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In vitro evaluation of antifungal activity of lemon peels (*Citrus limon* L. Osbeck) extract against agriculturally important soil-borne plant pathogenic fungi

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Abstract
Plant fungal diseases are responsible for substantial agricultural crop loss. To control plant fungal pathogens, farmers rely on synthetic fungicides with major concerns like phytotoxicity, vertebrate toxicity, pest resistance, pest resurgence, extensive environmental risks and high cost, etc. Plant-derived botanicals can be safely used to control and manage crop fungal diseases to combat aforesaid hazardous issues. Citrus peels are renowned for their abundance in bio active phytochemicals with potential for plant disease management. In this study, the phytochemicals present in the peels of *Citrus limon* was extracted by using methanol. The crude methanolic extract was studied for its antifungal property against agriculturally important soil-borne fungi such as *Sclerotium rolfsii, Fusarium oxysporum* f. sp. *cubense, Macrophomina phaseolina* and *Rhizoctonia solani*. *In vitro* assay shows 100% inhibition of *R. solani* at 750 ppm. On the other hand, the mycelial growth of *S. rolfsii*, *M. phaseolina* and *F. oxysporum* f. sp. *cubense* was inhibited by 94.79%, 94.16%, and 80.65% at 1000 ppm Hence, the present study clearly indicated the *Citrus limon* peels which is bio waste can be employed as bio resource in the development of green botanical fungicides.

Keywords: Phytotoxicity, phytochemicals, *Citrus* peel, soil-borne fungal pathogens

1. Introduction
Citrus has its primary origin in Southeast Asia and is widely distributed throughout the tropical and subtropical regions of the world (Moore *et al.*,2001, National Horticulture Board, 2010) [11,13]. Citrus is one of most prominent fruit crops cultivated throughout the world due their high nutraceutical value (Liu *et al*., 2012) [7]. India is exceptionally rich in both cultivated and wild citrus genetic resources (Nair and Nayar 1997) [12].

Citrus fruits (pulp and peels) are well-known for their flavour, nutritional and medicinal values. These medicinal properties are attributed to the bio-active secondary metabolites such monoterpenes (Limonene), citric acid and phenolics compounds, etc. present in the flavedo and albedo of the peels. (Lv *et al*., 2015; Patil *et al*., 2017; Zou *et al*., 2016) [9, 15, 25]. It is recognised that phenolic compounds, such as flavonoids, have anti-inflammatory, anticarcinogenic, antiviral, antibacterial, and anti-allergenic properties (Yashaswini *et al*., 2018) [24]. *C. limon* fruit is a valuable medicinal plant belonging to the family of Rutaceae (Order: Sapindales). The phytochemical constituents present in different parts of the plant, viz, leaves, stem, juice, peels, and flower, have anticancer, antifungal, antiviral, and anti-bacterial potentials (Akhilesh *et al*., 2012) [1]. But citrus processing industries produce 40 million tons of agro-industrial waste worldwide annually. The bio-waste contains almost 50% of the original fruit mass, of which ~40–55% are fruit peels. Citrus peels are readily available, cost-effective, and attractive source of bioactive ingredients.

Plant fungal pathogens significantly influence agricultural crop profitability, quality, and yield (Shuping *et al*., 2017) [19]. Synthetic chemical fungicide has deteriorated soil health and environmental quality (Mahmood *et al*., 2016 and Tripathi *et al*., 2020) [10, 22] and their residual toxicity has significant implication on non-target organisms, human health and global economy (Grewal *et al*., 2017; Yang *et al*., 2017) [5, 23]. Plant-based products are abundant and less precarious compared to synthetic pesticides and hence can be used as a substitute for synthetic antifungal compounds. Scientific interest has been drawn towards the variety of botanicals for their anti-microbial property (Shweta Singh *et al*., 2021) [20]. With this positivity towards botanicals in pest management and for the effective reuse of citrus peels, the present

study was carried out to evaluate the antifungal activity of methanolic extracts of lemon peel against important soil-borne pathogens such as *Rhizoctonia solani* (Ceratobasidiaceae: Cantharellales), *Fusarium oxysporum* f. sp. *Cubense* (Hypocreales: Nectriaceae), *Macrophomina phaseolina* (Botryosphaeriales: Botryosphaeriaceae), *Sclerotium rolfsii* (Polyporales: Atheliaceae).

2. Methods and Materials

2.1 Collection and preparation of plant material

Citrus limon fruits were collected from Baulpai village in Kolasib district of Mizoram, India (24.066074 N, 92.41 3910 E). The fruit was washed thoroughly, peeled and the peels shade dried for 4-5 days. The dried peels were processed into a fine powder using a blender and stored for future use in airtight containers. Thirty grams of fine peel powder was soaked in 150 ml (1:5, w/v) of methanol (HPLC grade) for three days. The solvent was filtered regularly at 24 hours interval and same quantity of fresh methanol was again poured after filtration. The filtrate was collected and concentrated. The concentrated crude extract was used for the *in vitro* antifungal assay.

2.2 Test fungi

Plant pathogenic fungi, namely *Rhizoctonia solani, Fusarium oxysporum* f. sp. *cubense, Macrophomina phaseolina and Sclerotium rolfsii* were preferred for this study. The isolates of fungus were obtained from the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. The isolated cultures of each fungus were maintained on Potato Dextrose Agar (PDA) medium and stored at 28±2 °C for further studies.

2.3 Antifungal Assay

The antifungal activity of *C. limon* peel methanolic extract was performed by the food poisoned technique (Pawar *et al*., 2018; Ramaiah *et al*., 2015) [16, 17] against all the test fungi. An appropriate quantity of filter sterilized *C. limon* peel concentrated methanolic extract was added to 45 ml of sterilized PDA medium to make different concentrations of diluted extracts viz., 250 ppm, 500 ppm, 750 ppm, 1000 ppm separately and transferred equally into three Petri plates. The media containing methanol was used as a negative control. Each replicate consists of 10 Petri plates per treatment. The media was allowed to solidify and after solidification, an actively growing fungal disc of 4 mm diameter was taken and positioned in the centre of the Petri plates containing a PDA medium fortified with *C. limon* peel methanolic extract. PDA medium without extract served as a negative control. All Petri plates were incubated at 28±2 °C for 2 days (*F. oxysporum* grows in 5 days). The radial growth of the fungus was measured after incubation. The percent growth inhibition of mycelial growth over untreated control was calculated by using the following formula (Gopalakrishnan *et al.*, 2014) [4],

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

2.4 Statistical analysis

The bioassay was carried out in three-replications in completely randomized design. The effect of several treatments on mycelial growth was studied using one-way analysis of variance (ANOVA). Duncan's multiple range test (DMRT) was used performed in Statistical Package for Social Sciences (SPSS version 16.0. Chicago, SPSS Inc, USA) to compare the treatment means at a 5% significance level.

3. Results

The antifungal properties of *C. limon* peel methanolic extract were evaluated against different soil-borne plant fungal pathogens at different concentrations viz., 250 ppm, 500 ppm, 750 ppm, 1000 ppm, by the food poisoned technique. Complete growth of fungal mycelium was observed in all the control plates. Methanolic peel extract of *Citrus limon* had significantly inhibited the growth of fungi. It showed 100 percent inhibition against *R. solani* at 750 ppm, whereas the mycelial growth of *S. rolfsii, M. phaseolina* and *F. oxysporum* f. sp. *cubense* was inhibited by 94.79%, 94.16%, and 80.65% at 1000 ppm (fig. 1). In all the different *in vitro* fungal assays, the increase in the concentration of extract increases the inhibition percentage. Hence, the action of *C. limon* peel methanolic extract is dose-dependent. *C. limon* peel methanolic extract have a high impact on *R. solani*, when compared with other fungi, namely *F. oxysporum* f. sp. *cubense, M. phaseolina*, and *S. rolfsii*. The result showed that the *C. limon* peel methanolic extract showed potent inhibitory activity against all the test fungi under study.

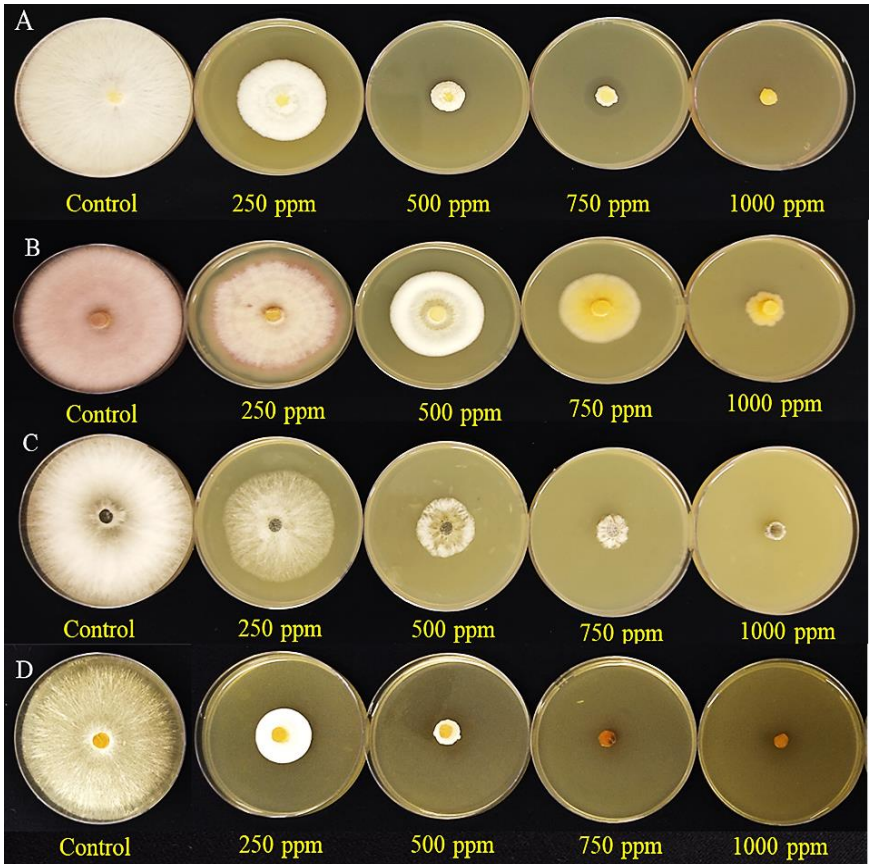

Fig 1: Antifungal activity of *C. limon* peel methanolic extract against A) *Sclerotium rolfsii* B) *Fusarium oxysporum* f. sp. *cubense* C) *Macrophomina phaseolina* D) *Rhizoctonia solani*

Table 1: Effect of *Citrus limon* peel methanolic extract at different concentrations against *S. rolfsii, F. oxysporum f. sp. cubense, M. phaseolina and R. solani*

Concentration of *Citrus limon* peel extract (ppm)	Per cent inhibition over control (%)			
	Sclerotium rolfsii	***Fusarium oxysporum* f. sp. *cubense***	***Macrophomina phaseolina***	***Rhizoctonia solani***
250	70.20±0.41^{d}	26.85±0.18^{d}	38.51±0.60^{d}	73.61±0.13^{c}
500	87.84±0.05^{c}	43.98±0.24^{c}	68.98±0.11^{c}	88.52±0.05^{b}
750	92.08±0.04^{b}	55.83±0.33^{b}	80.74±0.10^{b}	100.00^{a}
1000	94.79±0.02^{a}	80.65±0.19^{a}	94.16±0.01^{a}	100.00^{a}
Methanol*	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{d}

*Negative control.

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.

4. Discussion

The inhibition of the mycelial growth of fungi is attributed to the phytochemicals present in the peel of *Citrus limon*. The peels of *C. limon* are rich in numerous nutrients and phytochemicals, including phenolic group substances like flavanones (hesperidin, eriocitrin, narirutin, naringin and didimin) (Barreca *et al*., 2017) [2], Flavones (C-glycosides such as 6,8-di-C-glucopyranosylapigenin and 6,8-di-C-glucopyranosyl-luteolin and diosmetin-7-rutinoside) (Shweta Singh *et al*., 2020) [20], flavonols (rutin, quercetin, myricetin, isocitrol, limocitrol and limocitrin) are present in peels of lemon. Tetraterpenoids such as carotenoids were exposed in a considerable amount on lemon rinds (Guimaraes *et al*., 2010) [6]. Mineral content such as Ca, K, Na, Mg, Zn and Fe may also be presented on lemon rinds (Ghanem *et al*., 2012) [3]. These compounds might also be responsible for the antifungal activity of methanolic extract of lemon peel.

C. limon peel methanolic extract completely inhibited the growth of *R. solani* at 750 ppm. Singh *et al*., in 2021 [21], tested the antifungal activity of *C. limon* leaf ethanolic extract against *R. solani* which showed 7.5 mm zone of inhibition at 500 mg/ml concentration. Okwu *et al*., (2012) [14] reported that diethyl ether peel extract of *Citrus aurantifolia* inhibited *F. oxysporum* proliferation by 71.10%, 10% diethyl ether peel extract of *Citrus reticulata* inhibited *F. oxysporum* growth by 68.14%. and 10% of *C. limon* peel extract, which reduced growth by 48.48 percent. But our findings demonstrated that 1000 ppm of *C. limon* peel methanolic extract was effective in reducing the growth of *F. oxysporum* up to 80.65 percent. *C. limon* peel methanolic extract inhibited the growth of *S. rolfsii* by 94.79% at 1000 ppm, which is found to be more effective than the systemic fungicides such as Azoxystrobin and Thiophanate methyl tested by Saravani *et al*., (2021) [18] which suppressed the growth of *S. rolfsii* by 65.92% and 80.74%, respectively. *M. phaseolina* growth was arrested by 94.16% at 1000 ppm similar to the findings by Lokesh *et al*., (2020) [8], systemic fungicides such as Carbendazim (WP), Tebuconazole (25.9 EC), Propiconazole (25 EC),

Azaxystrobin (23 SC), Hexaconazole (5 EC) and Thiophanate methyl (70 WP) all significantly reduced the growth of *M. phaseolina* by 90.59, 49.41, 38.82, 80.00 and 84.71 per cent respectively. Similarly, at 1000 ppm concentrations, non-systemic fungicides such as Copper oxychloride (50WP), Mancozeb (75WP), Chlorothalonil (75WP), Thiram (75WP), and Zineb (75WP) prevented the fungus growth by 36.08%, 82.74%, 78.82%, 64.71% and 70.59% respectively. The above results clearly indicated that the *C. limon* peel methanolic extract was more useful in controlling *M. phaseolina* than synthetic and non-systemic fungicides.

5. Conclusion

The prospect of using the *C. limon* peel extract for the development of natural fungicides is appealing and acceptable due to its widely availability, safety, pest resistance, innocuous to non-target organisms, less negative effect on plant growth and cost effective. This study demonstrated that the methanolic extract of *C. limon* has *in vitro* fungicidal property. Henceforth, a further study is required to extract and purify bio active anti-fungal metabolites and their mode of action from the *C. limon* for green control of soil-borne plant fungal diseases.

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