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THE ANTIBACTERIAL ACTIVITY OF CRUDE LEAF EXTRACT OF *CITRUS* SINENSIS (SWEET ORANGE)

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ABSTRACT

The antibacterial activity of *Citrus sinensis* was evaluated on bacteria strains like *Escherichia coli, Pseudomonas aeruginosa , Klebsiella pneumoniae, and Staphylococcus aureus.* Water and ethanol were used for the extraction. The in vitro antibacterial activity was performed by agar disc diffusion method. The antibacterial effect of aqueous extract showed a zone of inhibition on *Escherichia coli,* which was seven millimeter in diameter, while on the other organisms it showed little or no zones of inhibition ranging from 0-3mm in diameter. The ethanol extract also showed little zones of inhibition on the test organisms ranging from 1-3mm in diameter. The minimum inhibitory concentration (MIC) evaluated on the ethanol and aqueous extracts using a two fold serial dilution showed no zones of inhibition .The results obtained in this study suggested that the extracts have low potential for use in the treatment of diseases caused by these test organisms.

KEYWORDS

aqueous extract, ethanol extract, zone of inhibition, disc diffusion method, test organisms

INTRODUCTION

Infectious diseases are the leading cause of death world wide. Antibiotic resistance has become a global concern (Westh *et al* .,2004).The clinical efficiency of many antibiotics in existence is being treated by the emergence of multi drug-resistant pathogen (Bandow *et al .,(*2003).Throughout the history of mankind, many infectious diseases have been known to be treated with herbal remedies .The natural herbal products either as pure compounds or as standardized plant extracts provided unlimited opportunities for new drug leads because of the un compared availability of diversities of chemical. This results to a never ending and urgent need to discover new antimicrobial compounds with different chemical structure and new mechanisms of action for re-emerging and new infectious diseases (Rojas *et al* .,2003)Therefore, researchers are increasingly turning their attention to folk medicine. Continuous search leads into developing better drugs against microbial infections (Benkeblia,2004).Several medicinal plants are being screened for their potential microbial activity based on increasing failure of chemotherapy and antibiotic resistance

exhibited by pathogenic agents (Colobo and Bosisio,1996; Iwu *et al*.,1996).The use of plants for medicinal purposes can be traced back to earlier civilization (Le Strange,1977).Medicinal plants have a global distribution although they are most abundant in the tropics (Calixto,2000)

The practice of herbal medicine in Nigeria is becoming so intense due to the fact that health officials and other personnel are coming to realize the potencies and efficacies of some of the indigenous plants .Some of the diseases that have been successfully managed traditionally include malaria, infantile convulsion, diarrhoea , dysentery, gonorrhoea, epilepsy, tonsilitis, fungal infections, mental illnesses and worm infections (Soforowa,1996).Undoubtedly, biodiversity studies still reveal that the plant kingdom has not been exhausted based on the species of medicinal plants which are yet to be discovered.

Some plants commonly used include shrubs, herbs, spices and trees for example, *Garcina biflavovone* has been found to be active against a wide variety of microorganisms such as *Escherichia coli, Staphylococcus aureus* (Iwu, 1993).*Citrus sinensis* belongs to a family Rutaceae. It's common name is sweet orange and in Igbo it is called "ape uta". In Latin American areas, the fruit is called "naranja de China". *Citrus sinensis* is widely distributed in the tropics. The fruit originated in Southern China, North Eastern India and Southern Asia formerly Indochina. It is a perennial tree. The entire part of the tree is useful medically.

Citrus sinensis has been found to be a valuable source of essential oil. The components include bioflavonoids, carbohydrates, terpernoids (Morton, 1987).Orange peel oil has lethal effect on fleas, fireants, houseflies due to its 90-95% limonene. Oranges are eaten to allay fever. The roasted pulp is prepared as poultice for skin diseases. The fresh peel is rubbed on acne. An infusion of the immature fruit is taken to relieve stomach and intestinal complaints. Orange flower water made in Italy and France as a cologne, is bitter and considered antispasmodic and sedative. A decoction of the dried leaves and flower is given in Italy as an antispasmodic, cardiac sedative and antiemetic. The inner bark macerated and infused in wine is taken as a tonic. A decoction of husked orange seeds is prescribed for urinary ailments in China. Orange peel is medically used against fungi (Strange *et al*.,1993).In Ecuado, the orange seed extract is given as a treatment for malaria. Although it is known to cause respiratory depression. In the Southeastern part of Nigeria, *Citrus sinensis* is used to manage malaria as well as skin diseases (Morton, 1987).

Based on history, plants have provided a good source of anti-infectious agents such as berberine, quinine, emetine which remain effective tools in the war against microbial infections. Plants that contain protoberberines, oligosaccharides, saponin, flavonoid, alkaloid, and phylates are used in the African traditional system and have been found to be active against a wide variety of microorganisms (Edeoga *et al*.,2005)

In as much as the period where medicinal plants were being used has been long, the infectious diseases are as old as the medicinal plants (Azoro,2002).the primary benefit of the utilization of plant derived medicine is that they are relatively safer than synthetic alternatives, offering very good therapeutic benefit and affordable treatment (Robbers *et al*.,1996).

Since time immemorial, herbs, trees and shrubs have served humans in many ways such as drugs, foods and flavours. This research is therefore aimed at the verification of the antibacterial activity of the leaves of *Citrus sinensis*, on some enteric and non-enteric organisms such as *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus* in vitro so as to provide a guide or direction on the concentration of plant extracts to the populace who utilize them to treat various diseases caused by these bacterial pathogens and also to prevent the side effects that would be associated with high doses by ascertaining if

the extracts would effect a clear zone of inhibition in vitro.

MATERIALS AND METHODS

Collection and Identification of Plant material

The plant material that was used in this work was freshly harvested leaves of *Citrus sinensis* obtained from Umuoru, Obi-Ngwa LGA, Abia State, Nigeria. Prof. H.O .Edeoga (Taxonomist) of the Department of Biological Sciences, Michael Okpara University of Agriculture, Umudike taxonomically authenticated the plant and voucher samples were deposited in the Departmental herbarium.

Preparation of the Plant Extract

The freshly collected plants were rinsed in water and were oven dried using a moisture extraction oven at 65^{o}C and the drying lasted for 36hours.The dried leaves were pulverized into powder using Thomas Wiley mill model E.D.5 (Oyagede *et al* .,1993) from Soil Science Laboratory, National Root Crops Research Institute (NRCRI).

Ethanol Extract Preparation

Ten grams of the pulverized leaves was weighed using Satoric AG Gottingen Electronic weighing balance. The weighed sample was soaked in 100mls of ethanol contained in a conical flask. The mixture was swirled. After 24hours elaption with interval stirring, the mixture was filtered using Whatman no.1 filter paper (Azoro,2002) into a clean beaker, the ethanol was recovered using a soxhlet apparatus and it was finally evaporated to dryness using a steam bath at 100^{o}C.

Aqueous Extract Preparation

Ten grams of the pulverized leaves was weighed and macerated in 100mls of distilled water. The mixtures were vigorously swirled. After the elaption of 24hours with interval stirring, the mixture was filtered using Whatman No.1 filter paper (Azoro,2002) into a clean beaker, and the filterate was concentrated to dryness by evaporation using the steam bath at 100^{o}C.

The filtrate had the following colour after filtration

Ethanol Extract was dark brown

Aqueous Extract was dark green

After evaporation the extracts were recovered and weighed.

Aqueous extract 1.6g

Ethanol extract 1.6g

The extracts were stored in the refrigerator at 8^{o}C for use. The yield was recovered as percentage of the quantity of initial plant material (10.0g) used.

$$(\%)\ \text{yield} = \frac{\text{yield in (g) x 100}}{\text{10.0g}}$$

Test for Purity and Sterilization of Materials

The dried extract was exposed to ultraviolet rays for 24hours and checked for sterility by streaking on a freshly prepared sterile nutrient agar which was incubated for 24hours at 37^{o}C(Baker and Pallister,1998).The materials used were sterilized. All the materials were washed with detergent and rinsed with water, they were drain dried, and the glasswares were foiled and carefully packaged into the autoclave for sterilization at 121^{o}C,115 atmospheric pressure for 15 minutes (Cheesbrough, 2000).

Bacterial Species Confirmation

Clinical strains of microorganisms used were *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa and Klebsiella pneumoniae* which were obtained from the Microbiology laboratory of Federal medical Centre(FMC),Umuahia, Abia State, Nigeria.

The test organisms were further identified and the biochemical and morphological characteristics were confirmed by standard methods (Cheesbrough, 2000).*Escherichia coli* was subcultured into MacConkey agar slant while the other organisms were subcultured into nutrient agar slant (Baker and

Palister,1998).All the microorganisms were maintained at 4°C on their respective slants.

Preparation of Plant Extract Concentration

The aqueous and ethanol crude extracts were reconstituted by weighing 0.2g of each and dissolving in 2mls of distilled water and 2mls of 50% dimethyl sulphoxide of 100mg/ml

Disc Diffusion Assay

The disc diffusion method reported by Baker and Pallister (1998) was adopted by the determination of the antibacterial activity of extracts. Whatman No.1 filter paper was used. The filter paper was cut into circular disc using a perforator giving a diameter of 6mm.The disc was treated by boiling for 30minutes so as to denature and destroy completely the entire chemical used in its preservation and also to prevent the inactivation of the extract when imbedded into the discs. After it has boiled, the disc was transferred into a glass Petri dish and kept in the oven until it became dry. After drying it was stored in a 121°C and at 115 atmospheric temperatures. It was stored for

Media Preparation and Antibacterial Activity

Muella Hinton agar was prepared by weighing 39grams of the powdered agar into 1000mls of distilled water in a clean conical flask. It was swirled until it became a mixture. It was then covered with a foil and was autoclaved at 121°C, 115 atmospheric pressure for 15 minutes. The medium was cooled at 47°C and 20ml of the molten medium was poured into a sterile glass petri dish and allowed to solidify. The sterility of the medium was tested by incubation for 8hours looking out for contaminants (Baker *et al* .,1985).A sterile wire loop was used to pick a colony of the test organism and placed into 2mls of peptone water. a sterile swab stick was dipped into the test tube containing the organisms and it was used to seed the organism on the solidified Muella Hinton agar in an inoculating chamber already set aseptically .The prepared disc was carefully transferred onto the inoculated culture plates using sterile forceps. The placed disc included 100mg/ml (DMSO) and gentamycin 2mg/ml.The plates were incubated for 24hours at 37°C, after which the zone of inhibition was measured and recorded. The test was carried out in duplicate.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentration is the concentration giving the least inhibitory activity and below which there is no further inhibition. It is therefore regarded as the concentration giving the lowest possible zones of inhibition. Two fold serial dilutions were prepared to obtain a 0.1-100mg/ml concentration range. Sterile discs were immersed into the different dilutions and absorption was allowed for 30 minutes.A pair of sterile forceps was used to pick the paper discs and placed on the inoculated plates and the portions were labeled. The plates were incubated at 37°C for 24hours.

Control Experiment Using Gentamycin and Dimethyl Sulphoxide (DMSO)

Gentamycin was used as the positive control in order to compare the diameter of zone of clearance from the extracts and already standardized antibiotic (Gentamycin) and it was carried out asceptically (Oyagede *et al*.,1993).This is to ensure the prescription of either antibiotics or plant herbs for antibacterial activities. Gentamycin (280mg) bottle with stock solution 80mg/2ml was used by diluting 1ml of gentamycin in 19mls of distilled water that is, 1:20 dilution (1+19mls) giving a final concentration of 2mg/ml.

Dimethly sulphoxide (DMSO) was used as a negative control impregnating the discs in 50% DMSO. These discs were placed on the inoculated culture medium along the aqueous and ethanol extract.

Phytochemical Screening

Preliminary phytochemical analysis of the crude powder of the plant was determined as follows:

***Tannins*:** 20mg of the pulverized plant materials was dissolved in one ml distilled water and was filtered. Two milliliters of the filterate was added to 2mls of $Fecl_3$.Blue black precipitate indicated the presence of tannins.

Alkaloids: Twenty milligammes of the pulverized material was dissolved in 1ml methanol and was filtered. A 2ml filterate was added to 1% HCl and was heated. One milliliter of filterate added to 6drops of Mayor's reagents resulted in a cream coloured precipitate which indicated the presence of alkaloids.

Saponins: 0.5ml filtrate was added to 5ml distilled water. Frothing persistence indicated presence of saponins.

***Cyanide glucosides*:** 2mls of the filtrate was added to 1ml glacial acetic acid and $FeCl_3$ and concentrated H_2SO_4. Absence of greenish-blue coloration was seen and this indicated the absence of cyanide glucosides.

Steroids: 20mg of the pulverized plant materials was dissolved in 10ml chloroform and was filtered. A 2ml filterate was added to 20ml acetic anhydride and concentrated H_2SO_4.Blue green ring indicated the presence of terpenoids.

***Flavonoids*:** 20mg of the pulverized plant materials was dissolved in 1ml ethanol and was filtered. A 2ml filterate was added to concentrated HCl and magnesium ribbon. Pink tomato red colour indicated the presence of flavonoids (Parekh *et al*.,2005}

RESULTS

Effects of Percentage Yield of Plant Extracts

Percentages of the yields of the plant extract (ethanol and aqueous) were recovered and calculated as percentages of the initial quantity of pulverished sample of plant material shown in Table 1.The ethanol extract of *Citrus sinensis* gave a yield (16g) representing 16% and while the aqueous gave a yield (1.5g) representing (15%).

Antibacterial Activity of Plant Extracts

The antibacterial activity of a *Citrus sinensis* extract was assayed in vitro by agar disc diffusion against 4 bacterial species. Table 2 summarizes the microbial growth inhibition of both aqueous and ethanol extracts of the screened plant species. The aqueous extract of *Citrus sinensis* had conspicuous zone of inhibition on *Escherichia coli* that is 7.00mm in diameter while on the other test organisms, it had little or no zone of inhibition as well as the effect of the ethanol extracts on all the test organisms whose zones of inhibition was not greater than 3.00mm.

On the contrary, 2mg/ml of gentamycin (positive control) showed wide zones of inhibition on all the test organisms which is incomparable to the 100mg/ml concentration of the plant extracts. Dimethyl sulphoxide (DMSO) negative control shows no zone of inhibition. It was completely resistant to all the test organisms.

Minimum Inhibitory concentration (MIC) Test

The MIC was determined with the ethanolic and aqueous extracts of *Citrus sinensis* of the two fold serial dilution tested on the organisms using disc diffusion method .As was observed, none of the dilutions showed any zone of inhibition, they were resistant. That is the MIC was completely nil.

Results of Phytochemical screening

Preliminary phytochemical screening of the test plant *Citrus sinensis* showed the presence of tannin, alkaloid, saponin, flavonoid, steroid, tripertenes and the absence of cyanide glucoside.

Table 1
Percentage yield of the crude extracts of Citrus sinensis

Plant species	Extract type	Weight of pulverized sample used	Weight of extract	Percentage of yield of extract
Citrus sinensi	Aqueous	10.0g	1.5g	15%
	Ethanol	10.0g	1.6g	16%

Table 2
Antibacterial Activity of the Extracts

Bacterial species	Aqueous	Ethanol
Escherichia coli	+	±
Pseudomonas aeruginosa	-	±
Klebsiella pneumoniae	±	±
Staphylococcus aureus	±	±

Key: + = Inhibition (>3.00)
± = Trace
- = Resistant

Table 3
Diameter of zones of inhibition of various extracts as well as controls in millimeter

	Citrus sinensis			
Bacterial species	**Aqueous extract**	**Ethanol extract**	**Gentamycin**	**DMSO**
E.coli	7.00	3.00	9.00	0.00
P.aeruginosa	0.00	1.00	4.00	0.00
K.pneumoniae	3.00	3.00	10.00	0.00
*S.*aureus	1.00	2.00	10.00	0.00

Table 4
Minimum inhibitory concentration of the extracts (Diameter in mm)

	Ethanol extract			Aqueous extract		
Bacterial species	100mg/ml	10mg/ml	0.1mg/ml	100mg/ml	10mg/ml	0.1mg/ml
E.coli	2.00	0	0	7	0	0
P.aeruginosa	1.00	0	0	0	0	0
K.pneumonia	3.00	0	0	3	0	0
S.aureus	2.00	0	0	1	0	0

Table 5
Preliminary Phytochemical analysis of Citrus sinensis

Test plant	Tannin	Saponins	Flavonoids	Steroids	Cyanideglucosides	Alkaloids
Citrus sinensis	+	+	++	+	-	+

Key: + = present - =absent ++ = highly present

DISCUSSION

The ethanol extracts exhibited inhibitory activities that were found to be a little higher than aqueous extract on all the test organisms except *Escherichia coli* on which the aqueous extract had no inhibitory activity. Although with a slight difference it can be therefore inferred that the active principles of the plant herb may be more soluble in ethanol as employed in ethnomedicine (Soforowa, 1984)

Despite the ethanol extract exhibited higher inhibitory activities than aqueous extract, the antibacterial activity was low. Results ranged from 1-3mm in diameter. Consequently, the minimum inhibitory concentration (MIC) determined did not show any zone of inhibition. The MIC result is traceable to the fact that the two fold serial dilution reduced its initial concentration of 100mg/ml to 10mg/ml and then to 0.1mg/ml which had no visible effect on the organisms resulting in no zone of inhibition.

A low level of activity at a low extract concentration may suggest that the concentrations of the active constituent in the extracts are too low for any appreciable antibacterial activity (Ashebir and Ashenati, 1999).

Further, low concentration of diffusible water soluble active constituents or excessive heating which often affect biologically active substances such as flavonoids, essential oils and other heterogeneous phytoconstituents present in the extract (Scalbert,1991) might also influence their respective activity. Preliminary phytochemical analysis revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids and terpenes. It is also possible that the plant showed low antibacterial potential because all the aforementioned secondary metabolites were present in low concentration and the concentration of plant extract used was also low (100mg/ml).

In addition, the positive control (gentamycin) had the widest zones of inhibition on all the organisms where the Dimetyl sulphoxide (DMSO) (negative control) had no effect on all the test organisms.

CONCLUSION

The results obtained with plant extracts continues the numerous searches for more effective drugs of plant origin which are less toxic and available for low socio-economic population in the treatment of diseases caused by pathogenic bacteria.

The potential for developing antimicrobial from higher plants appears rewarding as it will result to the development of a phytomedicine to act against microbes. Plant based antimicrobials have enormous therapeutic potentials as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. There is need for further exploration of plant-derived antimicrobials. The present study of in vitro microbial evaluation of some plants form a primary platform for further phytochemical and pharmacological studies .Also further studies will be needed to purify the bioactive compounds of the ethanol extracts and characterize the aqueous fraction of the plants and their phytochemical mode of action should be further investigated.

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