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In vitro anti-fungal and antioxidant studies on the essential oil: *Citrus macroptera* (Wild orange) of Northeast India

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Abstract
Citrus macroptera, commonly known as 'Wild Lime,' is an underexplored fruit with promising medicinal properties. This study aimed to assess the antifungal and antioxidant activities of *Citrus macroptera* essential oil against three prevalent Plant fungal pathogens, namely *Macrophomina phaseolina*, *Fusarium oxysporum* f. sp. *cubense* and *Colletotrichum gloeosporioides*. *In vitro* assay results indicated that *Colletotrichum gloeosporioides* (98.88%) showed the highest percentage of inhibition, followed by *Macrophomina phaseolina* (92.96%) and *Fusarium oxysporum* (77.40%). The essential oil of *Citrus macroptera* exhibited good antioxidant potential with an IC_{50} value of 195.96 µg/ml, which revealed its extent of application in both the pharmaceutical and agricultural sectors. Future research is likely to integrate and contrast the identification of the chemical composition of the essential oil, as well as the study of the active component responsible for its antifungal and antioxidant effects.

Keywords: *Citrus macroptera*, Lunglei-Mizoram, essential oil, antifungal, antioxidant activity

1. Introduction
Citrus is one of the most important horticultural crops grown and traded globally. Citrus has its basic origin in Southeast Asia and is extensively distributed throughout the tropical and subtropical parts of the world. (Moore 2001; National Horticulture Board, 2010) [11, 13]. Citrus fruits are beneficial with potential health-promoting chemical components and the key sources of vitamins and minerals (Teigiserova *et al.*, 2021) [21]. India is exceptionally rich in both cultivated and wild citrus genetic resources (Nair and Nayar 1997) [12].

Citrus peels, the dominant waste in the citrus processing units, are normally discarded. However, recent studies indicated that citrus peels contain a great number of flavonoids, and glycosides, which have excellent anti-inflammatory, anti-carcinogenic, and anti-microbial properties. Apart from the fruit, the citrus peel essential oil contains terpene-based compounds with D– limonene as a major compound which is known for its aroma health benefits and nutritional content. (Han *et al*., 2021) [4]

The medicinal properties of citrus are attributed to the bio-active secondary metabolites found in the flavedo and albedo of the fruit peels, including monoterpenes (Limonene), citric acid, phenolic compounds, etc. (Lv *et al*., 2015; Patil *et al*., 2017; Zou *et al*., 2016) [8, 15, 24]. The flavanoids and the phenolic compounds present in the citrus peel are responsible for the anti-inflammatory, anti-carcinogenic, antiviral, antibacterial, and anti-allergenic effects (Yashaswini *et al*., 2018) [23].

Citrus macroptera belonging to the family of Rutaceae is commonly known as SatKara, a pharmacologically diverse medicinal plant. This plant's parts, especially the fruit, have a wide variety of traditional medical applications for a wide range of health problems. Numerous active phytochemical components of this plant, including D-limonene, beta-caryophyllene, beta-pinene, geranial edulinine, ribalinine, isoplatydesmine, and terpene-based compounds have been identified (Waikedre *et al*., 2010) [5]. Studies indicated that *C. macroptera* fruit, peel and leaves have anti-inflammatory, anti-tumour, anti-microbial, antioxidant, thrombolytic, hypoglycemic, anxiolytic, antidepressant, cardioprotective, and hepatoprotective properties. (Aktar *et al*., 2017) [1]

Plant fungi affect crop yield, quality, and profitability (Shuping *et al*., 2017) [19]. Synthetic chemical fungicides have damaged the condition of the environment and the health of the soil, and their residual toxicity has a substantial impact on non-target creatures, people's health, and the global economy. (Mahmood *et al*., 2016; Tripathi *et al*., 2020) [9, 22]. Synthetic fungicides can be replaced with plant-based compounds since they are more abundant and safer than their synthetic counterparts. The wide range of naturally occurring plant-based compounds has attracted the attention of researchers. (Shweta Singh *et al*., 2021) [20]. With this favourable perception towards natural compounds in pest control and for the efficient reuse of citrus peels, the current study was conducted to assess the activity of citrus peel essential oils against significant fungal species, including *Macrophomina phaseolina*, *Fusarium oxysporum*, and *Colletotrichum gloeosporioides*. As citrus peel has antioxidant activity and there is no report on the antioxidant activity of *Citrus macroptera* essential oil. Consequently, it was subjected to screen the *Citrus macroptera* essential oil for its antioxidant properties (Miguel, 2010) [10].

2. Methods and Materials

2.1 Collection and preparation of plant material

Citrus macroptera fruits were collected from the Lunglei district of Mizoram, India (22° 79'51.02" N 92° 22' 47.38" E 1162 m). The fruit was washed thoroughly, peeled and the peels were shade dried for 4-5 days. The dried peels were cut into small pieces and stored for future use in airtight containers.

2.2 Extraction of Essential oils

Peels were powdered, weighed and then taken in a round-bottomed flask. The flask was then filled with twenty times the volume of distilled water as the weight of the peels (1:20 W/V) and placed over the heating mantle. The clevenger apparatus was attached to the round bottomed flask and was boiled at 70 °C for six hours. The essential oil, along with the steam, was distilled in a graduated cylinder. The condensed water that was accumulated in the graduated cylinder was collected at regular intervals. After six hours, the aqueous layer and the oil that had accumulated in the graduated cylinder were separated. The oil obtained was then refrigerated for further investigation.

2.3 Test fungi

The plant pathogenic fungi *Colletotrichum gloeosporioides, Fusarium oxysporum and Macrophomina phaseolina* were obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. These cultures were sub-cultured at regular intervals for bioassay experiments.

2.4 Antifungal assay

The antifungal activity of *C. macroptera* essential oil was performed by the poisoned food technique against all the test fungi. Sterile PDA medium was mixed with the *C. macroptera* essential oil (CaMaLu) to prepare varying concentrations ranging from 250 ppm, 500 ppm, 750 ppm, and 1000 ppm, which were then equally distributed to all the Petri plates. The media without treatment was taken as the negative control. All the treatments were done in triplicate. After the media had solidified, an actively growing fungal disc of 4 mm-diameter was removed from the maintained mother culture and placed in the middle of the Petri plates containing PDA medium that had been enriched with *C. macroptera* essential oil. All Petri plates were subjected to an incubation period of their respective growth periods (till the hyphae reaches the edge of the petri plates in the control concentration) at 28±2 °C. After incubation, the fungal mycelial growth was measured. The following formula was used for calculating the percentage of mycelial growth that was inhibited compared to the control (Gopalakrishnan *et al*., 2014) [3].

$$\text{Inhibition}(\%) = \frac{\text{Growth of pathogen mycelium in control} - \text{Growth of pathogen mycelium in treatment}}{\text{Growth of pathogen mycelium in control}} \times 100$$

2.5 DPPH antioxidant activity

DPPH antioxidant activity was calculated using a method adapted by Shimada *et al*. (1992) [17]. Different concentrations (20-100 µg/ml) of ascorbic acid were prepared with methanol and are used as standards. 50µl of different concentrations (20-100 µg/ml) of *C. macroptera* essential oil were added and mixed with 450 µl of 50 mM Tris HCl (50 mmol/L, pH 7.4). 50 µl of methanol in place of the test sample was used as the blank. To this, 1 ml of 0.1 mM DPPH (0.1 mmol/L in methanol), was added, mixed thoroughly, and incubated for 30 minutes at dark (RT). After incubation, the Free Radical Scavenging activity of essential oil (E-CMaLu) against stable DPPH was determined spectrophotometrically and the UV absorbance was read at 517 nm.

2.5.1 The scavenging effect was calculated using the following equation

$$\text{Scavenging effect (\%)} = \frac{(A0 - A1)}{A0} \times 100$$

where A_0 was the absorbance of the control (blank, without essential oil) and A_1 was the absorbance in the presence of the essential oil. Experiments were done in triplicate and IC_{50} was measured based on the inhibition percentage of DPPH radicals scavenged.

2.6 Statistical analysis

Three replications of the bioassay were performed using a completely randomized design. Using one-way analysis of variance (ANOVA), the results of several treatments on mycelial growth were investigated. To compare the treatment means at a 5% significance level, Duncan's multiple range test (DMRT) was employed in the Statistical Package for Social Sciences (SPSS version 16.0. Chicago, SPSS Inc. USA).

3. *Results*

3.1 *In vitro* Antifungal assay

The antifungal activity of *C. macroptera* essential oil indicated that the growth of the agriculturally important soil-borne pathogens *Fusarium oxysporum* f. sp. *cubense, Macrophomina phaseolina*, and *Colletotrichum gloeosporioides* were significantly inhibited in a dose dependent manner at different concentrations, namely 250 ppm, 500 ppm, 750 ppm and 1000 ppm by poisoned food technique Complete growth of fungal mycelium was observed in all the control plates. The results indicated that *Citrus* *macroptera* essential oil peel extract exerted strong antifungal activity in a dose-dependent manner, where percentage inhibition increased with the increase in concentration of the doses.

In vitro antifungal assay of the essential oil of E-CMaLu hindered the growth of *Colletotrichum gloeosporioides* and suppressed the mycelial growth of *Col* by 98.88% at 1000 ppm, which is higher than the other fungal species. The minimum inhibition percentage observed in *Fusarium oxysporum* f. sp. *cubense* 77.4% at 1000 ppm concentration of E-CMaLu and *Macrophomina phaseolina* growth was inhibited at a rate of 92.96%. (Fig 1, Table 1) C. MaLu peel essential oil has a high impact on *C. gloeosporioides*, when compared with other fungi, namely *M. Phaseolina* and *F. oxysporum* f. sp. *Cubense*. The results clearly indicated that, *Citrus macroptera* essential oil has got highest inhibitory activity against C. *gloeosporioides* and *M. phaseolina* and the lowest activity against *F. oxysporum*.

3.2 DPPH antioxidant activity assay

The antioxidant activity of E-CMaLu improved as the volume of essential oil used for the assay was increased from 20 to 100 µl. In the DPPH assay, E-CMaLu essential oil (20, 40, 60, 80 and 100 µl) showed an IC_{50} value of 195.96 µg/ml. Furthermore, the essential oil of E-CMaLu was able to scavenge DPPH radicals in a concentration dependent manner.

Fig 1: Antifungal activity of *C. macroptera lunglei* essential oil against A) *Fusarium oxysporum* f. sp. *cubense* B) *Colletotrichum gloeosporioides* C) *Macrophomina* phaseolina

Table 1: Effect of *Citrus macroptera* essential oil at different concentrations against *F. oxysporum, M. phaseolina and C. gloeosporioides*

Concentration of *Citrus macroptera* Essential oil (ppm)	Per cent inhibition over control (%)		
	F. oxysporum	*M. phaseolina*	*C. gloeosporioides*
250	26.85±0.14^{d}	12.5±0.58^{d}	10±0.15^{b}
500	42.2±0.1^{b}	31.38±0.03^{a}	32.27±0.67^{d}
750	50.92±0.11^{c}	48.61±0.15^{c}	87.28±0.38^{c}
1000	77.40±0.03^{a}	92.98±0.11^{b}	98.88±0.09^{a}
Control	0	0	0

Data represented as mean percentage ± SD and values followed by the same letter along the column are not significantly different (p<0.05) from each other.

Table 2: Evaluation of DPPH antioxidant activity of the *Citrus macroptera* lunglei ESO

Sample	Concentration (µg/ml)	IC_{50} (µg/ml)
E-CMaLu	100 ppm	195.96

4. Discussion

D-Limonene is a prominent constituent found in the essential oils of citrus plants and recognized as one of the most abundant naturally occurring monocyclic monoterpenes (Kim *et al*., 2013) [7]. In this study, the inhibition of the mycelial growth of fungi is attributed to the phytochemicals present in the essential oil of E-CMaLu. The antifungal efficacy of *Colletotrichum gloeosporioides, Fusarium oxysporum and Macrophomina phaseolina* on E-CMaLu is primarily reported in this study. Our findings showed that 1000 ppm of E-CMaLu essential oil extract reduced *C. gloeosporioides* growth by 98.88 percent and *M. phaseolina* growth by 92.96 percent compared to systemic (Saravani *et al*., 2021; Pallavi *et*., *al*. 2022) [17, 14]. The earlier literature reports suggest that the efficacy of *C. maxima, C. sinensis* essential oil as free radical scavengers may be due to the antioxidant activity of DL-limonene, the oil's principal component (Priyanka *et al*., 2010; Junior *et al*., 2009) [16, 6]. The essential oil from *Citrus macroptera* peel extract showed more potential antioxidant activity than the pulp (Sadia *et al*., (2008) [2].

5. Conclusion

The findings of the present study demonstrated that the essential oil isolated from the peel of *Citrus macroptera* exhibits effective *in vitro* fungicidal and antioxidant activities and can be used in crop protection due to its wide availability, safety, resistance to pests, benevolent nature towards non-target species, less adverse impact on plant growth, and low cost. In addition, the antioxidant property of the *C. macroptera* essential oil can be utilized as a natural preservative in the agrofood and in the cosmetic industries.

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