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Effect of Citrus Aurantifolia Leaf Extract on Mycelial Growth and Spore Germination of Different Plant Pathogenic Fungi

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Abstract
The effect of cold water extract of leaves of *Citrus aurantifolia* (Christm.) swingle (lime) at 10%, 20%, 30% and 40% concentration on the *in vitro* growth and germination of *Botryodiplodia theobromae* Pat., *Aspergillus niger* Van Tiegh, *Aspergillus flavus* Link. and *Penicillium oxalicum* currie Thom. was investigated. Phytochemical studies on the pulverized leaves of the medicinal plant revealed the presence of flavonoids, glycosides, tannins and phlobatannins. The leaf extract of the plant significantly ($P < 0.05$) reduced the mycelial growth and spore germination of all the test pathogens at the different levels of concentration. The inhibitory action of the extract on mycelia growth and spore germination increased with increasing concentration, giving a toxicity profile of 40% > 30% > 20% > 10%. *Penicillium oxalicum* was the most sensitive fungi to *C. aurantifolia* extract, while *Aspergillus flavus* was the least. These findings indicate a promising potential of *C. aurantifolia* in controlling the test pathogens in plants.
Keywords: *Citrus aurantifolia*, mycelial inhibition, spore inhibition, fungitoxic, pathogenic fungi.

INTRODUCTION

The necessity to develop non-toxic, safe and easily biodegradable alternatives to synthetic pesticides has prompted investigations on exploiting pesticides of plant origin. Studies have shown the importance of natural chemicals as possible source of non toxic and environmentally friendly alternative pesticides (Singh, 1994; Ghorbany and Salary, 2004; Stompor – Chrzan, 2004). Pesticides of plant origin are available and cost effective in countries where synthetic pesticides are expensive and difficult to obtain (Mason and Mathew, 1996).

Numerous natural products of plant origin are pesticidal and have the potentials to control fungal pathogens of crops (Okwu *et al.,* 2007). However Earnsworth (1990), reported that just very few plants; about 10% have been investigated for their antimicrobial activity. Therefore, a large reservoir of potential sources of botanical fungicides such as *Citrus aurantifolia* still exists especially in tropical forests awaiting exploitation.

Citrus aurantifolia (Christm.) swingle (lime) belongs to the family Rutaceae. It is a dense and irregularly branched tree. The stem is spreading and woody, brown in colour, with short stiff spines on twigs. The leaves are acute, entire elliptic, oblong-ovate, dark green above, pale-green below, alternate with narrowly winged leaf petioles. Flowers are white and stand from leaf corners. The fruit is round, greenish-yellow with thin skin, juicy, fragrant and very acidic. The seeds are small, avoid and pale (Aliyu, 2006). Lime and other related species have been variously utilized in a number of folk medicine (Gill, 1992; Ethnobot, 2005). It is claimed by local traditional healers to be used in the treatment of headache, stomach ache, cough, dysentery, fever, gonorrhea, hepatitis, jaundice etc. Although, many workers have reported antimicrobial activity of plant extracts (Onifade, 2000; Nwachukwu and Umechuruba, 2001; Okigbo and Igwe, 2007; Ijato *et al*., 2011; Gupta and Tripathi, 2011), there is paucity of information on the use of *Citrus aurantifolia* as pesticides of plant origin. This study aims at determining *in vitro*, the effect of cold water extract of leaves of *C. aurantifolia* on mycelia growth and spore germination of different plant pathogenic fungi.

MATERIALS AND METHODS

Plant material

The leaves of the plant were collected from Asaba, Nigeria in April, 2012. It was identified by a taxonomist in the Department of Forestry and Wildlife, Delta State University, Asaba Campus, Asaba, Nigeria.

Preparation of extract

Fresh leaves were washed thoroughly under running tap water, surface-sterilized (10% NaOCL for 2 mins), rinsed in three changes of sterile distilled water, air dried for 7 days and ground using a Tower blender (Model BL-NC- 6802D, Italy). Ten grammes, 20g, 30g and 40g of the ground material were soaked separately in 100 ml of sterile distilled water in 250 ml conical flasks, to give 10%, 20%, 30% and 40% concentrations of plant extract respectively. Each suspension was hand shaken for two minutes and allowed to stand for 12 hours before being filtered into a fresh 250 ml flask using four fold cheese cloth. Streptopenicillin was added at the rate of 12.5 mgl^{-1} to each extract to check bacterial contamination.

Phytochemical screening
The crude pulverized leaves of the plant were screened for plant metabolites using the methods of Trease and Evans (1989) and Poongothai *et al.*, (2011).

Pathogens
Botryopiplodia theobromae Pat, *Aspergillus niger* Van Tiegh, *Aspergillus flavus* Link and *Penicillium oxalicum* currie and Thom used in this study were obtained from the Department of Microbiology, Delta State University, Abraka, Nigeria. The pathogens were maintained on Potato Dextrose Agar (PDA) slant and revived twice on fresh PDA each time before use.

Effect of extract on mycelia growth and spore germination of the four plantpathogenic fungi
The effect of the lime extract on fungal growth and germination was determined using the food poisoning technique described by Okigbo *et al.,* (2009). One milliliter of the extract at 10%, 20%, 30% and 40% concentration was pipette separately and aseptically into 9ml of molten PDA medium in Petri dishes. Each plate was gently swirled on the table to ensure even dispersion of the extract and left to solidify. Five millilitre mycelial discs on agar were taken from the margin of an actively growing culture of each test fungus and one disc was placed upside down in the centre of each of three plates for each concentration. Plates without extract but similarly inoculated with mycelia discs were included as controls. The plates were incubated at room temperature (28 ± 2°C). Colony diameters were measured daily until control plates were fully grown. Colony diameter was taken as the mean growth along two pre-drawn, perpendicular lines on the reverse side of each plate.

To determine the toxicity of extract against spores, plates with each concentration were inoculated with one drop (0.1 ml) of conidial suspension (5.0×10^4 coindial ml^{-1}) of each fungal pathogen. Spore suspensions were obtained from 10 day – old cultures of the fungi and adjusted to a final concentration of 5.0×10^4 conidial ml^{-1}. Spore concentration was measured using a haemocytometer. Plates were inoculated at the centre of each of four sectors. Each extract concentration was tested in there plates of each fungus. Plates without extract but similarly inoculated were included as controls. Percentage inhibition of mycelial growth or spore germination was calculated using the formula:

$$\% \text{ inhibition} = \frac{dc - dt}{dc} \times \frac{100}{1}$$

Where:
dc = average diameter of fungal colony/average number of spore germination in control plates.
dt = average diameter of fungal colony/average number of spore germination in treated plates

Data analysis
Data collected were subjected to Analysis of Variance (ANOVA) using SAS 2002. Significant means were separated using Duncan's Multiple Range Tests (DMRT) ($P<0.05$

RESULTS
Phytochemical screening of the leaves of *C. aurantifolia* revealed that the plant material contained flavonoids, glycosides, tannins and phlobatannins. The components saponins and anthraquinones were not detected in the plant material (Table 1).

The plant crude extract showed significant ($P < 0.05$) difference in the reduction of mycelia growth of *B. theobromae, A. niger, A. flavus* and *P. oxalicum* at the different concentration (10 – 40%) used in the study. As the concentration increased the fungal growth reduced when compared with the control (Table 2). Hence, higher fungitoxicity was observed at higher concentration of the extract for all pathogens. *Penicillium oxalicum* was the most sensitive to *C. aurantifolia* extract, followed by *B. theobromae, A. niger* and the least was *A. flavus*.

Table 3 showed the inhibitory effect of leaf extract of *C. aurantifolia* on spore germination of the four pathogens at different concentration levels. *Citrus aurantifolia* leaf extract has significant ($P < 0.05$) inhibitory effect. *Penicillium oxalicum* was the most sensitive fungi to the plant extract, while *A. flavus* was the least. Fungitoxicity of the extract against spores increased with increase in concentration.

Table 1: Phytochemical components of *Citrus aurantifolia* leaves

Phytochemical component	Inference
Flavonoids	+
Glycosides	+
Alkaloids	-
Saponins	-
Anthraquinones	-
Tannins	+
Phlobatannins	+

Key: + = Present
- = Absent

Table 2: Percentage inhibition of mycelial growth of four plant pathogenic fungi at different oncentrations of *Citrus aurantifolia* leaf extract after 5 days incubation at 28°C

Extract concentration (%)	*Botryodiplodia theobromae*	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium oxalicum*
0	0.0^{d}	0.0^{d}	0.0^{d}	0.0^{d}
10	16.51^{c}	8.62^{c}	6.41^{c}	24.96^{c}
20	18.25^{c}	16.70^{b}	11.13^{b}	45.21^{b}
30	28.15^{b}	18.28^{b}	13.58^{b}	48.38^{b}
40	45.14^{a}	26.35^{a}	24.17^{a}	58.31^{a}

Means in the same column with different superscripts are significantly different ($P < 0.05$)

Table 3: Percentage inhibition of spore germination of four plant pathogenic fungi at different concentrations of *Citrus aurantifolia* leaf extract after 24 hours incubation at 28°C

Extract concentration (%)	*Botryodiplodia theobromae*	*Aspergillus niger*	*Aspergillus flavus*	*Penicillium oxalicum*
0	0.0^{d}	0.0^{c}	0.0^{c}	0.0^{d}
10	10.37^{c}	7.31^{b}	4.32^{b}	20.83^{c}
20	13.24^{c}	8.62^{b}	5.58^{b}	34.33^{b}
30	23.51^{b}	10.25^{b}	7.63^{b}	37.50^{b}
40	42.54^{a}	17.84^{a}	11.05^{a}	51.03^{a}

Means in the same column with different superscripts are significantly different ($P < 0.05$)

DISCUSSION

The leaves of *C. aurantifolia* studied were found to contain the following phytochemical compounds, flavonoids, glycosides, tannins and phlobatannins. Other investigators have reported the presence of some of these compounds in members of the family, Rutaceae, to which the plant used in the present study belong (Gill, 1992; Okwu *et al*., 2007). The inhibitory effect of this medicinal plant on the fungi tested may therefore be due to the presence of the above phytochemical components. Scientists have shown that these metabolites play defensive roles in the plants producing them. For example, Haralampidis *et al.* (2001) reported that secondary metabolites have been implicated as chemical defense against attack by soil fungi. In the same paper, they further reported that many plants synthesize secondary metabolites as part of their normal programme of growth and development, often sequestering them in tissues which for protection against microbial attack.

The outcome of this study showed that leaf extract of *Citrus aurantifolia* significantly inhibited mycelial growth and spore germination of *Botryodiplodia theobromae, Aspergillus niger, Aspergillus flavus* and *Penicillium oxalicum* on PDA, suggesting the presence of antifungal substances in the plant tissue. This is in agreement with the findings of several workers (Amadioha, 2000; Okigbo and Nmeka, 2005; Pawar and Thaker, 2006). The fungicidal effects of plant extract on different pathogens of crop plants have been widely reported (Onifade, 2000; Okigbo and Emoghene, 2003; Elaigwu, 2006; Okungbowa and Edema, 2007; Nduagu, *et al*., 2008). Several reports on the anti-fungal activities of extract *C. aurantifolia* have been documented (Okwu *et al,* 2007: Dongono *et al*. 2009). It was also observed that the inhibitory effect of the extract on mycelial growth and spore germination of the pathogens increased with increasing concentration of the extract. This is in conformity with observations made by Olufolayi (1999) and Ilondu (2011).

CONCLUSION

The present study has shown that extract from the leaves of *Citrus aurantifolia* contain fungitoxic compounds and effective inhibited the mycelia growth and spore germination of *B. theobromae, A. niger, A. flavus* and *P. oxalicum* on PDA. The study also revealed that the antifungal potency of the extract was concentration

dependent, increasing as the concentration was increased. *Penicillium oxalicum* was the most sensitive fungi, while *A. flavus* was the least based on these findings, *C. aurantifolia* leaf extract is viable and could be used as a biological fungicide to control the test pathogens in plants.

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