inoculation treatment, 30 µl ml^{-1} concentration of spray solution was required for the 100% control of rotting (Table 4) and in Figure 1 showed. The fungicidal spray was found cost effective and free from any side effect.

DISCUSSION

Although many plants belonging to different angiospermic families have been screened for their antifungal activity, *E. citriodora* belonging to the family Myrtaceae is reported for its antifungal activity against post harvest fungal pathogens probably for the first time. Substances may inhibit the growth of fungi of either temporarily (fungistatic) or permanently (fungicidal). Essential oils obtained from the leaves of *Cymbopogon martinii* var. motia (Dikshit et al., 1980), *Hyptis suaveolens* (Pandey et al., 1982), *Melaleuca leucodendron* (Dubey et al., 1983) and the rhizome of *Alpinia galganga* (Tripathi et al, 1983) was found to have fungistatic activity. Whereas essential oils from *Cymbopogon pendulus* (Pandey et al., 1996) that have fungicidal. However, in the present investigation the oil of *E. citriodora* like those of *C. flexuosus* (Shahi et al., 2003) prove to have fungistatic activity at lower concentration and fungicidal at higher concentration. A fungicide must not be affected by extremes of temperature. Only a few workers have studied the effect of temperature on antifungal activity of the oils, but the oil of *Pepromia pellucida* was reported to

Table 2. Minimum bioactive concentrations of the oil of *Eucalyptus citriodora* against fungal pathogens.

Fungi	Percentage mycelial growth inhibition at different concentration (µl ml^{-1}) after 7th day of inoculation							
	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6
Alternaria alternata	54.2	100^{s}	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Aspergillus flavus	41.2	70.6	91.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Aspergillus fumigatus	55.5	74.3	90.1	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Aspergillus niger	45.2	89.0	100^{s}	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Aspergillus parasiticus	42.0	91.0	100^{s}	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Botrytis cinerea	54.0	98.0	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Cladosporium cladosporioides	71.0	82.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Colletotrichum capsici	76.2	91.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Colletotrichum falcatum	56.2	69.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Curvularia lunata	76.2	81.0	90.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Fusarium cerealis	67.1	90.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Fusarium culmorum	69.2	81.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Fusarium oxysporium	70.1	89.3	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}	100^{c}
Fusarium udum	67.2	76.0	81.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Gloeosporium fructigenum	45.2	76.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Helmenthosporium maydis	79.2	89.2	98.9	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Helmenthosporium oryzae	75.1	92.2	95.4	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Penicillium digitatum	61.2	75.1	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Penicillium expansum	53.1	81.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Penicillium italicum	65.2	93.1	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Penicillium implicatum	71.2	80.1	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Penicillum minio-luteum	69.0	78.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}	100^{c}
Penicillum variable	50.1	71.2	81.2	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Phoma violacea	40.2	70.1	83.1	100^{s}	100^{s}	100^{c}	100^{c}	100^{c}
Rhizopus nigricans	60.2	81.2	91.0	92.8	100^{s}	100^{s}	100^{c}	100^{c}

s, Fungistatic action; c, fungicidal action.

Table 3. Phytotoxicity of oil on fruit skin (epicarp).

Concentration (µl/ml)	Phototoxic effect on different concentration					
	Colour	Odour	Weight	Size	Taste	Changes in epicarp
10	-	-	-	-	-	-
20	-	-	-	-	-	-
30	-	-	-	-	-	-
40	-	-	-	-	-	-
50	-	-	-	-	-	-
60	+	-	-	-	+	-
70	+	+	-	-	++	+
80	+	++	-	-	++	+
90	++	++	-	-	++	+
100	++	+++	-	-	++	+

-, No effect; +, mild; ++, moderate; +++, significant.

be active up to 80°C (Singh et al., 1984), in the present study the oil of *E. citriodiora* retained activity up to 100°C. A substance may be fungicidal against certain fungi yet ineffective against other pathogens.

Therefore, a clear picture of the toxicity of a fungicide comes only after it is tested against a large number of fungi. The literature shows that the essential oils have been found to exhibit a narrow or wide range of activity

Table 4. *In vivo* efficacy of the oil for the inhibition of inoculum in *Malus pumilo.*

Concentrations (µl ml^{-1})	Percentage inoculum growth inhibition at different treatments	
	Pre-inoculation treatment	Post-inoculation treatment
10	76.2	59.2
20	100	68.2
30	100	100
40	100	100
50	100	100
Variance	113.288	405.432
Std. Dev.	10.6437	20.1353
Std. Err.	4.76	9.0048

ANOVA summary

Source	SS	df	MS	F	P
Treatment (between groups)	238.144	1	238.144	0.92	0.365562
Error	2074.88	8	259.36		
Total	2313.024	9			

This test will be performed only if K>2 and the analysis of variance yields a significant F-ratio.

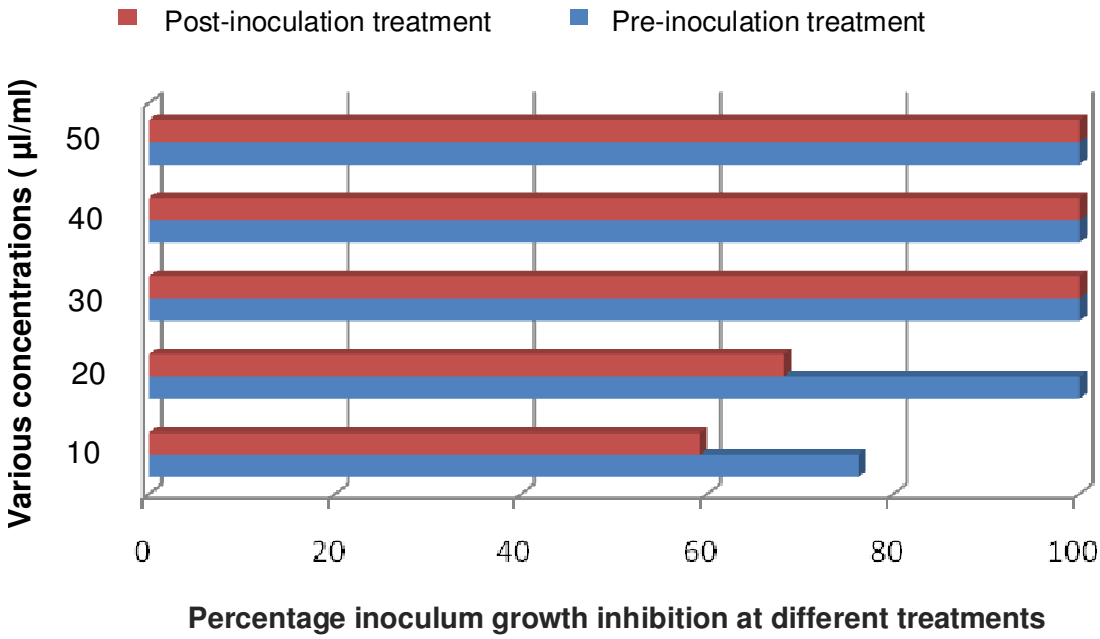

Figure 1. Bar graph showing percent fruit loss protected by the application of formulated fungicidal spray.

(Singh et al., 1980; Pandey et al., 1982; Dubey et al., 1983), but in the present study the *E. citriodora* oil exhibited a broad antifungal spectrum. Antifungal active oils derived from plants are generally non-phytotoxic (Pandey et al., 1982; Tripathi et al., 1983). In the present study, the oil was found to be non-phytotoxic at morphological level. Additionally, in preliminary *in vivo* trials, it has also been found effective in the control of fruit rot of *M. pumilo*. A chemical should be tested under both *in vitro* and *in vivo* conditions in order to prove its potential as promising antifungals for the control of disease. Since, detailed *in vitro* studies on the essential oil of *E. citriodora* indicate its potential as ideal antifungal compounds against post harvest spoilage fungi; it was further subjected to *in vivo* investigation, so as to confirm their efficacy as a natural product for the control of rotting in fruits. The present study clearly demonstrates that oil of *E. citriodora* holds a good promise as an antifungal against post harvest spoilage an account of their following virtues namely, strong efficacy against fungi

with fungicidal action, potentiality against heavy fungal inoculum, long shelf life, thermostable, wide range of antifungal activity and absence of any phytotoxic effects and better result during *in vivo* trials. The oil in the form of fungicidal spray can be exploited commercially after undergoing successful completion of wide range of field trial and to find out their economic viability.

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