



Phytochemical screening and antifungal potentials of *Citrus limon* peels against *Fusarium oxysporum* and *Rhizopus stolonifer* causing rots in water melon (*Citrullus lanatus* L.)

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ABSTRACT

This study investigated the phytochemical constituents and antifungal potential of *Citrus limon* peels using aqueous and methanol extracts against *Fusarium oxysporum* and *Rhizopus stolonifer* causing rots in water melon. The result of phytochemical screening revealed the presence of Saponin, Tannin, Phenols, Alkaloid and Flavanoid. Pathogenicity test conducted showed that *F. oxysporum* and *R. stolonifer* causes rots of *Citrullus lanatus*. The extracts inhibited the growth of *F. oxysporum* (76.67% and 60.00%) methanol and aqueous respectively. *R. stolonifer* growth was inhibited (85.93% and 71.11%) methanol and aqueous extracts. The fungitoxicity of synthetic fungicide Benomyl was 95.56%. The methanol extracts has highest inhibitory activity than aqueous extract. The result of the findings indicates the Biofungicide potentials of *Citrus limon* peels as alternative to synthetic fungicide in management of phyto-pathogens of water lemon.

KEYWORDS: Antifungal, phytochemical, fungicide, pathogenicity

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INTRODUCTION

The persistent and promiscuous utilization of synthetic fungicides have exerted a serious threat to ecological balance and human health resulting in carcinogenic occurrences and resistance to the chemicals by the pathogenic fungi which poses a serious threat to plant and agricultural products. There is an increasing demand for the reduction in use of chemicals as antifungal agents in the field of agriculture and to combat various infectious plant diseases due to increasingly aggressiveness of endogenous phyto-pathogens that are resistant to the use of synthetic antifungal agents. With regards to the negative effects imposed by the use of synthetic chemicals in management of plant diseases, utilization of plants extracts in the course of managing plant diseases is gaining importance [6]. The perceived negative effect caused by synthetic fungicide, bactericide, herbicide, nematicide and pesticide/insecticide on agricultural land and water bodies as well as the risks involved in such activities, much attention was focused towards alternative method of pathogen control [5].

Citrus limon fruit is an important medicinal plants of the family Rutaceae, it is used mainly for its phytochemical constituents

which are having anticancer and anti bacterial potentials in crude extracts of different parts viz, Leaves, stem, juice, peels and flower[18]. The fruits has wide spectrum of biological activities which include antibacterial, antifungal, antiviral activities due large constituents of phytochemicals [3]. The presence of arrays of phytochemicals such as saponins, steroids, tannins, glycosides, alkaloids, flavanoids and phenols in plants are responsible for antimicrobial activities [9,17].

The water melon *Citrullus lanatus* is an important vegetable crop plant in Nigeria, cultivated mainly for its commercial edible fruits of high water and nutritional contents [12]. The origin of watermelon has been traced to Africa and the Middle East where it was cultivated for thousands of years, and in China since at least 900 AD. Watermelon was brought to the New World in the 1500s. Watermelon is the fruit of a plant originally from a vine of southern Africa. It is a member of the cucurbitaceae family. The crop is grown commercially for its high water and nutritional contents of the fruits in areas with long frost-free warm periods[22]. The watermelon fruit has a smooth exterior rind and a juicy, sweet, usually red interior flesh. Watermelon (*Citrullus lanatus* L.) is a warm season crop in the Cucurbit family. Despite the commercial and nutritional importance of *Citrullus lanatus*

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L. fruits, its production is beset by many phyto-pathogenic fungi such as *F. oxysporum*, *F. solani*, *S. rofsii*, *A. niger*, *R. stolonifer*, *Rhizoctonia spp*, *Pythium spp*. among others [10]. The aim of this study is to identify the phytochemical constituents of *Citrus limon* peels and to determine its antifungal effects on Growth inhibition of *F. oxysporum* and *R. stolonifer* causing fruit rot of *Citrullus lanatus* L.

MATERIALS AND METHODS

Collection of Plant Materials

Fresh *Citrus limon* were purchased from Nai'bawa yanlemo market and were identified and authenticated at the herbarium of plant pathology laboratory of Bayero University, Kano. A voucher specimen was deposited at the University herbarium with voucher number [18].

Preparation of Extract of *Citrus Limon* Peels

Fresh peels of *C. limon* were washed with tap water and air dried under natural conditions in the shade for a period of 7-10 days at an ambient room temperature. The dried materials were grounded to fine powder using sterile mortar and pestle to obtain the powdered form and kept in a sterile plastic bag for further use [8]. 25g of the sample were subjected to successive extraction with water and methanol using Soxhlet apparatus for 5 hours at a temperature not exceeding the boiling point of the solvent [21]. The solvent extracts were removed by evaporation using water bath at 45°C, the crude extracts obtained were weight and directly used for qualitative phytochemical screening and determination of antifungal activity [23].

Collection of Sample

Citrullus lanatus fruits (Water melon) showing symptoms of rots were randomly selected from different market stalls and local storage facilities in Naibawa Yanlemo market of Kano metropolis, healthy fresh blemish free Water melons were also collected and packaged in different sterile polythene bags and taken to Plant pathology laboratory of Department of Biological Sciences Bayero University, for microbial analysis.

Isolation and Identification and of Rot Fungi

To isolate the pathogens responsible for the rots on the affected water melon fruits, the fruits showing discolouration and sign of rots were cut into smaller pieces and surface sterilized in 1% Sodium hypochlorite solution for 60 sec. These were then rinsed in three successive changes of sterile distilled water and blotted dry with sterile filter paper. Small segments of tissues (3mm³) from the margins of rotted lesions were cut out with a sterile scalpel and plated on potato dextrose agar (PDA) in 90mm Petri – dishes. The plates were incubated at room temperature (28 ± 3°C) for 7 days. Developing fungal colonies were sub – cultured continuously on fresh PDA plates to obtain pure culture of the isolates. Fungal isolates, *F. oxysporum* and

R. stolonifer were identified based on cultural and morphological characteristics using mycological atlas [7,19].

Pathogenicity Test

Fresh, healthy blemish free water melon fruits onion bulbs were washed with tap water, rinsed with distilled water and surface sterilized with 70% ethanol. Cylindrical discs were removed from the fruits with a sterile 4mm cork borer. A disc of a five days old culture of the isolated *F.oxysporum* was transferred into hole created in the fruits. The plug was carefully placed and the wounded area sealed with Vaseline to prevent extraneous infection. The inoculated fruits were placed in separate air tight containers and incubated for 14 days at room temperature (28 ± 2°C). The same procedure was used for the control except that discs of uninoculated PDA were placed in the holes created in the fruits [4]. Three replications were prepared for treatment and control. After incubation period, the water melon fruits were examined for infection and disease development. The causal agents were re-isolated from the infected fruits and compared with the original isolates, the same procedure were done for *R. stolonifer*.

In Vitro Antifungal Activities of Plant Extracts

Poison food technique was used to determine the antifungal effects of the different concentrations of the plant extracts. For testing the antifungal effects of aqueous and methanol extracts of peels of *C. limon* four different concentrations were used; 500mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml. 100ml sized conical flask was used for the experiment in each of the flask, 15mls of the media was poured, plugged with cotton wool and capped with aluminum foil. 5ml each of varying concentration of the peels extract were incorporated into each flask containing 15ml each of the media, these were then poured into pre-sterilized Petri-dishes and kept at room temperatures of 25°C ± 2°C, the seven days purified cultures were punched with sterile cork borer (2 × 2mm) and impregnation was made with the aid of sterile inoculating needle and then deposited at the center of the petri-dishes containing varying concentrations. The plates which contained synthetic Benomyl as well as 20ml of media and no treatment serve as positive and negative control. Impregnation were made and the seven days purified cultures were punched with sterile cork borer (2 × 2mm) which was deposited at the center of the plate. The result was measured in millimeter (mm) by measuring the fungal growth from two lines (vertical and horizontal) and the mean were recorded [30]. For each treatment, three (3) replicates were maintained. The antifungal activities in terms of percentage growth inhibition (PGI) of the mycelial growth was calculated [29].

$$PGI = \frac{dc - dt}{dc} \times 100$$

Where *dc* = Average mycelial growth in control plates
dt = Average mycelial growth in treated plates
 PGI = Percentage growth inhibition

Phytochemical Screening of *C. Limon* Peels Extract

Phytochemical screening were conducted to qualitatively determine the presence or absence of the following secondary metabolites which are alkaloids, tannins, flavonoids, saponins and phenol using methods outlined [33,14,15,31].

Test for Saponins

- 5ml of distilled water was added to 2ml of the extracts in a test tube and shaken thoroughly. The formation of foams or stable frothing after shaken indicates the presence of saponins [14].
- 0.5g of each extracts was separately shaken with distilled water in a test tube followed by heating in a water bath to a boiling point. Persistent frothing on warming confirms the presence of saponins [33].

Test for Tannins

Few drops of FeCl_2 (5% W/V) solution was added to 2-3ml of the extracts in a test tube followed by shaking. A dirty green or dark blue coloration confirm the presence of Tannins [14].

Test for Phenols

One milliliter of the extract was added to 1ml of 10% FeCl_3 and mixed together. The presence of blue precipitate confirms the presence of Phenols [14].

Test for Alkaloid

0.5g of each extracts were stirred with 10cm³ of 10% hydrochloric acid and was allowed to stand overnight and then divided into two parts for the following tests.

- 2 drops of meyer's reagent was added to 1cm³ of the extracts. Appearance of a creamy precipitate was taken as the evidence of the presence of Alkaloids [15].
- 2 drops of wagner's reagent was added to 1cm³ of the extracts. A reddish brown precipitate observed in each test tube indicates the presence of Alkaloids [14].

Test for Flavanoids

One milliliter of the extracts was treated with 1ml of dilute NaOH. The presence of a cloudy precipitate confirms the presence of flavonoids [14].

Data analysis

All data obtained were statistically analyzed for significant difference ($P < 0.05$) by analysis of variance (ANOVA) and means were separated using Duncan Multiple Range Test (DMRT)[32].

RESULTS AND DISCUSSIONS

The phytochemical analysis of *Citrus limon* peels extracts are shown in Table 1. The result showed that Saponins, Tannins,

Phenols, alkaloids and flavanoids were present in methanol extract but only flavanoid was not observed in aqueous extracts, this may be due to solubility of the methanol is much more than that of aqueous solvent. Junab and Trideep[18] screened the phytochemical constituents of lemon peels the result revealed the presence of alkaloids, saponin, flavanoid, tannins glycosides and steroids which is in agreement with the results of this study where by most of the bioactive components screened were present.

Rutaceae family generally contain host of active phytochemicals like limonin, nomilin, octanol, cineole and naringin that inhibit fungal pathogens, this compounds occur in high concentrations in grape, lemon and orange fruits and are responsible for that bitter taste of the fruits[1,24]. Saponins were found in preliminary studies of the *C. limon* extracts, the presence of saponins may also be responsible for bitter taste and antifungal properties of this plant[25]. The presence of tannins and phenolic compounds in *C. limon* peels indicates that this plant have antimicrobial properties since phenols and phenolic compounds are extensively used in disease prevention and remain a standard with which other bactericides or fungicides are compared [24]. Okwu *et al.* [25] reported high percentage of alkaloids and flavanoids in *C. limon* peels extracts which are in conformity with the preliminary screening of *C. limon* peels extracts in this study. Phenolics forms large group of naturally occurring, diverse and widespread compounds responsible for antifungal or bactericidal properties (Okwu *et al.*, 2007).

The percentage growth inhibition of *F. oxysporum* aqueous and methanol *C. limon* peel extracts was shown in Table 2. The aqueous and methanol extracts at (500mg/ml) showed highest % inhibitory activities with (60% and 76%) respectively, synthetic antifungal component Benomyl had (95.5%) inhibition, low inhibitory activities were recorded at (62.5mg/ml) with (14.8 and 39.9%) aqueous and methanol extracts. The high inhibitory activities of methanol extract could be due to solvent solubility extraction of antifungal moieties and could be linked

Table 1: Phytochemical Analysis of aqueous and methanol peels extracts of *Citrus limon* L.

Plant/Solvent	Saponin	Tannin	Phenol	Alkaloid	Flavonoid
Aqueous (<i>C. limon</i>)	+	+	+	+	-
Methanol (<i>C. limon</i>)	+	+	+	+	+

(+) = presence; (-) = Absence

Table 2: Antifungal activities (% Growth Inhibition) of aqueous and methanol extracts of *F. oxysporum* (*C. limon*)

Concentration (mg/ml)	<i>F. oxysporum</i> (<i>C. limon</i>)	
	Aqueous extract	Methanol extract
500	60.00 ^a ±1.92	76.67 ^b ±1.70
250	44.81 ^c ±1.85	60.74 ^c ±1.96
125	25.56 ^d ±0.64	45.19 ^d ±0.98
62.5	14.81 ^e ±2.06	39.63 ^e ±2.06
Benomyl	95.56 ^a ±0.00	95.56 ^a ±0.00

Values are mean ± standard error of 3 replicates

^{a,b,c}Means in a column with different superscripts are significantly different ($P < 0.05$)

to the fact that *C. limon* has large deposit of alkaloids and phenolic compounds which have antifungal properties [28]. The antimicrobial properties of plants extracts have been largely attributed to the presence of flavanoid and alkaloid compounds [13].

The percentage growth inhibition of *R. stolonifer* aqueous and methanol extract were presented in Table 3. The aqueous and methanol extract at 500mg/ml had highest % percentage inhibition of (71% and 85%) aqueous and methanol extracts respectively. The synthetic fungicide Benomyl had (95.6) inhibition. Relatively moderate inhibition were recorded at (250mg/ml) with (60 and 68%) aqueous and methanol extracts respectively. Lowest inhibitory activities were recorded at (62.5mg/ml) with aqueous extract (16.7%) inhibition.

Citrus plants contain phytochemical compounds such as limolin, nomilin, octanol, cineole and terpenoids that possesses antifungal properties, this components of phytochemical which occur in high concentrations in lemon, grapes and oranges are responsible for bitter taste in this fruits and inhibits the growth of bacteria and fungi [24]. The mechanism of inhibitory actions of phytochemicals such as alkaloids and phenolic compounds on micro organisms may be due to impairments of variety of enzymes systems in micro organisms including those involved in energy production, interference with the integrity of the cell membranes and structural components synthesis (Huang and Chung, 2003).

According to Okwu *et al.* [25] 10% concentrations of peels and leaf extracts of citrus and synthetic Benomyl have inhibited the growth of *F. oxysporum* by (83.55% and 83.04%) respectively, this findings is in agreement with the present study which showed that 500mg/ml lemon peels methanol extracts inhibited the growth of *F. oxysporum* by 76.67% and Benomyl 95.56%. Citrus flavanoid have a large spectrum of biological activities including anti bacterial, antifungal, anti diabetic, anti cancer and anti viral activities [27]. Phenolic compounds are also considered to be bacteriostatic and fungistatic. These compounds caused swelling of hyphal tips, plasma seeping around huphae, cell wall devastation, abnormal branching or fusion of hyphae and consequently wrinkling of hyphal surface [16]. Cowen [11] reported that lipophilic flavanoids may disrupt microbial membrane, Ahonkhai *et al.* [2] reported that volatile oils of Citrus have anti microbial effects against bacteria and fungi. Lemon peels

Table 3: Antifungal activities (% Growth Inhibition) of aqueous and methanol extracts on *R. stolonifer* (*C. limon*)

Concentration (mg/ml)	<i>R. stolonifer</i> (<i>C. limon</i>)	
	Aqueous extract	Methanol extract
500	71.11 ^b ±1.70	85.93 ^b ±0.37
250	60.00 ^c ±1.92	68.52 ^c ±1.61
125	32.96 ^d ±1.34	51.11 ^d ±1.11
62.5	16.67 ^a ±2.31	38.89 ^a ±1.70
Benomyl	95.56 ^a ±1.28	95.56 ^a ±1.28

Values are mean±standard error of 3 replicates

^{a,b,c} Means in a column with different superscripts are significantly different ($P < 0.05$)

have high quantity of saponin with lytic properties against many strain of bacteria and fungi [20].

Plants stores different antifungal, anti bacterial and anti viral phytochemicals on the bark, leaves and exocarp of fruits for protection especially to preserve the fruits and protect the seeds from microbial attack. This is in agreement with the findings of Okwu and Emenike [26] who reported that phytochemicals are reserved in plants to protect the plants against invasion by pathogenic micro organisms. The extracts of peels of *Citrus limon* inhibited the growth of *F. oxysporum* and *R. stolonifer* significantly which is in agreement with the work of Okwu *et al.* [25]. Extracts of *Citrus limon* peels contains antifungal compounds that can be use as Biofungicides. The prospect of using Citrus peels as natural fungicides is encouraging as it is biodegradable, non toxic, environmentally friendly and safe to non target micro and macro flora and fauna, less resistance development in pathogenic pest and above all cheap and readily available. Based on these findings *Citrus limon* peels that are waste by-products, its extracts can be use to control and manage fungal disease of plants as alternative to synthetic fungicides.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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