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Antifungal activity and chemical composition of *Citrus reticulata* Blanco essential oil against phytopathogens from North East India

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ABSTRACT

The essential oil (EO) isolated by hydro-distillation from the peel of fully matured ripen fruits of *Citrus reticulata* Blanco were analyzed by GC and GC–MS. Thirty seven different components were identified constituting approximately ≥99% of the oil. The major components were limonene (46.7%), geranial (19.0%), neral (14.5%), geranyl acetate (3.9%), geraniol (3.5%), β-caryophyllene (2.6%), nerol (2.3%), neryl acetate (1.1%) etc. The antifungal activity of the oil was tested by poisoned food (PF) technique and the volatile activity (VA) assay against five plant pathogenic fungi viz *Alternaria alternata* (*Aa*), *Rhizoctonia solani* (*Rs*), *Curvularia lunata* (*Cl*), *Fusarium oxysporum* (*Fo*) and *Helminthosporium oryzae* (*Ho*). The oil showed better activity in VA assay. The Minimum inhibitory concentration (MIC) for *Aa*, *Rs* and *Cl* was 0.2 ml/100 ml whereas >0.2 ml/100 ml for *Fo* and *Ho* in PF technique. Fungal sporulation was also completely inhibited at 2 ml/100 ml of the oil except for *Cl* and *Ho*, which was only 0.5% (±0.5) and 0.25% (±0.25) respectively as compared to control.

1. Introduction

The genus *Citrus* of the family Rutaceae includes about 17 species distributed throughout the tropical and temperate regions (Davies & Albrigo, 1994; Shaw, 1977). More than 52 varieties of *Citrus* are found in home gardens and also in wild in North east part of India up to an altitude of 1200 m in the hilly states. Some common species are *Citrus indica*, *Citrus ichangensis*, *Citrus macroptera*, *Citrus latipes*, *Citrus aurantium*, *Citrus megaloxycarpa*, *Citrus jambhiri* and *Citrus reticulata*. *C. reticulata*, one of the commercially important species, is grown and traditionally used by different ethnic groups and local people in North East India.

Although, the fruits are mainly used for dessert, it has significant economic value for its essential oil (EO) due to their aromatic compounds (Minh Tu, Thanh, Une, Ukeda, & Sawamura, 2002). Lime flavours are used in beverage, confectionary, cookies and desserts (Buchel, 1989; Dharmawan, Kasapis, Curran, & Johnson, 2007). The exocarp of *C. reticulata* and *Citrus sinensis* is used for flavorings of liquor. Few studies have already reported the chemical composition of *C. reticulata* peel oil (Lawrence, 1992; Shaw, 1979). In fact, the composition of the oil is significantly affected by the ripeness of fruits, vegetative stage of plant, storage condition and extraction method (Njoroge, Mungal, Koaze, Phi, & Sawamura, 2006; Venkateshwarlu & Selvaraj, 2000). The quality and the odor of the oil are influenced by the limonene content which may vary in the different agro-climatic conditions (Dharmawan et al., 2007). Significant contributions were made on the composition of *C. reticulata* EO (Shaw, 1979; Slater, 1961). There is no report on the Citrus EO from North East India even though the oil has significant commercial value in the country.

The EO preparations that possess antimicrobial activities have been the subject of many investigations resulting in the screening of a wide variety of plant species and have revealed structurally unique biologically active compounds (Matasyoh, Kiplimo, Karubiu, & Hailstorks, 2007). Again, EOs of some plants have recently been proven to be successful eco-friendly bio-control agent (Chutia, Mahanta, Saikia, Baruah, & Sarma, 2006; Sokovic & Griensven, 2006). Many authors have reported antimicrobial, antifungal, antioxidant and radical-scavenging properties of EOs (Sacchetti et al., 2005; Sokovic & Griensven, 2006) and in some cases, a direct food-related application also (Madsen & Bertelsen, 1995). It was observed that EOs from *Citrus limon* and *Citrus aurantifolia* (tolerant varieties to leaf and fruit spot disease) strongly inhibited fungal growth as compared to EOs from very susceptible varieties like *Citrus paradise* and *C. sinensis* (Kuate et al., 2006). But there is no report of the antifungal activity of *C. reticulata* EO. Therefore, the present study was made to determine the chemical compositions and antifungal activity of *C. reticulata* peel oil against some common food borne and phytopathogenic fungal species viz *Alternaria alternata* (*Aa*), *Rhizoctonia solani* (*Rs*), *Curvularia lunata*

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(*Cl*), *Fusarium oxysporum* (*Fo*) and *Helminthosporium oryzae* (*Ho*) with emphasis on the possible future application of the EO as alternative antifungal agents.

2. Materials and methods

2.1. The plant material

C. reticulata Blanco is commonly known as mandarin fruit. The fresh fully matured ripe fruits were collected from home gardens. The fruits had been washed and cut into equal portions to remove the peels. The fruit albedo layers were peeled off carefully and discarded. The peel oils were isolated by hydro-distillation in Clevenger's apparatus as described by Sharma and Tripathi (2006).

2.2. Oil isolation and GC–MS analysis

The EO was collected by hydro-distillation for 6–8 h. The yield of oil was recorded (0.6 ml/100 g peel) and stored in air-tight sealed glass vials covered with aluminum foil at 4 °C for further use. The major constituents were analyzed by GC and GC–MS.

GC analysis was carried out on a Chemito 8510 GC instrument equipped with a data processor. A BP-5 wide-bore capillary column (30 m × 0.53 mm i.d., 1.0 μm film thickness) was used for the separation of the sample components (sample size 0.03 μl, measured using a Hamilton GC syringe of 1.0 μl cap.). Hydrogen was used as the carrier gas at a flow rate of 5 ml/min and 20 p.s.i. inlet pressure. The GC column oven temperature was from 70 °C to 210 °C at a rate of 2.5 °C/min, with a final hold time of 5 min. Both injector and detector (FID) temperatures were maintained at 230 °C. GC–MS analysis was carried out on a Trace DSQ MS (Thermo Electron Corporation), using a BP-5 capillary column (30 m × 0.25 mm i.d., 0.5 μm film thickness); with helium as the carrier gas at a flow rate of 1 ml/min; split ratio 1:20. The column temperature was from 65 °C to 210 °C (10 min hold) at 3 °C/min. Mass spectra were recorded in the range 50–450 amu, operating at 70 eV, and the ion source temperature was maintained at 200 °C. The constituents of the oil were identified by using standard reference compounds and also by matching the mass spectra fragmentation pattern with NIST Mass Spectra Library stored in the GC–MS database.

2.3. Fungal strains used

Pure cultures of *A. alternata* (*Aa*), *R. solani* (*Rs*), *C. lunata* (*Cl*), *F. oxysporum* (*Fo*) and *H. oryzae* (*Ho*) were obtained from the Mycology and Plant Pathology Laboratory, NEIST, Jorhat (India). The isolates were collected from the diseased samples and were maintained on Potato Dextrose Agar (PDA) at 4 °C.

2.4. Antifungal assay

The antifungal activity against the test pathogens was determined by the poisoned food (PF) technique of Grover and Moore (1962) and the volatile activity (VA) assay.

In PF technique, 20 ml of Potato Dextrose Agar (PDA) was poured into sterilized Petri dishes and measured amount of oil was added to get the required concentrations viz 0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 ml/100 ml sterile molten PDA (Feng & Zheng, 2007). In media, 0.05 ml/100 ml Tween-80 was added for even distribution of the oil. The test fungi were inoculated with 5 mm mycelial plugs from 7-days-old cultures and incubated at 25 ± 2 °C. The growth of fungal species was recorded after one week of incubation and the percentage inhibition was computed after comparison with the control.

In VA assay, Petri dishes were filled with 20 ml of PDA and one disc (0.5 cm diameter) of mycelial plug was taken from the edge of a 4–6 day old fungal culture and was placed on PDA in the Petri dishes (Sharma & Tripathi, 2006). The Petri dishes were inverted and sterile filter paper discs (4 mm diameter) impregnated with the above concentrations (0.01, 0.025, 0.05, 0.075, 0.1, 0.15, 0.2 ml/100 ml distilled water with 5% Tween 20) of EO were attached to the inverted lid (1 disc per lid). The Petri dishes were then wrapped with parafilm along the rim to check the release of the volatile components, inverted and incubated for 7 days at 25 ± 2 °C. The radial growth of the mycelium was recorded and results were expressed as percentage fungal colony growth by the formula described by Pandey, Tripathi, Tripathi, and Dixit (1982).

The fungistatic nature of the oil in VA assay was tested by using the modified technique of Mahanta et al. (2007). The inhibited fungal discs were re-inoculated into fresh medium and revival of their growth was observed. The spores of the previously exposed colonies were collected by adding 5 ml sterile water containing 0.1 ml/100 ml Tween-80 to each Petri dishes and rubbing the surface three times with the sterile L-shaped spreader. The spore suspension was collected and then centrifuged. A haemocytometer slide was used to count spore concentration.

2.5. Statistical analysis

All the experiments were repeated four times. Significant differences between values were determined by using Duncan's multiple range test ($p < 0.05$), following one-way ANOVA. Statistical analysis was performed using MS Excel and graphs were produced using Origin Pro 7.5.

3. Results and discussion

3.1. Oil compositions

The major constituents of the EO are shown in Table 1. A total of 37 components were identified and the major components were limonene (46.7%), geranial (19.0%), neral (14.5%), geranyl acetate (3.9%), geraniol (3.5%), β-caryophyllene (2.6%), nerol (2.3%), citronellal (1.3%), neryl acetate (1.1%) etc. Limonene contributes to the aromatic odor of the oil and hence the plant belongs to the limonene chemotype. Some other compounds were linalool (0.7%), 6-methyl-5 hepton-2 one (0.7%), decanol (0.6%), β-bisobolene (0.6%) etc. These compositions of *C. reticulata* significantly vary from the other studies reported earlier (Sawamura, Tu, Onishi, Ogawa, & Choi, 2004).

Limonene, neral and geranial were the major oil components of four different varieties of *C. sinensis* EOs (Sawamura, Tu, Yu, & Xu, 2005); while β-pinene and γ-terpinene were completely absent in *C. reticulata*. Minh Tu et al. (2002) observed maximum limonene (95.1%) content in *C. reticulata* Blanco *var. tangerine* EO from Vietnam and Dharmawan et al. (2007) observed 89.6% limonene content in freshly-squeezed juice also.

3.2. Antifungal assay

In PF technique, the antifungal activities of *C. reticulata* oil against the test pathogens at different concentration are shown in Fig. 1. The oil at 0.1 ml/100 ml concentration significantly reduced ($p < 0.05$) the colony growth of *Aa* (84%), *Rs* (80%), *Cl* (93.25%), *Fo* (42%) and *Ho* (54%) in PF technique. Complete inhibition of fungal growth was observed at 0.2 ml/100 ml for *Aa*, *Rs* and *Cl*. The MIC of *Fo* and *Ho* was >0.2 ml/100 ml. However, EO at 0.15 ml/100 ml completely inhibited the growth of *Cl* whereas, only 80–98% in other species. EO at ≤0.1 ml/100 ml showed little effect against the tested organisms.

The MIC in VA assay was 0.2 ml/100 ml for *Aa*, *Cl* and *Fo*. The MIC for *Rs* and *Ho* was >0.2 ml/100 ml (Fig. 2). However, VA assay was

Table 1
Chemical compositions of *Citrus reticulata* essential oil.

Sl No	Compound	RI[a]	Percentage[b]
1	α-Pinene	928	0.1
2	6-Methyl-5 hepten-2 one	961	0.7
3	Sabinene	962	0.1
4	Myrcene	982	0.3
5	1,8-Cineole	1016	Trace*
6	Limonene	1019	46.7
7	(Z)-β-ocimene	1027	Trace*
8	(E)-β-ocimene	1038	0.4
9	Nonanal	1082	0.4
10	Linalool	1084	0.7
11	Cis-limonene oxide	1118	Trace*
12	Trans-limonene oxide	1120	Trace**
13	Citronellal	1130	1.3
14	Rose furan epoxide	1154	Trace*
15	α-Terpineol	1169	Trace*
16	Decanol	1184	0.6
17	Nerol	1209	2.3
18	Carvone	1211	Trace*
19	Neral	1212	14.5
20	Geraniol	1234	3.5
21	Geranial	1241	19.0
22	Neryl formate	1262	Trace**
23	Geranyl formate	1282	Trace*
24	Undecanal	1286	Trace**
25	δ-Elemene	1330	Trace**
26	Citronellyl acetate	1335	Trace*
27	Neryl acetate	1344	1.1
29	Geranyl acetate	1362	3.9
29	Dodecanal	1388	Trace*
30	β-Caryophyllene	1411	2.6
31	Trans-α-bergamotene	1429	0.1
32	α-Humulene	1444	0.3
33	β-Bisabolene	1499	0.2
34	Germacrene B	1543	Trace*
35	(E)-nerolidol	1547	Trace**
36	Caryophyllene oxide	1562	0.2
37	Trans-phytol	2097	Trace*
38	Other compound	–	0.6

*<0.1%; **<0.01%.
[a] Retention Indices relative to *n*-alkanes.
[b] Relative Area percentage without using FID response correction factor.

more effective than the PF technique where complete inhibition of *Fo was* being observed at 0.2 ml/100 ml (MIC > 0.2 ml/100 ml in PF technique). *Fo* was found to be the most resistant strain whereas *Cl* was the most sensitive one against *C. reticulata* EO. The MIC of *C. reticulata* EO was different from the other citrus oils against the tested pathogens. This may be due to the variation of chemical components from species to species. The antimicrobial activity of EO is believed to be associated with phytochemical components such as monoterpenes (Matasyoh et al., 2007) which diffuse into and damage cell membrane structures. Sokovic and Griensven (2006) observed antifungal activity of limonene and α-pinene (MIC 4.0–9.0 μl/ml) against *Verticillium fungicola* and *Trichoderma harzianum* which are found at different amount in different plant EOs. The EOs and its related substances act to make the cell membrane of the fungus permeable, causing leakage (Piper, Calderon, Hatzixanthis, & Mollapour, 2001).

Spore production and germination of the fungal pathogens was strongly inhibited in the presence of EO (Mahanta et al., 2007). Fungal sporulation in PDA was significantly ($p < 0.05$) inhibited by the EO of *C. reticulata* with spore production of 74% (*Aa*), 75.5% (*Rs*), 67% (*Cl*), 83% (*Fo*) and 70.5% (*Ho*) at 0.05 ml/100 ml (Fig. 3). Moreover, spore production was completely inhibited at 0.2 ml/100 ml for *Aa*, *Rs* and *Cl* whereas 0.5% (±0.5) for *Fo* and 0.25% (±0.25) for *Ho*. Hammer, Carson, and Riley (1999) reported that sporulation of *Candida albicans* was completely inhibited by *C. reticulata* oil at 0.2 ml/100 ml.

The chemical compounds like linalool, caryophyllene oxide, α-pinene, α-terpineol have antifungal and antibacterial activity (Matasyoh et al., 2007) which are found in appreciable amounts in *C. reticulata* oil. Kurita, Miyaji, Kurane, and Takahara (1981) have shown that citral acts as a fungicidal agent because it is able to form a charge transfer complex with an electron donor to fungal cells, which results in fungal death. The germination of *Penicillium digitatum* conidia was stimulated by certain combination of the volatiles such as limonene, β-pinene, sabiene, β-myrcene, acetaldehyde, ethanol and CO_2 (Filtenborg, Frisvad, & Thrane, 1996). This might be due to the mechanism developed by some fungal pathogens using the secondary metabolites as a signal to initiate germination,

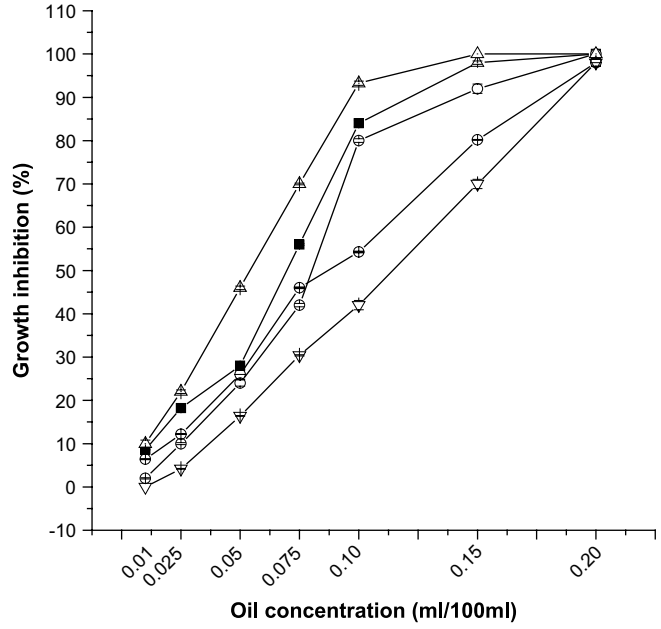

Fig. 1. Effect of *Citrus reticulata* oil on fungal growth at different concentrations on PDA (significant difference at $p < 0.05$, ANOVA test); (■) *Alternaria alternata*, (○) *Rhizoctonia solani*, (△) *Curvularia lunata*, (▽) *Fusarium oxysporum*, (⊖) *Helminthosporium oryzae*. Error bars indicate the mean ± standard error.

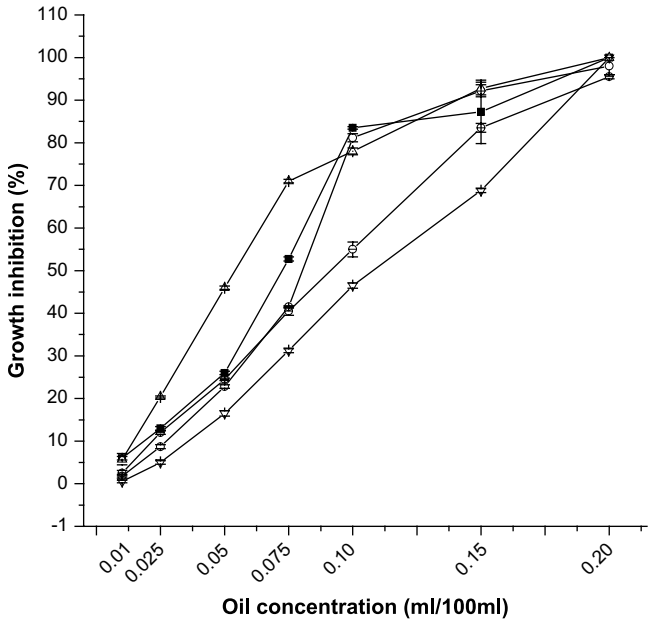

Fig. 2. Antifungal activity of *Citrus reticulata* oil in Volatile Assay against the fungal isolates (significant difference at $p < 0.05$, ANOVA test); (■) *Alternaria alternata*, (○) *Rhizoctonia solani*, (△) *Curvularia lunata*, (▽) *Fusarium oxysporum*, (⊖) *Helminthosporium oryzae*. Error bars indicate the mean ± standard error.

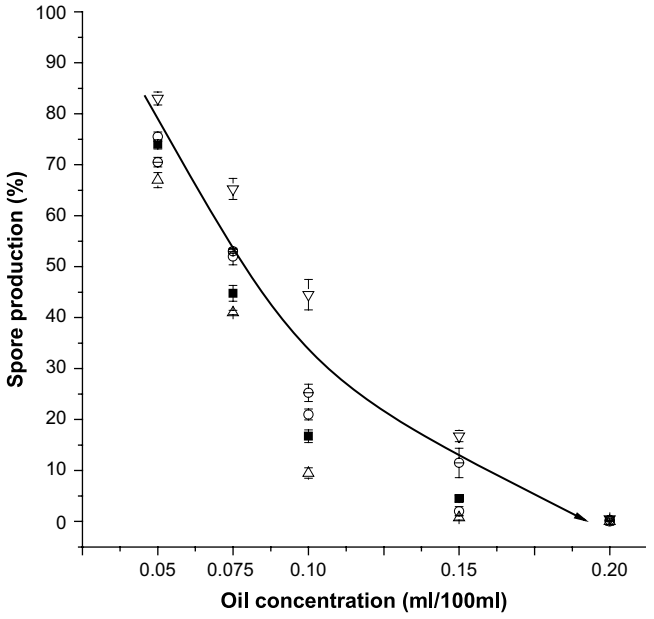

Fig. 3. Effect of *Citrus reticulata* oil on the fungal spore production at different concentrations (significant difference at $p < 0.05$, ANOVA test); (■) *Alternaria alternata*, (○) *Rhizoctonia solani*, (△) *Curvularia lunata*, (▽) *Fusarium oxysporum*, (⊖) *Helminthosporium oryzae*. Error bars indicate the mean ± standard error.

appressorium formation and infection (Flaishman & Kolattukudy, 1994). Even in vapour treatment, the oil was effective in a very low concentration and hence could make a major advantage to limiting the spread of the pathogen by lowering the spore load. However, there is a limitation to the potential application of EO in diseased plants or as food preservatives (Nychas & Tassou, 2000). The availability of oxygen also affects the antimicrobial efficacy of the oil in the field condition. Paster et al. (1990) observed that the activity of oregano EO on *Staphylococcus aureus* and *Staphylococcus enteritidis* was greatly enhanced when these organisms were incubated under microaerobic or anaerobic conditions.

4. Conclusion

The results indicated that *C. reticulata* oil and its components may be used as an alternative of synthetic fungicides or preservatives. Further studies are undertaken for the individual components and their antifungal activity against these pathogens for their possible application in the field or as natural preservatives.

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