

Antibacterial Effects of Aqueous Ethanolic Leaf Extracts of *Ocimum gratissimum*, *Cymbopogon citratus*, *Vernonia amygdalina* and *Annona muricata* on the Isolated Pathogenic Organism of Citrus Canker from *Citrus sinensis* (Sweet Orange)

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Abstract

The aqueous ethanolic extracts of *Vernonia amygdalina*, *Ocimum gratissimum*, *Cymbopogon citratus* and *Annona muricata* were screened for antibacterial activities at 200mg/ml concentration. Antibacterial efficacy of extracts against the bacteria were identified by gram staining techniques, coagulase and catalase tests and the bacterial isolate was indicated by the appearance of clear zones of inhibition around the disks. The extracts of *Vernonia amygdalina* and *Ocimum gratissimum* showed inhibitory activities against the bacterial isolate with zones of inhibition of 12mm and 8.5mm respectively while extracts of *C.citratus* and *A.muricata* showed no zone of inhibition. Result of the antimicrobial activity of the extracts compared effectively with the activity of standard fumigant with extract of *V. amygdalina* showing the highest potency which was used in preparation of a local fumigant. The minimum inhibitory concentration of *V.amygdalina* and *O.gratissimum* were 25mg/ml and 50mg/ml respectively which were not determined in *C.citratus* and *A.muricata*. In conclusion, the results of this investigation showed that the aqueous ethanolic leaf extracts of *V. amygdalina* and *O. gratissimum* can be used as an effective control measure for the elimination of citrus canker of *Citrus sinensis*.

Keywords: Citrus canker; Gram staining; Biochemical tests; Zone of inhibition; Antibacterial activity.



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1. Introduction

Citrus canker, caused by *Xanthomonas axonopodis* sp. *citri* (XC) (synonym *X. campestris* sp. *citri*), is a serious disease reducing the external quality of citrus fruits. It affects all types of citrus and severely infects on *Citrus sinensis* (sweet orange).

Canker occurs in all areas where sweet orange grows. Typical symptom on leaves is a raised necrotic lesions surrounding with yellow halo but on fruit and stem halo seldom occurs. Control of the disease requires integrated cultural practices and chemical sprays. Copper compound products are recommended for canker control. One major limitation of using chemical control agents is that phytopathogenic bacteria frequently develop a resistance to these compounds [1].

In recent years, much interest has been developed in the antimicrobial effects of medicinal plants for plant disease control. Some plant extracts were reported as effective inhibitors of phytopathogenic bacterial growth and *X. axonopodis* was also suppressed by plant extracts [2].

There is growing interest in exploiting plants for medicinal purposes especially in Africa, as microorganisms are developing resistance to many drugs thereby creating situations where some of the common and less expensive antimicrobial agents are losing effectiveness [3]. Herbal medicine which uses medicinal plants primarily presents as an alternative to such situation [4]. These medicinal plants have immensely contributed to the development of human health and welfare. Concomitantly, there is an increase in data and huge patronage to herbal products round the world [5]. Medicinal plants such as *Ocimum gratissimum*, *Cymbopogon citratus*, *Vernonia amygdalina* and *Annona muricata* have been asserted to provide various culinary and medicinal properties. These medicinal properties exert bacteriostatic and bacteriocidal effects on some bacteria. These effects have been attributed to the peptides, alkaloids, essential oils, phenols and flavonols which are major components in those plants mentioned above [6].

Citrus sinensis is widely grown in Nigeria and many other tropical and subtropical regions [7]. In terms of volume in production, citrus ranks after banana as the world second fruit crop with more than 108 million tons [8]. Sweet orange (*Citrus sinensis* L. Osbeck) commonly called orange is a member of this family and a major source of vitamins, especially vitamin C, sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium [9].

Economically, sweet oranges are important fruit crops, with an estimated 60 million metric tonnes produced worldwide as at 2005 for a total value of 9 billion dollars. Of this total, half came from Brazil and the United States of America [10].

Sweet orange originated from south East Asia, but is consumed all over the world as an excellent source of vitamin C, a powerful natural antioxidant that builds the body immune system. Important phytochemicals like limonoids, synephrine, hesperidin flavonoid, polyphenols, pectin, and sufficient amount of folacin, calcium, potassium, thiamine, niacin and magnesium are also present. These biologically active compounds prevent arteriosclerosis, cancer, kidney stones, stomach ulcers and reduce cholesterol level and high blood pressure, thereby promoting human health. These diseases can be controlled through chemical treatment of fruits, use of biological control agents, proper packaging and storage facilities and other disease management practices to reduce postharvest damage.

2. Material and Methods

2.1. Sources of Plant Samples

The infected sweet oranges were collected at Abia community in udi Local Government Area of Enugu State and the Fresh samples of *Ocimum gratissimum*, and *Annona muricata* leaves were collected from a bush at Umuagu Village in Nnobi community, Idemili South local Government Area while *Cymbopogon citratus*, and *Vernonia amygdalina* leaves were collected from a bush at Umuegbu village in Nnobi community, Idemili South Local Government Area in Anambra State between the hours of 6-8 am at a prevailing temperature of about 28±2oC. All the collections were done in the month of May.

The plant leaves and the infected sweet oranges were identified and authenticated by Mr. Nwatu, the taxonomist at Botany Department of Biological Sciences Faculty, Nnamdi Azikiwe University, Awka, Anambra State.

2.2. Extraction of Samples

The leaves were collected in bulk, washed under running tap water to remove dirt and rinsed with distilled water. The harvested leaves were air dried for fourteen days in the Applied Biochemistry laboratory and the leaves pulverized in a mechanical grinder.

The powdered leaves (50g) were extracted with 250 ml of 70% ethanol which were done by soaking the powdered leaves in 70% ethanol for 32hours, and then filtered by first , using a clean muslin cloth and then, No. 1 Whatman filter paper. The filtrates were concentrated to dryness.

2.3. Isolation of Micro-Organism from the Infected Sweet Orange

Procedures: The infected part was cleaned with 70% ethanol to disinfect the infected part. The infected part was excised using an ethanol-cleaned knife and cut into tiny bits with a spatula. The fragmented sample (0.1g) was placed in a test tube containing 10ml of distilled water and serially diluted using ten-fold dilution for 10,000 times (serially diluted for four times).

A quantity (1ml) of 10,000 times diluted test organism was transferred to the various prepared agar medium and spread evenly using spread plate method described by Madigan, *et al.* [11].

The petri-dishes were incubated at 37°C for 16 – 24hours for growth.

2.4. Morphological Characterization

2.4.1. Gram Staining Technique

Procedures: The test organism was heat-fixed (a suspension of the test organism was passed over a flame from a Bunsen burner) on a slide and the slide flooded with 1% aqueous solution of crystal violet and allowed to stand for a minute. The crystal violet stain was poured off, washed with water and the slide flooded with gram Iodine solution and allowed a contact time of a minute. The slide was gently rinsed with water from a squirt bottle and blotted by air-drying to remove excess water without allowing smear to become dry.

The slide was discolored with ethanol, washed, air dried and counterstained using 2% solution of safranin (dye), which was then washed briefly with water, blotted (air dried) and viewed with a light microscope. Oil immersion lens was used (x100).

2.5. Biochemical Tests

2.5.1. Slide coagulase test

2.5.1.1. Principle

Coagulase test is used to differentiate *Staphylococcus aureus* (positive) from coagulase negative *Staphylococcus* (CONS). Coagulase is an enzyme produced by *S.aureus* that converts (soluble) fibrinogen in plasma to (insoluble) fibrin.

Procedures: The slide was divided into two sections with grease pencil, one labeled as “test” and other as “control”. A small drop of distilled water was placed on each area. A colony of the test organism was emulsified on each drop (placed on the water) to make a smooth suspension. The test suspension was treated with a drop of plasma (from human blood) and mixed well with a loop and observed for clumping. The control suspension serves to rule out false positivity due to agglutination.

2.6. Catalase Test (Slide Method)

2.6.1. Principle

Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize toxic forms of oxygen metabolites; H₂O₂. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them by mediating the breakdown of hydrogen peroxide into oxygen and water.

Procedures: Some colony of the test organism was gently transferred onto a slide using a sterile wire loop and 4 – 5 drops (200µl) of a 3% H₂O₂ added to it. The slide was covered against a dark background and observed for effervescence.

2.7. Plasma Preparation

Procedures: Human blood (4ml) was added in a centrifuge tube containing 0.8ml of Ethylene diaminetetraacetate (EDTA). It was centrifuged for 15minutes at 1000 – 2000xg using an electric centrifuge. The resulting supernatant (plasma) was transferred into a clean tube using a Pasteur pipette.

2.7.1. Determination of Antibacterial Activities of Plant Extracts Using Disk Diffusion Method

Procedures: Disk of 6mm in diameter which was cut out from Whatman No.1 filter paper, was impregnated in various concentrations of each plant extract, dried in an oven (at 55°C) under an aseptic condition.

The Nutrient agar medium was prepared and kept to gel. Nutrient broth (5ml) was placed in a test tube and labeled. Suspension of test organism was made in the nutrient broth with reference to 0.5M McFarland turbidity. A swab was dipped in the nutrient broth containing the test organism and streaked uniformly on the prepared solid agar. Disks containing various fixed concentration (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml) were placed on the center of the plate with the growing organism and incubated at 37°C for 16 – 24 hours. Diameters of zones of inhibition were measured in millimeters.

2.7.2. Determination of Minimum Inhibitory Concentration (mic)

This was carried out using disk diffusion method as described by [Olutiola, et al. \[12\]](#).

Procedures: Different concentrations (200mg/ml, 100mg/ml, 50mg/ml, 25mg/ml) of the extracts were used. Plates were incubated for growth at 37°C for 24hours, after which they were observed for clear zones around the disk, indicating inhibition. The concentration above that in which there is no inhibition was noted as minimum inhibitory concentration (MIC).

2.7.3. Standard Fumigant Sensitivity Assay

Procedures: An 18 – 24hrs old culture of the test organism on nutrient agar was uniformly smeared on a solid-fixed 20ml nutrient agar.

This was carried out using disk diffusion method. Disk impregnated in 200mg/ml of standard fumigant, 2,3-dichlorovinyl diphosphate, DDVP was placed on a nutrient agar containing the test organism and allowed to stand on the bench for 1hr for proper diffusion of the standard fumigant and thereafter, incubated at 37°C for 24hours.

The resulting diameter of inhibition zones were measured in (mm).

2.7.4. Umigant Preparation Using Vernoniaamygdalinaethanolic Leaf Extracts

The powdered *V. amygdalina* (240g) was soaked in 1 litre of 70% ethanol for 32hours. The extracts were first filtered using a clean muslin cloth, and then, filtered using a No.1 Whatman filter paper. The filtrate was concentrated to dryness in a waterbath (at 80°C)

About 1gm of the dry extract was dissolved in 200ml of propan-2-ol, giving a concentration of 80mg/ml which was packaged in a fumigant bottle, ready for spraying.

2.7.5. Test for Toxicity of the Local Fumigant on Plant Leaves

The prepared fumigant was sprayed on leaves (fronds) of Coconut and Orange. After spraying, the fumigant stuck on their leaves. Their leaves were observed for seven days for effects such as leaf folding and change of colour.

3. Results and Discussion

This study was conducted to isolate, identify and characterize the organism in an infected sweet orange (*C. sinensis*) and to determine the sensitivity of the organism to the aqueous ethanolic leaf extracts *V. amygdalina*, *C. citratus*, *A. muricata*, and *O. gratissimum*, and commercial fumigant.

Isolation, identification and characterization of the organism from an infected sweet orange, showed that a rod-like gram-negative organism which was positive to catalase test (it can breakdown hydrogen peroxide into oxygen and water), negative to slide coagulase test (it cannot convert fibrinogen in plasma into fibrin) is responsible for the disease of the sweet orange (Table 1). The organism was identified as *Xanthomonas axonopodis*. The above observation was in accordance to that of Islam, *et al.* [13].

Table-1. Result showing biochemical tests of microorganism isolated from infected sweet orange

S/N	TEST	RUSLTS	INFERENCE
1	Catalase	+ve	Can break down hydrogen peroxide into water and oxugen.
2	Slide coagulase	- ve	Cannot convert fibrinogen in plasma into fibrin

Table-2. Results of Diameter of zones of inhibition (in mm) at different concentrations

Concentration of Extracts (mg/ml)	<i>V. amygdalina</i>	<i>C.citrates</i>	<i>O.gratissiman</i>	<i>A.muricata</i>
200	12	-	8.5	-
100	12	-	7	-
50	10	-	7	-
25	8	-	-	-

Key (-): No zone of inhibition

3.1. Result for Antimicrobial Sensitivity Test

Table-3. Diameter of Zone of inhibition (mm) at Concentration of 200mg/ml

Sources of lant extracts in hibitor	<i>V. amygdalina</i>	<i>C.citrates</i>	<i>O.gratissiman</i>	<i>A.muricata</i>	2,3 Dichlorovinylidihoshate (DDV)
Zones of inhibition	12mm	-	8.5mm	-	14mm

Key: - = No zone of inhibition

The leaf extracts used in this study showed that only *V.amygdalina* and *O.gratissimum* inhibited the organism (Table 2). The ethanolic extract of *V. amygdalina* inhibited the organism with a zone of inhibition of 12mm. However, *O. gratissimum* extracts recorded a zone of inhibition of 8.5mm.

The outcome of this study has shown that leaf extracts of *V. amygdalina* and *O. gratissimum* possess inhibitory potentials against the organism with the leaf extracts of *V. amygdalin* showing the highest potency while the leaf extracts of *C. citratus* and *A. muricata* exhibited no antagonism against the test organism (Table 2).

Table-4. Results for minimum inhibitory concentration of plant extracts

Plant Extracts	<i>V. amygdalina</i>	<i>C.citrates</i>	<i>O.gratissiman</i>	<i>A.muricata</i>
MIC	25mg/ml	ND	50 mg/ml	ND

Key: ND = Not Determined

: MIC =Minimum Inhibitory Concentration

Antimicrobial activities in plants have been reported to be as a result of bioactive components present in the plants, such as alkaloids, saponins, tannins, flavonoids, steroids etc [14]. The minimum inhibitory concentration (MIC) values of the extracts were 25mg/ml to 50mg/m for *V. amygdalina* and *O. gratissimum* respectively while they were not determined for both *C. tratus* and *A. muricata* (Table4). This shows that the organism was more sensitive to *V. amygdalina* than *O. gratissimum* and also that *V. amygdalina* was more potent in inhibiting *Xanthomonas axonopodis* than *O. gratissimum*.

The extracts of *V. amygdalina* compared favourably with commercial fumigant, 2, 3-dichlorovinyl diphosphate (DDVP), in its bacterial inhibitory potentials. This was seen as recorded in Table 3. This result is a pointer to the fact that, if the crude extracts were subjected to purification, the active components could record same or a higher zone of inhibition than the commercial fumigant. Similarly, combinations of extracts from different plants could produce greater inhibition of microbial growth through synergy than the commercial fumigant, DDVP. It is also possible that some impurities could have lowered the potency of the crude extracts, which when removed may exhibit higher potency.

3.2. Results for Toxicity Test

The sprayed coconut leaves (fronds), guava leaves and Orange leaves incurred no harm as no leaf color change or leaf folding was seen. This shows that the prepared fumigant might not be toxic to the plants.

4. Conclusion

The ethanolic leaf extract of *Vernonia amygdalina* can be used as an effective measure for controlling plant diseases like citrus canker or in combination with *Ocimum gratissimum* for synergism. These two plants are edible and also cannot deposit toxins in plants, agro-products and the environment when utilized as commercial fumigants contrary to synthetic chemical fumigants available in the markets and have good potentials of being cultivated in larger proportions for possible application in the fumigation industry as biological fumigants.

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