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Bioactivity of Leaf Extract of *Phyllantus amarus* against Fungal Pathogens Associated with Sweet Orange Rot Disease

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study was carried out to determine disease incidence and control of fungal pathogens associated with rot of sweet orange using *Phyllantus amarus* leaf extract. Forty sweet oranges (*Citrus sinensis* var. Valencia) were collected from the markets and taken to the laboratory. Isolation and identification of the fungi were carried out using standard procedures and the efficacy of the leaf extracts of *Phyllantus amarus* was determined on the isolated fungi. The result of this study showed that four pathogens (*Aspergillus niger, Aspergillus flavus, Penicillum expansum* and *Rhizopus stolonifer*) were identified to be associated with the rot. Rot incidence was upto of 35%. Pathogenicity confirmed the characteristic features similar to the original diseased samples. The effect of the leaf extract of *Phyllantus amarus* on the isolated fungi shows that the rate of inhibition increases with increase in concentration of the *P. amarus* extract, The *P. amarus* extract was effective against *A. flavus* had the highest zone of inhibition (10.667). The inhibition zone was lesser for *R. stolonifer* among the pathogens (8.333). Sweet orange should be consumed after harvest or can be stored in refrigerator for period of 1=2 weeks. The fruit should be discarded if any alteration in color or taste of the fruit is noticed as this can be hazardous to human health.

Keywords: Phyllantus amarus; laboratory; orange; pathogens; fruit.

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1. INTRODUCTION

"Fruits are very important for the wellbeing of human due to their dietary and nutritional qualities. The consumption of fruit and vegetable products has drastically increased by more than 30% during the past few decades" [1]. "They are good source of nutrients for growth, repair and control of body processes as most of them contain sugar, vitamins, mineral elements and small quantities of protein and oil" [2]. "*Citrus sinensis,* known as sweet orange, is the most popular of the citrus fruits. It is widely cultivated in most regions of the world" [3]. "Oranges contain a rich source of vitamin C, flavonoids, phenolic compounds and pectins. The main flavonoids found in citrus species are hesperidine, narirutin, naringin and eriocitrin" [4]. "Just one orange provides 116% of the daily requirement for vitamin C. Vitamin C is the primary water-soluble antioxidant, which prevents free radical generation in the body and damage to the tissues in the aqueous environment both inside and outside cells" [5]. "Drinking of orange juice without salt and sugar is associated with reduced severity of inflammatory conditions, like asthma, osteo-arthritis, and rheumatoid arthritis. Vitamin C is also necessary for the proper functioning of immune system. Vitamin C is good for preventing cold, cough and recurrent ear infections" [6]. Studies have shown that oranges have the following medicinal values such as protect against cardiovascular diseases [5], possess anti-carcinogenic properties, reduce the risk of kidney stones [7], possess anti-ulcer properties, antianxiety effect, anti-typhoid activity [8], antibacterial activity [5] and antifungal activities [9].

"The improper handling, packaging, storage and transportation may result in decay and growth of microorganisms, which become activated because of the changing physiological state of the fruits and vegetables. Fruit, due to their low pH, higher moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which in addition to causing rots, may also make them unfit for consumption due to mycotoxins" [10]. "A single infected orange fruit can be the source of infection to other orange fruits during storage and on transit" [11]. "The infection fungi and bacteria on fruits take place at the time or just before harvesting. Infection may occur also during post-harvest handling or storage. Common air molds such as *Penicillium* species may gain entry into the susceptible tissue and cause spoilage during packaging. *Penicillium digitatum* and *Penicillium italicum* causes green and blue mold diseases respectively which are universal post-harvest diseases of citrus. The extensive spore production nature of these pathogens ensures its spread in field, packing house, equipment, de-greening, storage rooms, transit containers and market place" [12].

Postharvest disease control methods must be ecofriendly, safer for human health, economically viable, and able to extend shelf life. Using antagonistic microorganisms, compounds of natural origin and physical measures are some of the effective alternatives for control of postharvest diseases. Utilization of natural antimicrobial agents could represent a low-cost alternative therapy as microorganisms become resistant to conventional antimicrobial agents. Chemical studies of antimicrobial plant extracts could reveal new substances with potential usefulness as antimicrobial drugs or as models for the development of new drugs. The leaves extract of *Phyllantus amarus* have been reported to have an antimicrobial effects. The aim of the study is to determine the fungal pathogen associated with post-harvest rot of sweet orange in Mubi and evaluation of *P. amarus* against the post harvest pathogens.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples of spoilt Citrus fruit and healthy ones were collected from two (2) markets in Mubi (Mubi main market and Kasuwan Kuturu). The leaves of *Phyllanthus amarus* were obtained from Sabon Gari, Digil ward Mubi North in a sterilized polythene bag and taken to the herbarium unit of the Department of Botany, for identification and authentication.

2.2 Preparation of Culture Medium

Sabourand Dextrose Agar (SDA) was used for the isolation of fungi from the orange fruit and for the preparation of pure culture. The medium was prepared from commercially produced dehydrated medium. 65g of SDA powder was dissolved in 1L of distilled water in a sterile conical flask covered with cotton wool and aluminum foil paper. It was mixed thoroughly and autoclaved at 121^0C for 15minute under a pressure of 15 pounds. The medium was allowed to cool after autoclaving to 50^0C; Chloramphenicol was added to inhibit the growth

of bacteria and was then dispensed aseptically into sterile petri dishes.

2.3 Determination of Disease Incidence

Oranges were collected at random in two markets in Mubi main market and Kasuwan kuturu. The incidence of disease was determined by counting the infected oranges using the following formula;

$$\text{Incidence} = \frac{Total\ number\ of\ diseased\ plants}{Total\ number\ of\ plants\ observed} \times 100$$

2.3.1 Isolation and identification of pathogen

The sliced *Citrus cinensis* fruits prepared were placed on Sabourand Dextrose Agar (SDA) aseptically and incubated at 28^{o}c for five days. A pure culture was obtained and maintained by sub-culturing each of the different colonies that emerged onto the SDA plates and incubated at 28^{0}C for five days. As a control, healthy fruits were sterilized with 75% ethanol. The fruits were cut in segments with a sterile blade, placed on SDA and incubated at 28^{0}C for five days. The fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology and pigmentation as described by [13].The technique of [14] was used for the identification of the isolated fungi with the use of safranin stain.

2.3.2 Pathogenicity test

Pathogenicity test was carried out in order to ascertain whether the isolated fungi were really responsible for the spoilage of citrus fruits. Healthy fruits were sterilized with ethanol cylindrical plug tissues were cut out from the fruits using a sterilized 2 mm sized cork borer. Agar plate containing a weak old fungal culture was aseptically placed in the holes; it was covered and sealed by means of petroleum jelly. The procedures were repeated separately across each of the fungal isolates. The inoculated samples and the control were placed in sterile polythene bags and were incubated in an oven for 5 days. The point of inoculation of each type of fungus were examined and recorded. The diameter of the rotten portion of the orange was measured. The fungi were then re-isolated from the inoculated fruits and compared with the initial isolates.

2.3.3 Preparation of leaf extract

The preparation of the leaf extract was carried out by the procedures described by [15]. Dried leaves of *Phyllanthus amarus* were grinded into fine powder and sieved. The powder was dissolved in the solvent (ethanol) and shaken vigorously for 5-10 minutes and left for 24 hours after which the extract was filtered and dried under reduced pressure. The extract was prepared into four different concentrations ranging 50, 100, 200 and 400 mg/ml. The extract concentration was prepared by weighing 3.4g of the extract into 3 ml of ethanol; a doubling dilution of the diluted extract was carried out into four different labeled bottles to obtain the concentrations.

2.3.4 Susceptibility testing

This was carried out using agar well diffusion method. The standardized organisms were dropped on the freshly prepared SDA. Four wells were made on the inoculated SDA plate using a sterile cork borer of 3 mm. The wells were properly labeled according to the different concentrations of the extract prepared. The punched wells were filled with 0.2 ml of each of the extract (50, 100, 200 and 400 mg/ml), the plate were then allowed to stay on the laboratory table for one hour for the extract to diffuse into the agar after which they were incubated at 28^{o}C for 24 hours. The plates were observed for any evidence of inhibition which appeared as clear zones that was completely devoid of growth around the wells. The diameter of the clear zones was measured with a transparent ruler calibrated in millimeter (mm). Ketoconazole was used as positive control.

2.4 Experimental Design /Data Analysis

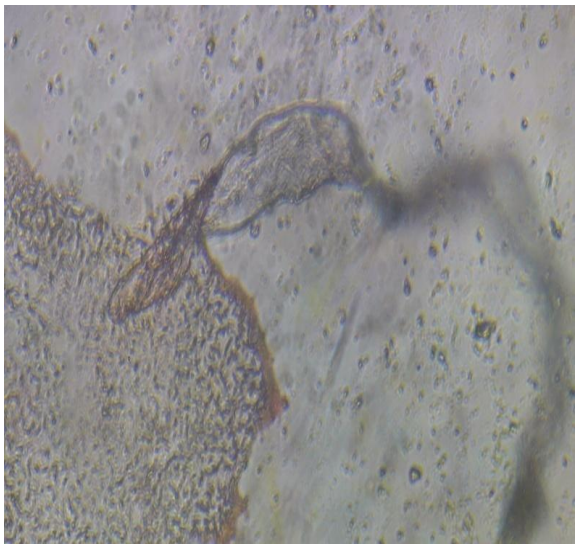
The treatments consisted of four concentrations (50, 100, 200 and 400 mg/ml) of *Phyllanthus amarus* leaf extracts replicated three times and laid out in a Completely Randomised Design (CRD). Data obtained from the study were subjected to one-way Analysis of Variance (ANOVA) using MINITAB Computer software programme. Means were separated using Duncan's Multiple Range Test (DMRT) at $p < 0.05$.

3. RESULTS

3.1 Isolation and Identification of Pathogens

Four fungal pathogens (*Aspergillus niger, Aspergillus flavus, Penicillum expansum* and *Rhizopus stolonifer*) were isolated and confirm through pathogenicity test to be responsible sweet orange fruit rot in the markets (plate i-iv).

Plate Ia: Pure culture of *A. flavus*



PlateIb: Photomicrograph of *A. flavus*

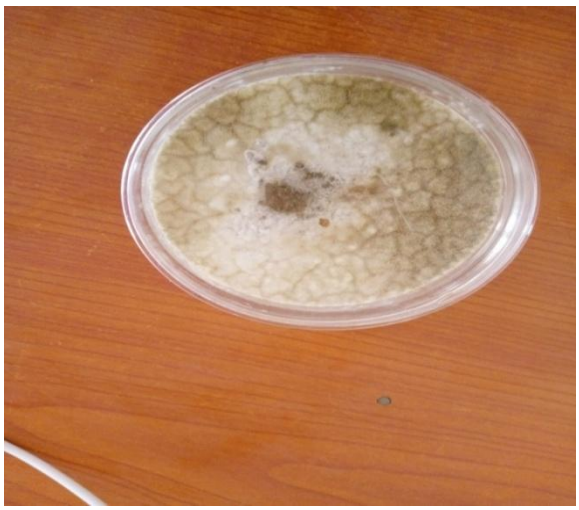


Plate IIa: Pure culture of *P. expansum*

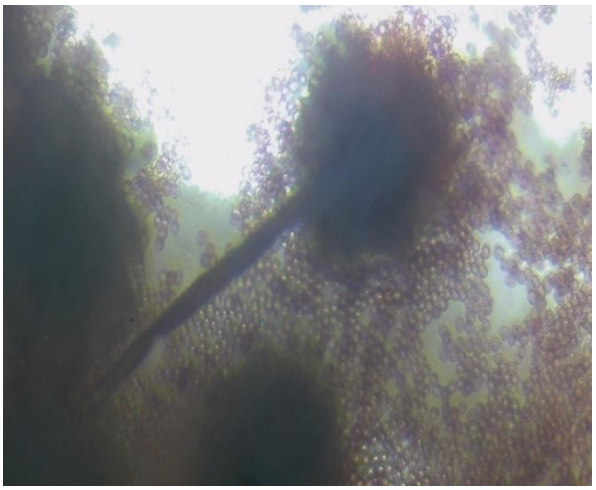


Plate IIb: Photomicrograph of *P. expansum*

Plate IIIa: Pure culture of *A. niger*

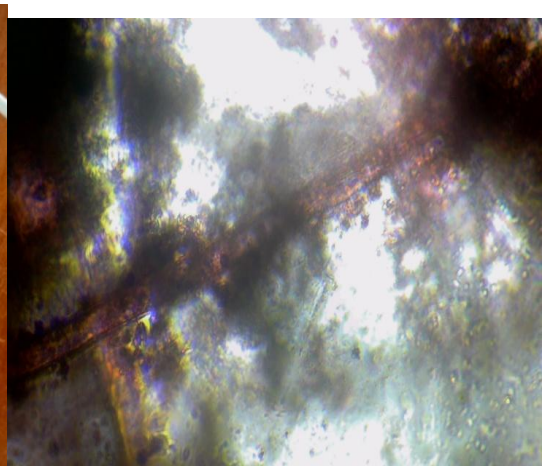


Plate IIIb: Photomicrograph of *A. niger*

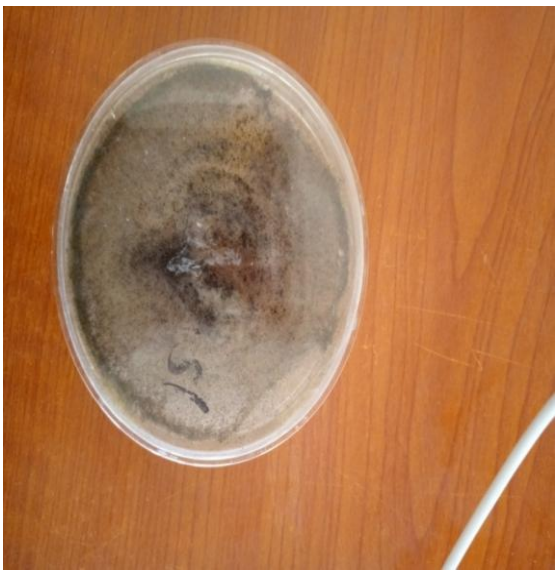


Plate IVa: Pure culture of *R. stonolifer*

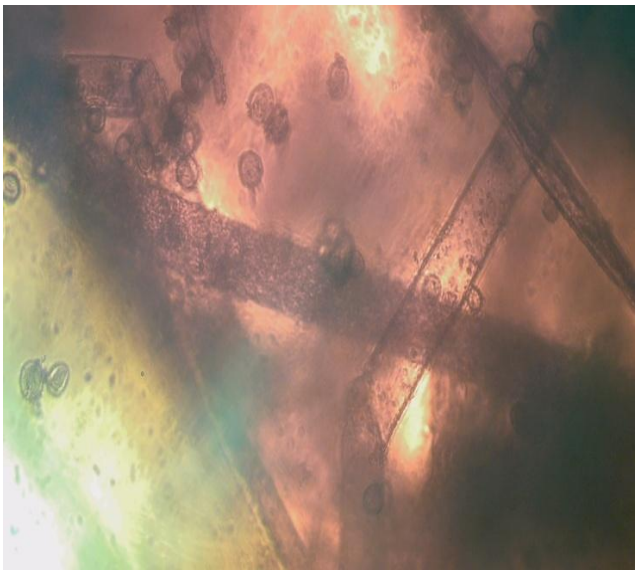


Plate IVb: Photomicrograph of *R. stonolifer*

3.2 Disease Incidence

The percentage (%) incidence of pathogens in the two markets surveyed shows that rot occurred in both market, whereby Mubi main market had the highest incidence with 35% and kasuwa kuturu with 25% (Table 1) respectively.

3.3 Pathogenicity Test

The pathogenicity test revealed that the pathogen isolated from the infected oranges were pathogenic (Table 2).The rate of decay caused by the isolated fungi *A. flavus* and *A. niger* to be most pathogenic with rapid disintegration of the healthy fruits. The pathogens having rot diameter of 53 mm and 45 mm respectively and shows similar growth characteristic features to the original diseased samples.

3.4 Effect of Leaf Extract on the Isolated Fungi

The effect of the leaf extract of Ph*yllantus amarus* on the isolated fungi is given below (Table 3). There was a significant inhibition of growth of pathogens treated with *P*. *amarus* leaf extract. The inhibition diameter was best in *A. flavus* at the highest concentration (400 mg/ml). The extract shows no inhibition at the least concentrations (50 mg/ml) in all the isolated fungi. There was significant difference between the control and all other concentrations at probability level of $p< 0.05$.

4. DISCUSSION

"Fruits and vegetables are very important and have high dietary and nutritional qualities. Consumption of fruit and vegetable products has dramatically increased by more than 30% during the past few decades" [1]. However, microorganisms have been causing serious threats to agricultural produce. This study revealed the natural incidence of *A. niger, A. flavus, P. expansum and R. stonolifer* sweet oranges in markets and their pathogencity was confirmed on *C. sinensis* (sweet orange) fruits sold in Mubi. This result agrees with the work of [16] where they reported *Aspergillus* spp, *Mucor* spp, *R. stonolifer* to be associated with rotten *Citrus* spp. It also corresponds with the work of [17,18] who also reported these pathogens to be causative agents of orange fruits rot. [19] also found out that *Aspergillus* was the common genus affecting different types of fruits, it appeared on 86.6% of orange fruits where *Aspergillus niger* and *Aspergillus flavus* were the dominant on the tested fruits (Apple, Orange, Mango and Grape). Nasiru *et al.* [16] in their work reported fungal pathogens responsible for the spoilage of orange include *Aspergillus niger, Aspergillus flavus, Aspergillus fumigates, Aspergillus terrus,Mucor heimalis, Mucor racemosis, Absidia, Carmbifera, Rhizophus stolonifer* and *Fusarium oxysporum* are associated with *Citrus* species.

Table 1. Occurrence and Incidence of Fungal Pathogens

Fungal isolates	Presence of isolates in each market	
	Mubi main market	Kasuwan kuturu
Aspergillus niger	+	+
Aspergillus flavus	+	-
Penicillum expansum	+	-
Rhizopus stolonifer	-	+
Incidence %	35	25

Key: + = Present, - = Absent

Table 2. Pathogenicity test

Test Organisms	Diameter of ROT/ (mm)
A. Niger	45.00
A. flavus	53.00
P. exapansum	21.00
R. stolonifer	15.00

Table 3. Effect of Leaf Extract of *Phyllantus amarus* on the Isolated Fungi

	Diameter of Zone of Inhibition (mm)			
(mg/ml)	***A. niger***	***A. flavus***	***P. expansum***	***R. stolonifer***
400	9.000^{d}	10.667^{c}	9.333^{c}	8.333^{c}
200	6.667^{c}	9.333^{c}	8.333^{c}	7.333^{c}
100	4.000^{b}	5.000^{b}	5.000^{b}	4.000^{b}
50	0.00^{a}	0.000^{a}	0.000^{a}	0.000^{a}
Control	11.667^{e}	11.333^{d}	11.333^{d}	11.667^{d}

NB:Means with different superscript along the column are statistically significantly different at p<0.05. SE± = Standard Error

The percentage (%) incidence of pathogens in the two markets surveyed, showed that rot occurred in both markets, main market had the highest incidence with 35% and kasuwa kuturu with 25% whereas [20] reported kasuwa kuturu having highest with 58.3% and central market the least 33.3% this might be either due to the method of sample collection, weather and also duration in which the research was carried out.

The efficacy of the ethanolic leaf extract of *Phyllantus amarus* on the isolated fungi shows that the rate of inhibition increases with increase in concentration of the extract. Ilondu et al. [21] reported that the leaf extract of *P. amarus* was effective on the growth inhibition of some fungal pathogens including *Aspergillus niger* and *A.flavus*. Senjobi et al. [22] also reported that "the leaf extract of *P. amarus* had a broad-spectrum activity against gram negative and gram positive bacteria as well as fungal species".

5. CONCLUSION

It can be concluded that *A. niger, A. flavus, P. expansum* and *R. stolonifer* are pathogens causing rots in *Citrus* fruits. However, incidence of rot varies in the two markets. Ethanolic leaf extract of *P. amarus* was found to be effective in inhibiting the growth of the pathogen. There was increase in inhibition of the pathogen with the increase in concentration of the extract.

6. RECOMMENDATION

Fruits and vegetables in general are known to be perishable and have a relatively short shelf-life causing them to deteriorate rapidly. Therefore, sweet orange should be consumed shortly after harvesting or properly refrigerated (or stored using other means) and should be discarded if any alteration in color or taste of the fruit is

noticed as this can be hazardous to human health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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