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Phytochemical Composition and *In Vitro* Antifungal Activity Screening of Extracts from Citrus Plants against *Fusarium oxysporum* of Okra Plant (*Hibiscus esculentus*)

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ABSTRACT

Phytochemical studies of five varieties of citrus species, sweet orange (*Citrus sinensis*), tangerine (*Citrus reticulata*), lemon (*Citrus limonum*), lime (*Citrus aurantifolia*) and grape (*Citrus vitis*) revealed the presence of bioactive compounds comprising alkaloids (0.22-1.60%), saponin (0.30-0.98%), flavonoids (0.30-0.89), phenols (0.02-0.64%) and tannins (0.23-1.45%). The growth of *Fusarium oxysporum* which causes damping-off diseases of okra (*Hibiscus esculentus*) was inhibited *in vitro* by the extracts of citrus species. The extracts from the peels of *C. sinensis*, *C. aurentifolia* and *C. reticulata* showed 83.55%, 71.10% and 68.14% inhibition activity, respectively. An analysis of chemical composition showed that the most active geranoxycumarine, triclosan, benzetonine, limonin and nomilin contained in grapefruit (*Citrus vitis*) and sweet orange (C. *sinensis*) have high alkaloids and phenolic phytoconstituents. The fungitoxicity of the extracts from the peels of *C. sinensis* was the same as that of benomyl, a synthetic fungicide.

Keywords: antifungal properties, *Citrus*, inhibition, phenolic compounds, synthetic fungicide

INTRODUCTION

Numerous natural products of plant origin are pesticidal and have the potentials to control fungal diseases of crops. Considerable effort has been directed and devoted to screening plants in order to develop new natural fungicides as alternatives to existing synthetic fungicides, which are associated with problems such as phytotoxicity, vertebrate toxicity, pest resistance and resurgence, widespread environmental hazards and high cost (Okwu 2003). This effort has resulted in the use of extracts from citrus plants as botanical fungicides.

Effects of oil extracts from various Citrus varieties on the *in vitro* mycelia growth of *Phaeoramularia angolensis* were evaluated and the result showed positive inhibition of *P. angolensis* (Dangmo *et al*. 2002). Research has shown (Saxena and Kidiavai 1997) that extracts of medicinal plants such as citrus tree, neem tree, pyrethrum and some herbs contain toxic substances and so have potential for use in the development of natural pest control products.

It is generally assumed that the active constituents contributing to these antifungal properties are the phytochemicals (Okwu 2004, 2005). Phytochemicals constitute one of the most numerous and widely distributed groups of substances in the plant kingdom. Woody plants and herbs synthesize and accumulate in their cells a great variety of phytochemicals including low molecular phenolics (hydroxylbenzoic and hydroxycinamic acids, acetophenone, flavonoids, stilbenes, and lignans) as well as oligo- or polymeric forms (hydrolysable and condensed tannins and lignins) (Close and McArthur 2002; Okwu 2004; Okwu and Omodamiro 2005). Plants essential oils have been reported to contain limonene, methol, linolool, octanol, acetophenone, camphor, cineole, terpinolene, decanal, monoterpenes, sesquiterpenoids, and aromatic hydrocarbonyl (Bagachi *et al*. 2006; Haider 2006).

Flavonoids belong to a group of polyphenolic compounds found in fruits and vegetables (Waladkhani and Clemens 2001). The family includes monomeric flavanols, flavanones, anthocyanidins, flavones and flavonols (Waladkhani and Clemens 2001). In addition to their free-radical scavenging activity (Kandaswni and Middleton 1994) flavonoids have multiple biological functions: antibacterial, antifungal and antiviral effects as well as being inhibitors of phospholipase A2, cycloxygenase and lipoxygenase (Ho *et al*. 1992; Middleton and Kandaswani 1992).

Inhibition of germination and viability of sclerotia of *M. phaseolina* and *Fusarium oxysporum* Schlechtend Fr *Sp Chrysanthemi* by the essential oils of *Citrus medica* and *Ocimum canum* was due to volatile and non-volatile substances such as citral, limonenes and dipentene found in *C. medica* and citral, citronellal, linalol, methyl cinnamats, α-camphor and traces of phenols and acetic acid available in *O. canum* (Huang and Chung 2003). Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils. These compounds were often considered as antifungal agents (Huang and Chung 2003), inhibiting the growth of microorganisms.

Okra (*Hibiscus esculentus*) is cultivated as a vegetable and its fruit pods are consumed as vegetables. It originated in tropical Africa and has now been widely spread throughout the tropics (Purseglove 1979; Thompson and Kelly 1987). Okra plants are attacked by a number of seed and soil diseases caused by different fungi. Rots, blights and wilts are caused by *Fusarium oxysporum*. The fungus is reported (Thompson and Kelly 1987) to reproduce the disease due to its presence in the soil and seed as well as on infected plants. Naturally-infected pods appear brown to black. The affected plants show a dark brown to black discoloration from the base of the stems. *F. oxysporum* caused damage, including flower and pod abortion, pod and seed rot, shrunken pods, pod and leaf necrosis, discoloration, reduced germination and reduction in plant vigor in cowpea

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(Awurum *et al*. 2005). Fusarium wilts, caused by *F. oxysporum* is one of the most widespread and destructive diseases of many ornamental and horticultural crops (Beckman 1987). The soil-borne fungus causes vascular wilts by infecting plants through the roots and growing internally through the cortex to the stele (Beckman 1987). The vascular tissue of the root and then the stem are colonized by growth of hyphae and movement of conidia in the transpiration. Initial symptoms appear as chlrosis and distortion of the lower leaves, often on one side of the plant. Foliar chlorosis, necrosis and plant stunting become more pronounced as the disease progresses. Witting occurs on the affected side of the plant, followed by vascular discoloration and stem necrosis. The entire plant wilts and dies as the pathogen moves into the stem (Beckman 1987). *F. oxysporum* have a high saprophytic survival potential in soil. It is necessary to explore the possibility of minimizing the mortality using plant extracts. In order to maximize yield in okra production, the plant should be healthy and disease-free.

Citrus fruits are well endowed with a variety of phytofungicides that are necessary to inhibit fungal growth and development. In this report, the antifungal properties of the extracts from the peels and leaves of citrus tree against *F. oxysporum* was evaluated *in vitro*. The present study was undertaken to evaluate the phytochemical composition of citrus peels and leaves and consequently to employ the extracts as a low cost fungicide for peasant farmers.

MATERIALS AND METHODS

The experiment was carried out in the Department of Chemistry and Plant Health Management laboratories, Michael Okpara University of Agriculture, Umudike, Nigeria.

Source of materials

The fruits and leaves of sweet orange (*Citrus sinensis*), lime (*C. aurantifolia*), grape (*C. vitis*), tangerine (*C. reticulata*) and lemon (*C. limonum*) were harvested from the National Root Crops Research Institute (NRCRI) orchard, Umudike, Nigeria. The citrus species were identified by Mr. John Ibe, the manager of the Forestry Department, NRCRI. An infected okra plant (*Hibiscus esculentus*) was collected from the research farm of Michael Okpara University of Agriculture, Umudike, Nigeria.

Preparation of plant extracts

The epicarps of the five citrus species were peeled off. The leaves and peels were air dried on the laboratory bench for 10 days and then ground into a uniform powder using a Thomas Wiley mill machine (model Ed-5, USA). The powdered materials (650 g powder for each sample) were stored in airtight bottles for chemical analysis.

Extraction

Each of the powdered plant materials (100 g) was packed into a Soxhlet apparatus (2L) and extracted exhaustively with 500 ml of diethyl ether (60-80°C) for 6 hours. The ether was evaporated using a water bath and then left overnight at laboratory temperature for evaporation of the remaining ether. The test solution of each extract was prepared by dissolving 10 g of crude plant extract separately in 100 ml sterile distilled water in a 250 ml Erlenmeyer flask in a water bath at 80°C for 2 h. Extracts were subsequently filtered through four folds of cheese cloth.

Isolation of the inoculum

An infected okra plant (*Hibiscus esculentus*) was collected from the research farm of Michael Okpara University of Agriculture Umudike, Nigeria. The petiole leaf and stem of the infected plant were cut in bits using a sharp blade and then placed in a Petri dish and were disinfected with 50 mL of 70% ethanol and finally rinsed with three changes of 500 mL of sterile water, after which the tissues were placed in a Petri dish containing a moist filter paper at room temperature of 27°C. After a week, a pronounced whitish growth was observed on the surface of the tissues. 300 g of potato were brought to the laboratory and were washed five times with tap water until adhered soil was completely removed. 200 g of the potato were peeled and boiled in 1 L of water and filtered. 20 g of agar and 20 g of dextrose were added to the filtrate and made up to 1 L with sterilized water and use to subculture the organism and obtained a pure culture, which was finally examined using a compound microscope. The identity of the organism confirmed to be *F. oxysporum* f. *sp Chrysanthemi* with the aid of an identification manual by Barnett and Huntter (1972).

In vitro experiment

Each of the Petri dishes contained potato (20 g), dextrose agar (20 g), (PDA) and a 10% concentration of 5 ml plant extract were mixed together and allowed to solidify. Dishes were inoculated with the fungus by cutting a 4 mm-diameter disc from a pure culture of *F. oxysporum* f. sp growing on the PDA using a cork borer. This was done for each of the extracts as well as for two controls: a plate containing 5 mL of benomyl mixed with PDA and another without plant extracts. The cultures were incubated at 27°C in an inoculation chamber for 9 days. Radial growth of the fungus for each treatment was measured at the 9th day of inoculation using a ruler and the percentage inhibition was calculated using the formula of Amadioha (2003), as shown below:

$$\% \text{ Growth inhibition} = \frac{(DC - DT)X100}{DT}$$

where DC = colony diameter of control and DT = colony diameter of treated plates.

Phytochemical analysis

Alkaloids and phenols were determined according to the method of Harborne (1973) while tannins were determined using the method of Van Burden and Robinson (1981). Saponin was determined using the method of Obadoni and Ochuko (2001). Flavonoids were determined according to the method of Boham and Kocipai (1994).

Statistical analysis

All measurements were replicated three times and standard deviations determined. The student's *t*-test at $P<0.05$ was applied to assess the difference between the means (Steel and Torrie 1980).

RESULTS AND DISCUSSION

The phytochemical contents of the citrus peels and leaves are shown in **Table 1**. The alkaloids content of the peels of

Table 1 Phytochemical composition of leaves and peels citrus fruits (%).

Species	Alkaloids		Phenols		Flavonoids		Tannins		Saponins	
	Leaf	Peel	Leaf	Peel	Leaf	Peel	Leaf	Peel	Leaf	Peel
C. reticulata	0.22 ± 0.11 b	1.20 ± 0.20 b	0.02 ± 0.10 b	0.23 ± 0.10 b	0.03 ± 0.02 b	0.07 ± 0.10 b	0.23 ± 0.10 b	0.45 ± 0.20 b	0.83 ± 0.10 a	0.56 ± 0.02 a
C. aurantifolia	0.44 ± 0.03 a	1.00 ± 0.20 b	0.05 ± 0.20 b	0.47 ± 0.22 b	0.07 ± 0.11 b	0.52 ± 0.02 a	1.45 ± 0.22 a	0.63 ± 0.20 a	0.66 ± 0.10 a	0.98 ± 0.12 a
C. limonum	0.54 ± 0.20 a	1.35 ± 0.11 a	0.25 ± 0.22 a	0.64 ± 0.11 a	0.64 ± 0.14 a	0.48 ± 0.20 a	1.31 ± 0.02 a	0.59 ± 0.10 a	0.34 ± 0.22 b	0.58 ± 0.22 a
C. sinensis	0.39 ± 0.05 a	1.60 ± 0.03 a	0.02 ± 0.11 b	0.40 ± 0.20 a	0.62 ± 0.33 a	0.34 ± 0.12 a	0.54 ± 0.11 a	0.92 ± 0.20 a	0.99 ± 0.10 a	0.72 ± 0.30 a
C. vitis	0.28 ± 0.11 b	1.20 ± 0.22 b	0.10 ± 0.02 a	0.56 ± 0.10 a	0.89 ± 0.20 a	0.15 ± 0.11 b	0.76 ± 0.20 a	0.50 ± 0.02 b	0.86 ± 0.20 a	0.30 ± 0.10 a

Data are means ± standard deviation of triplicate determination on a dry weight basis. Values with the same superscript in each column are not significantly different at $P<0.05$.

C. sinensis was very high (1.60%), followed by *C. limonum* which contained 1.35% of alkaloids while the peels of *C. aurantifolia* contained 1.0% of alkaloids. The content of alkaloids was low in the leaves compared to the peels. This observation was also made in the results of the phenolic content where the values of phenol in the peels are higher than those in the leaves. The reason may be that during fruiting, more alkaloids and phenolic constituents are produced in the peels in order to protect and preserve the seeds from microbial attack (Okwu and Emenike 2006). Orange and lemon oil contain substantial amounts of limonene, a terpenoid that also posses antiviral, antifungal and antibacterial properties (Patkowska 2006). Citrus contain a host of active phytochemicals like limonin, nomilin, octanol, cineole and naringin that inhibits fungal pathogens. These compounds which occur in high concentrations in grape fruits, lemons and oranges is responsible for the bitter taste of these fruits (Angion *et al*. 1998; Alias and Linden 1999, Okwu and Morah 2007a, 2007b). Limonoids possesses the ability to inhibit the development of bacteria and fungi (Angion *et al*. 1998; Woedtke *et al*. 1999; Patkowska 2006). Grape-fruit also contains 7-geranoxycumarine, triclosan or benzetonine chloride which inhibits the development of bacteria and fungi (Angion *et al*. 1998; Woedtke *et al*. 1999; Patkowska 2006). The presence of phenolic compounds in citrus indicates that these plants may be antimicrobial agent since phenols and phenolic compounds are extensively used in disease preventions and remain the standard with which other bactericides or fungicides are compared (Okwu 2003, 2005; Okwu and Morah 2007a). Phenolics form a large group of naturally occurring, diverse and widespread compounds. They are characterized by the presence of an aromatic ring with one or more hydroxyl groups. These phenolic compounds in citrus seed may be responsible for the antiseptic, antifungal or bactericides properties of the plants (Okwu and Morah 2007b). The mechanism of inhibitory action of these alkaloids and phenolic compounds on microorganisms may be due to impairment of a variety of enzyme systems, including those involved in energy production, interference with the integrity of the cell membranes and structural component synthesis (Huang and Chung 2003). The antimicrobial activities of phenols are further evidenced by their active role in plant disease resistance and prevention (Matern and Kneusel 1988; Russel and Chopra 1990). Moreover, phenolic compounds from plant extracts act as antimicrobial agents (Okwu 2005). Phenolic compounds are also considered to be bacteriostatic and fungistatic. These compounds caused swelling of hyphal tips, plasma seeping around hyphae leaking of plasma, cell wall distortion, abnormal branching or fusion of hyphae and consequently wrinkling of hyphae surface (Huang and Chung 2003).

Furthermore, the effects of grape fruits, lemons, orange and lime extracts on fungi may be due to inhibition in the formation of zoosporangia and germination of the pathogens zoospores thereby limiting the growth of mycelium (Orlikowski 2001). Besides, grape fruit, lime and lemon fruits extracts inhibited the growth of mycelium, the formation of conidial spores and chlamydospores of *F. oxysporum* thereby reducing the number of propagation units of this fungus in the medium (Patkowska 2006). There is inhibition in the formation of mycelial filaments and conidial spores of the fungus (**Table 2**). The citrus extracts were effective inhibiting mycelial growth, spormulation and formation of sclerotia of fungi pathogens towards *H. esculentus*.

Flavonoids are another phytochemical found in citrus samples. The results revealed the presence of 0.03% and 0.07% (w/v) flavonoids in the peels and leaves, respectively of *C. reticulata*, 0.52% and 0.07% for *C. aurantifolia* while *C. limonum* leaves contained 0.64% of flavonoids. Flavonoids are widely distributed in plants fulfilling many functions including protection from attack by microbes and insects. Quercitin, Myricitin, rutin, tangeritin, naringin and hesperidin (**Fig. 1**) are found amongst the common flavonoids in citrus fruits. These flavonoids are responsible for the bitter taste of some grape fruits, lemons and oranges. Hesperidine is believed to play a role in plant defense. It acts as an antioxidant according to *in vitro* studies (Alias and Linden 1999; Okwu and Morah 2007a). Quercetin is found to be the most active of the flavonoids and many medicinal plants owe much of their activity to their high quercetin content. Tangertin is a polymethoxylated flavones that is found in tangerine and other citrus peels. The presence of phenolic compounds in citrus fruits enhanced their inhibitory function on microorganism. The citrus flavonoids functions as antioxidants and radical scavenger in the medium.

Table 2 Inhibitory effects of 10% concentration of leaf and peel extracts of citrus and benomyl on *in vitro* growth of *Fusarium oxysporum*.

Treatment	**% Inhibition**	
Benomyl	83.64 ± 0.10 a	
Control	0.00 c	
Citrus samples	**Leaf extract**	**Peel extract**
C. reticulata	44.25 ± 0.05 a	68.14 ± 0.17 a
C. aurantifolia	44.25 ± 0.05 a	71.10 ± 0.09 a
C. limonum	27.75 ± 0.13 b	48.48 ± 0.07 a
C. sinensis	26.12 ± 0.13 b	83.55 ± 0.13 a
C. vitis	24.56 ± 0.15 b	42.15 ± 0.13 b

Data are means ± standard deviation of triplicate determinations on dry weight basis. Values with superscript that are the same are not significantly different at P<0.05.

Tannins content were found more in *C. aurentifolia* leaf (1.45%) and *C. limonum* leaf contained (1.31%) of tannins, followed by *C sinensis* peels which contained 0.92% of tannins. The presence of tannins may be a reason for the bitter and astringent taste of unripe citrus fruits particularly lime, lemons and grapes. Saponins were found to be available at 0.99% in *C. sinensis* leaf, *C. aurantifolia* peel contained 0.98% of saponins and *C. vitis* leaf contained 0.86% while *C. limonum* leaf contained 0.34% of saponins. The presence of saponins may also be responsible for bitter taste and antifungal properties these plants exhibits particularly lemons, lime and grapes.

Table 2 shows the inhibitory effects of 10% concentration peel and leaf extracts of citrus and benomyl on *in vitro* growth of *F. oxysporum*. Both *C. sinensis* and synthetic fungicide (benomyl) have 83.55% and 83.64% inhibition respectively. This could be linked to the fact that *C. sinensis* has enormous deposit of alkaloids and phenolic content. Better inhibition of *F. oxysporum f. sp chrysanthemi* was obtained with the peels than on the leaf extracts. This could also be attributed to the fact that more phytochemicals were deposited on the peel particularly alkaloids and phenols. The extracts showed a remarkable effectiveness in controlling *F. oxysporum f. sp chrysanthemi*. According to Patkowska (2006), grape fruit extract also inhibited the development of *Phytophthora crytogea*, *P. cinnamoni* and *F. oxysporum f. sp chclaminis*. On the other hand, grape fruit extract protected *chrysanthemums* from the infection by *Puccinia horiana* and willow-form melampsora epitea (Orlikowski 2001; Patkowska 2006).

Plants store these antifungal, antibacterial and antiviral chemicals on the peels to protect and preserve the seeds from microbial attack. This agreed with the findings of Okwu and Emenike (2006) who reported that phytochemicals are reserved in plants to protect the plant against the attack and inversion of micro-organisms. The extracts and synthetic fungicide (benomyl) inhibited the growth of the organism, *F. oxysporum f. sp chrysanthemi*. This is in agreement with the work of Amadioha and Obi (1998). In the *in vitro* experiment, all the extracts were highly effective in the inhibition of the organism. Extracts of citrus plants contain antifungal compounds that can be used as alternative to synthetic fungicides, including fumigants and contact pesticides. The prospect of using citrus plant extracts for development of natural fungicides is appealing and acceptable. This is because citrus peels and leaves are readily available, environmentally safe, and less risky for developing resistance in

pests, less hazardous to non target organisms and pest resurgence, less adverse effect on plant growth, less harmful to seed viability and quality and above all less expensive (Prakash and Rao 1997). Based on these findings, citrus plant extracts are viable and can be possible alternative to synthetic pesticides for control of fungal diseases.

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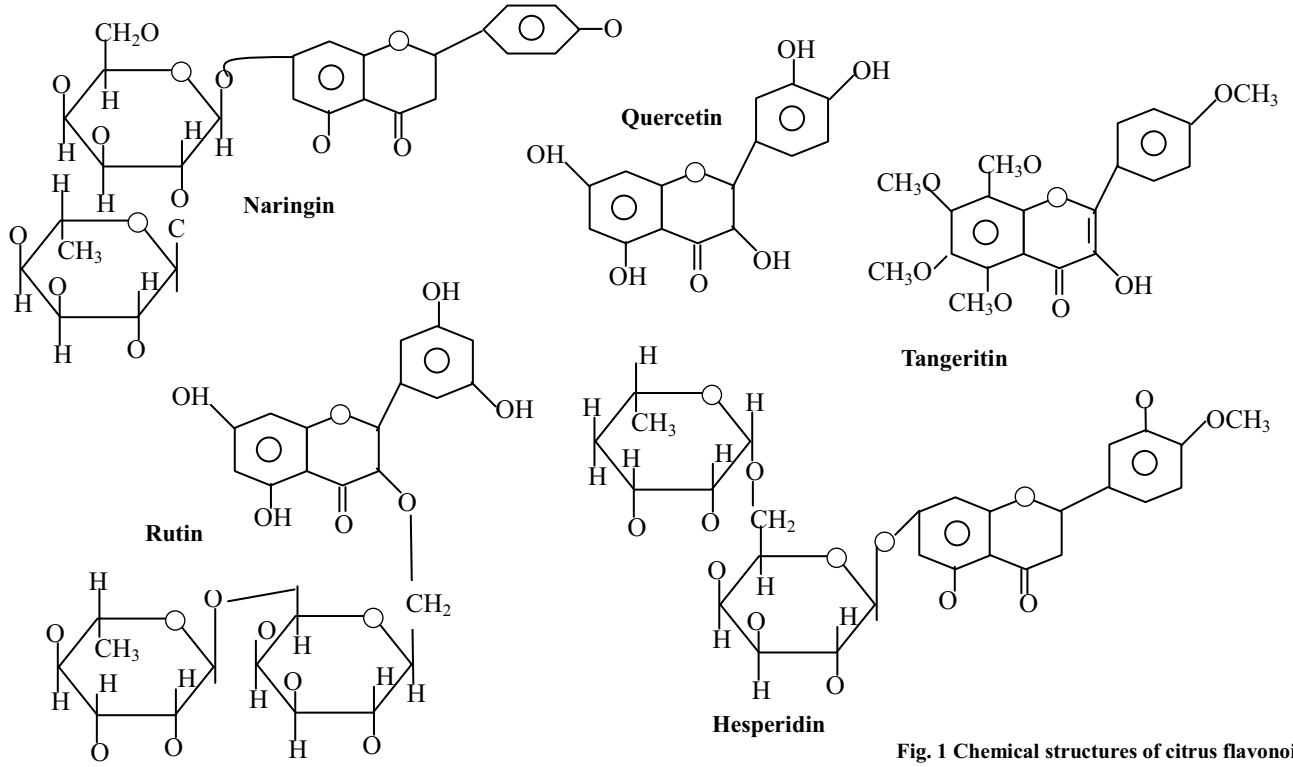

Fig. 1 Chemical structures of citrus flavonoids.