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Inhibition of Growth of Fungi Isolated From Deteriorating Melon Seed By Extracts of *Punica Granatum* and *Cymbopogon Citratus*

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Abstract
A study was conducted to investigate the effect of extracts of *Punica granatum* and *Cymbopogon citratus* on *Aspergillus nivale, Rhizopus stolonifer* , *Mucor mucido* and *Aspergillus fumigatus* isolated from deteriorating melon seed using radial growth technique. Phytochemical screening revealed that extracts the plants contained saponins, glycosides, alkaloids and tannins. Steroids were also detected in *Punica granatum..*The plant extracts inhibited the growth of all the test fungi. The minimum inhibitory concentration (MIC) of the extracts ranged between 0.06µg/ml. and 0.07µg/ml while the minimum fungicidal concentration ranged between 0.06µg/ml and 0.08µg/ml. Aqueous extract of *Punica granatum* appears to be more effective as an antifungal agent than the extract of *Cymbopogon citratus* Extracts of *Punica granatum* and *Cymbopogon citratus* may be important sources of preservative of melon seed.

Key words: Investigate, deteriorating, phytochemical, fungicidal, concentration, preservative.

Introduction

Curcubitacea is a large family which includes many economic species such as melon, water melon, pumpkin and cucumber (Rushing *et al.,* 1999). Melon seeds are widely grown in Nigeria. Melon (*Cucumeropsis mannii*) is a herb, annual crawling plant characterized by a ramifying but shallow root system. The leaves are large, alternate simple long –petiole and palmately lobed. The inflorescence consists of flowers borne in leaf axis with a coiled tendril. The flowers are predominantly yellow and large occurring singly. The fruits are large with light brown seeds arranged in false axial plancentation. Melon seeds are flat, oval with papery coat, which are thicker at the margins. The seeds are used in soup preparation for various foods; it is used as thickening and emulsifying agent in soup. It is eaten as snacks or fermented traditionally to produce a flavouring agent "Ogiri" for use as a soup condiment (Akindele, 1978; Ogundana, 1972). It is believed that melon has a good taste.

Melon is very abundant immediately after the rains but become scarce during the dry season in Nigeria. Spoilage of melon seed is due to the activity of microorganisms, yeasts, moulds and bacteria. In addition insects, rodents and enzymes may attack melon.

Punica granatum (*Pomegranate*) seeds dried with the pulp are commonly used as a spice in many dishes. The seeds are used as a garnish on rice dishes, potatoes and apple sauce. The infusion of the young flowers of *Punica granatum* is astringent and is given in chronic diarrhea; dysentery and bronchitis (Gill, 1992). The bark of the root and stem are used as anthelmintic. It is very effective in the treatment of tapeworm infection. Information on the antimicrobial activity of *Punica granatum* is scanty.

Cymbopogan citratus (Lemon grass) is a tall tropical grass. The fresh stalks and leaves have a clean lemonlike odour. The plant is used as a spice, the dried spice is available in several forms; chopped in slices, cut and sifted and powdered. Decoction of the leaves along with onion and honey is used to cure cough, malaria fever and chest pain among the Yoruba speaking people of Western Nigeria hence there is need to

investigate the antimicrobial property of *Cymbopogon citratus*. A paste of the leaves is used to cure ringworm infection (Gill, 1992). The aim of this study is to determine the extent to which the extracts of *Punica granatunm* and *Cybopogon citratus* could inhibit the growth of fungi isolated from deteriorating melon seed.

Materials and Methods

Plant material: Seeds of melon (*Cucumeropsis mannii*), leaves of *Punica granatum* and *Cympogon citrate used in this study were collected in* Bida, Nigeria. They were identified at forestry research institute, Ibadan Nigeria in accordance with the criteria stipulated by International Committee for Botanical Nomenclature (ICBN).

Test microorganisms: The microorganisms used in this study were *A. nivale*, *R. stolonifer, Mucor mucido* and *A. fumigatus*. The fungi were isolated from deteriorating melon seeds and identified by standard microbiology procedures of Ainsworth *et al.* (1993).

Preparation of plant extract: Seeds of *Punica granatum* and *Cymbopogon citratus* were air dried at room temperature (28±2°C) for 4 weeks and pulverized to powder using a clean electric blender (Model Phillips 190). A 50 g sample of the pulverized leaves was soaked in 200ml of distilled water and allowed to stand for 72 h with intermittent starring. This was filtered through a Whatman No 1 filter paper and the filtrate obtained was evaporated to a dry mass using a rotary evaporator at 80°C. The dried extract was exposed to uv light for 24h and cheeked for sterility by streaking on nutrient agar plates. The extract was assayed against the test fungi to determine the antifungal properties (Bauer *et al*., 1966).

Phytochemical screening of extract: The method described by Odebiyi and Sofowora (1978) were adopted to test for the presence of steroids, saponins, glycosides, flavonoids alkaloids and tannins.

Test for steroids: Chloroform extract of the test plant seeds (10ml) was evaporated to a dry mass and the mass dissolved in 0.5ml of chloroform**.** Acetic anhydride (0.5ml) and 2ml of concentrated sulphuric acid were added. A blue of green colour or a mixture of these two shades was regarded as positive for the presence of steroidal compounds.

Test for saponins: The plant extract (0.5g) was stirred in a test tube, foaming which persisted on warming was taken as evidence for the presence of saponins.

Test for glycosides: The powdered plant seeds (1g) were introduced into different beakers. To one of the beakers was added Sulphuric acid (5ml) while water (5ml) was added to the other beaker. The two beakers were heated for 3min and the contents filtered into abelled test tubes. The filtrate was made alkaline with sodium hydroxide (0.5ml) and allowed to stand for 3min. The presence of reddish brown precipitate in the filtrate was regarded as positive for glycosides.

Test for flavonoids: A small piece of magnesium ribbon was added to ethanol extract of the seeds. This was followed by dropwise addition of concentrated hydrochloric acid. Colours ranging from orange to red indicated flavones, red to crimson indicated flavonoids, crimson to magenta indicated flavones.

Test for alkaloids: Extract of seed samples (0.5g) were stirred with 5ml of 1% hydrochloric acid (HCl) on a steam bath. The solution obtained was filtered and 1ml of the filtrate was treated with 0.2ml of Mayer's reagent. The two solutions were mixed and made up to 100ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as evidence for the presence of alkaloid in the extract.

Test for tannins: Powdered seed sample of the test plants (10g) were weighed into different beakers and 10ml of distilled water added. The mixtures were boiled for 5min. 5% $FeCl_3$ (0.2ml) was then added. Production of greenish precipitate indicated the presence of tannins.

Antifungal assay: The antifungal properties of the extracts were determined using the radial growth method of Banso and Ngbede (2006). Graded concentrations of the extracts (0.01 µg/ml – 0.06 µg/ml) of the extract were introduced into different

McCartney bottles containing 18ml sterile malt extract agar. The mixture was poured into different Petri dishes and allowed to solidify. Each plate was inoculated with 5mm diameter of the fungal culture .Control experiments were performed without the extracts. Plates were incubated at 25°C for 72h. Antifungal activities were expressed in terms of diameter of growth.

Determination of minimum inhibitory concentration (MIC): Graded concentrations (0.04 µg/ml, 0.05 µg/ml, 0.06 µg/ml, 0.07 µg/ml, 0.08 µg/ml) of the extracts were prepared. Each of these was added to 18mls of malt extract in test tubes. Each tube was then inoculated with 0.1 ml of spore suspension of *A. nivale, R. stolonifer* , *Mucor mucido* or *A. fumigatus* dilutions to give a final spore suspension of 10^6 spores per ml. The tubes were incubated at 28±2°C and examined for growth after 7 days. The least concentration of the plant extract that does not permit any visible growth of the inoculated test organism in the broth medium was regarded as the MIC in each case .Control experiments were performed without the plant extracts.

Determination of minimum fungicidal concentration (mfc): The contents of the tubes that showed no visible fungal growth or turbidity in the minimum inhibitory concentration experiment were cultured into freshly prepared potato dextrose agar plates to assay for the fungicidal effect of the extracts. The plates containing the test organisms were incubated at 25°Cfor7 days. The minimum fungicidal concentration was regarded as the lowest concentration that did not yield any fungal growth on the solid medium used.

Results

P. granatum and *C. citratus* contain saponins, glycosides, alkaloids, and tannins. Flavonoids were not detected in the samples tested (Table 1). Aqueous extract of *P.granatum* and *C. citratus* exhibited antifungal activity against *A. niger*, *R. stolonifer*, *Mucor* species and *A. fumigatus* (Tables 2 and 3). The largest growth diameters ranging from 18.0 0.1 mm to 15.0 0.1 mm were recorded against assays containing *P. granatum* extract (Table 2). *R. stolonifer* appears to be the most resistant to the effect of extracts of *P. granatum* and *C. citrates* while *A. fumigatus* which had lower ranges of mean diameter of growth (3.0 ±0.2 mm to 2.5± 0.5 mm) appears to be the most susceptible (Table 2). Lower ranges of mean growth diameters (2.5± 0.5 mm to 17.5 ± 0.2 mm) were recorded against *C. citratus* while higher ranges of mean growth diameters (3.0 ± 0.2 mm to 18.0 ±0.1mm) were obtained against *P.granatum*. The minimum inhibitory concentration of *P. granatum* and *C. citratus* extracts against the test fungi ranged between 0.04 µg/ml and 0.08 µg/ml (Table 3). The minimum inhibitory concentration of extract of *P. granatum* against *A. niger*, *R. stolonifer*, *Mucor* species and *A. fumigatus* were 0.06 µg/ml, 0.07µg/ml, 0.07 µg/ml , 0.08 µg/ml , respectively . Values ranging from 0.07 µg/ml, 0.07 µg/ml, 0.08 µg/ml , and 0.07 µg/ml were recorded against *A.niger, R. stolonifer*, *Mucor* species and *A. fumigatus* respectively when *C. citratus* was assayed against the fungi.

Minimum fungicidal concentration values of 0.06 µg/ml was recorded against *A.niger, R.stolonifer* and *Mucor* species when extract of *P. granatum* was tested against the fungi while a value of 0.07 µg/ml was recorded against *A. fumigatus*. Minimum fungicidal concentration values of 0.08 µg/ml was recorded against *R. stolonifer* and *Mucor* species when *C. citratus* extract was assayed against the fungi (Table 4).

Table 1. Phytochemical constituent of seed extracts

Active principle	*P. granatum*	*Cymbopogon citratus*
Steroids	+	-
Saponins	+	+
Glycosides	+	+
Flavonoids	-	-
Alkaloids	+	+
Tannins	+	+

Discussion

The fact that the results of this study showed that extracts of *P. granatum* and *C. citratus* exhibit antifungal properties justifies

their traditional use as medicinal plants and spices . This may be due to the presence of active principles in the plant materials. Plants generally produce many secondary metabolites which constitute an important source of microbiocides, pesticides and pharmaceutical drugs (Ibrahim, 1997; Ogundipe *et al.,* 1998). Spices contain phenolic and essential oils, which are inhibitory to microorganisms (Nakatani, 1994). The effect on microorganisms may depend on the type as well as the medium (Giese, 1994). It was reported that fats and proteins bind or solubilise phenolic compounds thereby reducing their availability for antimicrobial activity (McCance and Widdowson, 1993; McNeil and Schmidt, 1993).

Table 2: Antifungal effect of *Punica granatum* and *Cymbopogon citratus*

Concentration (µg/ml)	Mean diameter of growth (mm)±SD							
	P.granatum				*C. citratus*			
	A. niger	R. stolonifer	Mucor species	A. fumigatus	A. niger	R. stolonifer	Mucor species	A. fumigatus
0.01	17.0±0.1	18.0±0.1	16.0±0.1	15.0±0.1	16.5±0.1	14.0±0.1	15.5±0.1	17.5±0.2
0.02	16.5±0.1	14.0±0.1	13.0±0.1	12.0±0.3	14.5±0.5	11.0±0.2	11.5±0.3	12.0±0.3
0.03	14.0±0.5	11.0±0.2	11.0±0.2	10.5±0.2	11.0±0.1	8.5±0.1	10.0±0.1	10.5±0.1
0.04	10.0±0.4	9.0±0.2	8.0±0.1	8.0±0.2	9.5±0.2	6.0±0.1	6.0±0.1	7.5±0.3
0.05	5.0±0.1	4.0±0.2	5.0±0.1	4.0±0.2	7.5±0.2	3.0±0.3	4.0±0.1	3.5±0.4
0.06	4.0±0.1	3.0±0.2	4.5±0.2	3.0±0.2	4.5±0.3	2.5±0.5	3.0±0.1	2.0±0.2

SD = Standard deviation; control treatment consisted of the test fungi without the extract

Table 3: Minimunm inhibitory concentration of extract

Organism	Concentration of extract (µg/ml)											
	P.granatum						*C. citratus*					
	0.08	0.07	0.06	0.05	0.04	MIC	0.08	0.07	0.06	0.05	0.04	MIC
A. niger	-	-	-	+	+	0.06	-	-	+	+	+	0.07
R. stolonifer	-	-	+	+	+	0.07	-	-	+	+	+	0.07
Mucor species	-	-	+	+	+	0.07	-	+	+	+	+	0.08
A. fumigatus	-	-	+	+	+	0.08	-	-	-	+	+	0.06

+ = presence of growth; - = absence of growth; MICs were presented as the minimum inhibitory concentrations that did not permit any visible growth of test organism in malt extract.

Table 4: Minimunm fungicidal concentration of extract

Organism	Concentration of extract (µg/ml)											
	P.granatum						*C. citratus*					
	0.08	0.07	0.06	0.05	0.04	MIC	0.08	0.07	0.06	0.05	0.04	MIC
A. niger	-	-	-	+	+	0.06	-	-	+	+	+	0.07
R. stolonifer	-	-	-	+	+	0.06	-	+	+	+	+	0.08
Mucor species	-	-	-	+	+	0.06	-	+	+	+	+	0.08
A. fumigatus	-	-	+	+	+	0.07	-	-	+	+	+	0.07

+ = Presence of growth; - = absence of growth; MFCs were presented as the lowest concentrations that did not permit any visible growth of test organisms in malt extract agar.

The minimum inhibitory concentration values of extracts of *P.granatum* and *C. citratus* against the test fungi showed that fungi vary widely in the degree of their susceptibility to antifungal agents. This agrees with the report that antimicrobial agents with low activity against an organism have high minimum inhibitory concentration while a highly antimicrobial agent has a low minimum inhibitory concentration (Banso, and Ngbede, 2006; Prescott *et al.,* 2002).

When the test fungi used in the minimum inhibitory concentration tests were subcultured on malt extract agar for the assessment of the minimum fungicidal concentration of the extracts, the result indicated that the minimum inhibitory concentration of the extracts were obtained

at higher concentrations than in minimum inhibitory concentration studies. This finding therefore suggests that the antifungal substances contained in the extracts were fungistatic at lower concentrations while becoming fungicidal at higher concentrations of the extracts. Similar observations have been reported by Amer *et al*. (1981). The results of this study show that aqueous extracts of *P.granatum* and *C. citratus* have activity against food spoilage fungi. Extracts of spices could be useful in the preservation of melon seed.

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