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Antimicrobial Assay of *Citrus limon, Citrus sinensis and Citrus aurantifolia* on *Staphylococcus aureus and Escherichia coli* isolated from Wound

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ABSTRACT

The Antibacterial activities of 3 Citrus species (*Citrus sinensis, Citrus aurantifolia and Citrus Limon*) were studied against *staphylococcus aureus* and *Escherichia coli* isolated from wound infections. A total number of 112 organisms were isolated from wound infections collected from different individuals from different Hospitals in Enugu, Esut Teaching Hospital parklane; National Orthopedic Hospital Enugu, Goodness and mercy hospital Emene, De graceful Touch Clinic, Emene Enugu. The samples were cultured using Mueller-Hinton agar (Oxoid, UK) and incubated for 24 hours at 37^{o}C. Sixty (60) samples of *Staphylococcus aureus* were isolated and fifty two (52) samples of *Escherichia coli* were isolated, subcultured, stored and preserved in readiness for sensitivity against the citrus extracts. Methanol and ethanol extracts were used for antimicrobial assay. Out of the 52 isolates of *E.coli* tested against the citrus extract, 40 isolates (76.9%) were susceptible to the citrus extracts , while 12 isolates (23.0%) were resistant ; Out of 60 isolates of *Staphylococcus aureus* tested against the extracts, 45 isolates (75%) were susceptible to the citrus extract while 15 (25%) were resistant. The 40 susceptible isolates of *E.coli* were from 10(55.55%) isolates from *Citrus sinensis* in methanol extract ,8(44.44%) isolates from *Citrus sinensis* in ethanol extract, 6 (50%) isolates from *Citrus aurantifolia* in methanol extract, 6(50%) isolates from *Citrus aurantifolia* in ethanol extract, 6(60%) isolates from *Citrus limon* in methanol extract, 4(40%) isolates from *Citrus limon* in ethanol extract. The 45 susceptible isolates of *Staphylococcus aureus* were from 12(60%) isolates from *Citrus sinensis* in methanol extract ,8(40%) isolates from *Citrus sinensis* in ethanol extract. 8(53.33%) isolates from *Citrus aurantifolia* in methanol extract, 7(46.66%) isolates from *Citrus aurantifolia* in ethanol extract, 5(50%) isolates from *Citrus limon* in methanol extract, 5(50%) isolates from *Citrus limon* in ethanol extract. *Citrus sinensis* showed the highest inhibition followed by *Citrus aurantifolia* and then *Citrus limon*. In this study, extracts from the plant exhibited antimicrobial activity against various pathogens including *S. aureus, E. coli.*

Keywords: Antimicrobial, *Citrus limon, Citrus sinensis, Citrus aurantifolia* on *Staphylococcus aureus and Escherichia coli*

INTRODUCTION

Citrus sinensis, Citrus aurantifolia and Citrus limon belong to Rutaceae, it is a polyembroyonic plant cultivated in several part of the world especially hot subtropical or tropical region such as India, USA, Nigeria, Mexico and Egypt [1,2,3,4,5]. The plant is shrub in nature and height of about 2 meter tall, evergreen with dense and irregular branches which possess short and stiff spines [6,7,8,9,10]. The *Citrus aurantifolia* and Citrus sinensis fruits are ovoid berry of about 3-6cm in diameter and sometimes possess apical papilla. When ripe, the fruits turn yellow from initial blue [11,12,13,14]. The plant is used in traditional medicine for treatment of several diseases such as cold and stomach ailment [15,16,17,18]. It can also be used as an antiseptic, mosquito repellant, antifungal, antibacterial and antiviral agent. The health benefits of *Citrus aurantifolia, Citrus sinensis and Citrus limon* plants are highly associated with the high amount of bioactive constituents it contained such as phenols, flavonoids, carotenoid, vitamins and minerals [19,20,21]. Limes contain unique flavonoid compounds that have antioxidant and anti-cancer properties [22,23]. The flavonoids help to inhibit cell division in many cancer cell lines in addition to its antimicrobial efficacy [24,25]. The plant also demonstrated bioactive activities for cold, fever, sinusitis, sore throats,

asthma and bronchitis [26,27]. Antibacterial assessment of *Citrus sinensis, Citrus aurantifolia and Citrus limon* in aqueous, ethanol, acetone, chloroform, ethanol and petroleum ether leaves extract conducted by [28,29,30].

AIM OF THE STUDY

The aim of this research was to determine Antimicrobial assay of *Citrus limon, Citrus sinensis and Citrus aurantifolia* on *Staphylococcus aureus and Escherichia coli* isolated from Wound.

MATERIALS AND METHODS

STERILIZATION OF GLASS WARES

All glass wares were sterilized in a hot air oven at 100°c for 1hour and all media prepared were sterilized in an autoclave at 121°C for 15 minutes.

COLLECTION AND IDENTIFICATION OF PLANT MATERIALS

Fresh fruits (Sweet orange, lemon, lime,) were purchased from different Markets, in Enugu State, Nigeria.

PREPARATION OF PLANT MATERIALS

The fruits were rinsed thoroughly with distilled water and were cut into halves. Zests were extracted by "peeling" using a clean knife in an aseptic condition. The Zests were suspended into Methanol, Ethanol, Ethyl Acetate, N- Hexane and Water

PREPARATION OF EXTRACTS

The blended plant (250g) was macerated in 500ml of Methanol, Ethanol, Ethyl Acetate, N- Hexane, and Water in different flat botton flask. The mixture were stirred, covered and allow to stand for 24hours. The mixture was filtered afterwards using sterile Whatman No. 1 filter paper in a Buchner funnel. The filtrate were subjected to concentration using water bath (Chem-index, WB500E, USA) at 30°c for 6h to obtain a concentrate. Thereafter the concentrate was allowed to stand for 24h for proper elimination of the solvent [8]. The extract obtained was stored in refrigerator at 40°c until needed for phytochemical and antimicrobial analysis.

IMPREGNATION OF THE CITRUS EXTRACT IN STERILE DISCS

Preparation of Paper Disks

Using an ordinary office two hole puncher, paper disks with approximate diameter of 6.3mm were punched out one by one from sheets of Whatman paper, precaution were taken to avoid overlapping of the holes.

Preparation of Antimicrobial Solutions Using the Citrus extracts In 3 Different Solvents

Antimicrobials used were powders obtained from Citrus peel powder and Citrus juice of various serial dilutions. These Powders were accurately weighed and dissolved in the appropriate diluents to yield the required concentration, using sterile glassware. Stock solutions were prepared using the formula (1000/P) X V X C=W, where

i. P= potency of the antibiotic base,
ii. V=volume in ml required,
iii. C=final concentration of solution and
iv. W=weight of the antimicrobial to be dissolved in V.W=weight of the antimicrobial to be dissolved in V. Standard strains of stock cultures were used to evaluate the antibiotic stock solution. The antibiotic powder were dissolved in their appropriate solvents and further diluted in distilled water while the Citrus juice were diluted using serial dilituion method.

Impregnation of the Disks

Immersion method was employed; blank disks were soaked in known concentration of Citrus extracts serially diluted and made from the three species used for this research in three different solvents and then allowed to dry for 2 hours.

Drying

Without covering the Petri dishes, the disks were allowed to dry in a clean incubator at 35℃ for 2-3 hours. After drying 50 to 100 disks were placed in small sterile air tight labeled containers with a desiccant at the bottom. A layer of sterile cotton or foam was placed over the desiccant to avoid contact with the disks. The disks were stored in a freezer at 14oc. Unopened containers were

removed from the freezer 1 or2 hours before use to equilibrate to room temperature before this opened to minimize the amount of condensation that may occur when warm room air reaches the cold containers.

INSTRUMENTS FOR SAMPLE COLLECTION

The instruments used for sample collection include sterile swab sticks, sterile sample containers, spatulas and hand gloves, nose masks.

COLLECTION OF SAMPLES FROM WOUND INFECTIONS

Using sterile swab sticks, exudates, discharges and puses from different wounds are collected from different hospitals in Enugu State.

INSTRUMENT FOR SAMPLE COLLECTION

The instruments used for sample collection include sterile swab sticks, sterile sample containers, spatulas and handgloves.

PREPARATION OF MEDIA

The media used were prepared according to the manufacturer's instructions stated below

ISOLATION OF PRIMARY CULTURES

Step 1: The samples from different hospitals and laboratories in Enugu were cultured in nutrient broth and were incubated at 37^{0}C for 24 hours.

After 24 hours they were stored in the refrigerator, in readiness to be sub cultured.

Step 2: Using an inoculation loop under a sterile or aseptic condition the organisms (primary culture) were sub cultured into Chromogenic agar and are incubated at 37^{0}C for 24 hours.

Step 3: Using an inoculation loop under a sterile or aseptic condition the organisms (primary culture) were sub cultured into Macconkey agar and are incubated at 37^{0}C for 24 hours.

Step 4: Using an inoculation loop under a sterile or aseptic condition the organisms (primary culture) were sub cultured into Mueller-Hinton agar and are incubated at 37^{0}C for 24 hours.

Step 5: Using an inoculation loop under a sterile or aseptic condition the organisms (primary culture) were sub cultured into Luria broth for *E.coli* and Mannitol Salt Agar for *S. aureus* and then incubated at 37^{0}C for 24 hours.

IDENTIFICATION OF THE ISOLATES (*Escherichia coli* AND *Staphlococcus aureus*).

IDENTIFICATION 'A'

After 24hrs, many colonies appeared and *Escherichia coli* and *Staphlococcus aureus* were identified using

→ The texture and appearance on the agar plate

→ The color and shape and size on the agar plate and the organisms were confirmed using Gram staining techniques

IDENTIFICATION 'B'

GRAM STAINING PROCEDURE

- Flood with crystals violet or methyl violet (primary stain)
- Flood the smear with lugols's iodine (mordant)
- Decolorize using 95% alcohol until the slide appear colorless
- Counter stain with safranin or carbol fuchsin for 20–30 sec
- Observe slide under oil immersion objectives lens.

Test organisms

A total number of 112 organisms were isolated from wound infections collected from different individuals from different Hospitals in Enugu, Esut Teaching Hospital parklane; National Orthopedic Hospital Enugu, Goodness and mercy hospital Emene, De graceful Touch Clinic, Emene Enugu. The samples were cultured using Mueller-Hinton agar (Oxoid, UK) and incubated for 24 hours at 37^{o}C. Sixty (60) samples of *Staphylococcus aureus* were isolated and fifty two (52) samples of *Escherichia coli* were isolated, subcultured, stored and preserved in readiness for sensitivity against the citrus extracts.

STANDARDISATION OF INOCULUM

Test organisms were subcultured onto fresh plates of nutrient agar (Oxoid, UK) and Muller-Hinton and all were incubated appropriately as specified for each organism.

BIOCHEMISTRY CONFIRMATION TEST

SUGAR TEST

REAGENT: Phenol red broth, (differential for gram negative organism)

This is a test used to identify gram negative enteric bacteria. The phenol red broth is prepared according to the manufacturer's instruction and poured in different test tubes and the isolated organisms were introduced into the test tube containing the phenol red broth and colour changes were observed from red to yellow indicating positive to sugar test.

CATALASE TEST

REAGENT: Hydrogen peroxide

This test is used to know or dictate the presence of catalase enzyme in a test isolates using hydrogen peroxide. In this test, drops of hydrogen peroxide were placed on the slides, and the isolates were introduced to the hydrogen peroxide and oxygen bubbles were observed for positive results.

OXIDASE TEST:

REAGENT: N, N,N^1, N^1 tetramethyl P-phenylenediamine (TMPD)This is a test used in Microbiology to determine isolates that produces certain cytochrome C oxidase; if positive, it means that the isolate contain cytochrome C Oxidase and can use oxygen for energy production by converting O_2 to H_2O_2 or H_2O with an electron transfer chain. If negative it means isolate does not contain cytochrome C Oxidase. The disks of this reagent were prepared using impregnation method and the disk were allowed to dry. After this, each of these disk were wet with about 4 drops of distilled water and using an aseptic wire loop, large mass of the isolated bacteria were transferred to the disk and color changes were observed after 3 minutes, if the area of the inoculation turns dark blue-maroon or black, then result is positive, if light pink- Negative.

INDOLE TEST:

REAGENT: Tryptophan broth, Kovac reagent. This test is used to determine the ability of an organism to split amino acid tryptophan to form the compound indole. The tryptophan broth was prepared according to the manufacturers instruction and poured into test tubes, and the broths in test tubes were inoculated each with the isolated bacteria and were incubated at 37oc for 28 hours, after the incubation, 0.5ml of Kovac's reagent were added to each of the test tubes and colour changes were observed.

METHYL RED TEST

REAGENT: MR-VP (methyl red-Voges proskauer) broth used for both methyl red and VP tests.This test determines whether the isolates perform mixed acid fermentation when supplied glucose. The broth was prepared according to the manufacturer's instruction and was poured into many test tubes, after this, each test tube was inoculated with the isolated bacteria and they were incubated at 35°c for 4 days. After the incubation, 5 drops of methyl red indicator were added to each test tube and the color changes were observed

VOGES-PROSKAUER TEST

REAGENT: MR-VP broth, alpha naphthol, potassium hydroxide. This is a test for identification of enterobacteriaceae, usually performed along side with methyl red test. It is used to detect acetoin in bacteria broth culture. The broth was prepared according to the manufacturer's instruction and was poured into many test tubes, after this, each test tube was inoculated with the isolated bacteria and they were incubated at 35°c for 4 days. After the incubation, Alpha naphthol and potassium hydrogen were added and the color changes were observed in the test tubes, for positive acetoin is converted to diacetyl, naphthol is catalysed to bring out red.

SERIAL DILUTION

A serial dilution is the stepwise dilution of a substance in solution. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion. Serial dilutions are widely used in experimental sciences, including biochemistry, pharmacology, and

microbiology. A 10 fold Serial dilution was used in preparation of antibiotic disc dilution. ... Serial dilutions are used to accurately create highly diluted solutions as well as solutions for experiments resulting in concentration curves with a logarithmic scale.

Antimicrobial assay

112 Microorganisms were used in this study, consisting of 52 *Escherichia coli* and 60 *Staphylococcus aureus*. Disk diffusion method was used for the Antimicrobial assay in this research.

Minimum Inhibitory concentration

The minimal inhibitory concentration was done using serial dilution method in 10 test tubes for each extract.The organisms that showed susceptibility to different solvent extracts were introduced into the broths containing different concentrations of each extract (serial dilutions of the extracts corresponding to 200, 100, 50, 25, and 12.5, 6.25, 3.125 μg/mL). The tubes were then incubated for 24 h at 37°C. The minimum inhibitory concentrations (MIC) were taken as the lowest concentration of the extracts that did not permit any visible growth.

STATISTICAL ANALYSIS

The data were expressed as mean ± SEM of three replicates. The data were subjected to one-way analysis of variance (ANOVA), and differences between means were determined by Duncan's multiple range test using the Statistical Analysis System (SPSS Statistics 17.0, SPSS Inc. Chicago, Illinois, USA) where applicable. $P \leq 0.05$ were regarded as significant.

RESULTS

TABLE 1:MICROOBIOLOGICAL IDENTIFICATION TEST

BIOCHEMISTRY TESTS	*E.coli*	*Staphylococcus aureus*
Microscopic Appearance	Rod shaped	Moderate circular shape which appear in clusters
Colony Shape	|Circular colony	large Circular colony
Gram stain	-ve (Negative)	+ve (positive)
Motility	+ ve(Positive)	- ve(Negative)
Flagella	+ ve(positive)	-ve(Negative)
Capsules	- ve(Negative)	+ve(Positive)
Sugar test	+ ve(Position)	+ve(Positive)
Carbohydrate	+ve (Positive)	+ ve(Positive)
Catalase	+ve (Positive)	+ve (Positive)
Oxidase	- ve(Negative)	+ ve(Positive)
Indole	+ ve(Positive)	-ve(Negative)
Lactose	+ ve(Positive)	-ve(Positive)
Voges proskauer	- ve(Negative)	-ve(Negative)
Methyl red	+ve(Positive)	- ve(Negative)
H_2SGas production	+ve(Positive)	-ve(Negativee)

TABLE 2:ANALYSIS OF PRIMARY METABOLITES

Constituents	*Experimental method*	*Zest* *Aqueous Solution*	*Zest* *Ethanol*	*Zest* *Methanol*
Citrus sinensis.				
Carbohydrate test	Molisch's test	++	++	++
Reducing Sugar test	Benedict's test	+	+	+
Protein test	Million's test	-	+	+
Citrus limon.				
Carbohydrate test	Molisch's test	++	++	++
Reducing Sugar test	Benedict's test	+	+	+
Protein test	Million's test	_	+	+
Citrus aurantifolia.				
Carbohydrate test	Molisch's test	++	++	++
Reducing Sugar test	Benedict's test	+	+	+
Protein test	Million's test	_	+	+

TABLE 3: ANTIMICROBIAL ASSAY OF CITRUS EXTRACTS ON ISOLATED ORGANISMS
CITRUS SINENSIS (SWEET ORANGE)

S/N	EXTRACTS	ZONES OF INHIBITION IN MM		Concentration (12.5umg/ml)			
		BURNT ULCER	LEG ULCER	CANCER EXUDATE	PUS	BED SORE	REMARKS
1	Methanol *E.coli*	22mm	21mm	19mm	19mm	17mm	+
2	Ethanol *E.coli*	16mm	16mm	18mm	17mm	18mm	+
1	Methanol *Staph*	19mm	17mm	16mm	19mm	17mm	+
2	Ethanol *Staph*	14mm	16mm	17mm	17mm	18mm	+

TABLE 4: ANTIMICROBIAL ASSAY OF CITRUS EXTRACTS ON ISOLATED ORGANISMS CITRUS LIMON (LEMON)

S/N	EXTRACTS	ZONES OF INHIBITION IN MM		Concentration (12.5umg/ml)			
		BURNT ULCER	LEG ULCER	CANCER EXUDATE	PUS	BED SORE	REMARKS
1	Methanol *E.coli*	20mm	20mm	19mm	19mm	17mm	+
2	Ethanol *E.coli*	16mm	16mm	18mm	17mm	18mm	+
1	Methanol *Staph*	19mm	17mm	16mm	19mm	17mm	+
2	Ethanol *Staph*	13mm	15mm	15m	16mm	16mm	

TABLE 5: ANTIMICROBIAL ASSAY OF CITRUS EXTRACTS ON ISOLATED ORGANISMS CITRUS LIME (AURANTIFOLIA)

S/N	EXTRACTS	ZONES OF INHIBITION IN MM		Concentration (12.5umg/ml)			
		BURNT ULCER	LEG ULCER	CANCER EXUDATE	PUS	BED SORE	REMARKS
1	Methanol *E.coli*	18mm	19mm	19mm	19mm	19mm	+
2	Ethanol *E.coli*	18mm	17mm	17mm	17mm	18mm	+
1	Methanol *Staph*	17mm	18mm	18mm	17mm	17mm	+
2	Ethanol *Staph*	15mm	16mm	14mm	14mm	16mm	+

DISCUSSION

Citrus sinensis, Citrus aurantifolia and Citrus limon belong to Rutaceae, it is a polyembroyonic plant cultivated in several part of the world especially hot subtropical or tropical region such as India, USA, Nigeria, Mexico and Egypt [9]. The *Citrus sinensis*, *Citrus aurantifolia* and *Citrus Limon* are ovoid berry of about 3-6cm in diameter and sometimes possess apical papilla. When ripe, the fruits turn yellow from initial blue. The plant is used in traditional medicine for treatment of several diseases such as cold and stomach ailment. It can also be used as an antiseptic, mosquito repellant, antifungal, antibacterial and antiviral

agent. The health benefits of *Citrus aurantifolia, Citrus sinensis and Citrus limon* plants are highly associated with the high amount of bioactive constituents it contained such as phenols, flavonoids, carotenoid, vitamins and minerals [13]. Sweet oranges and lemons contain unique flavonoid compounds that have antioxidant and anti-cancer properties. The flavonoids help to inhibit cell division in many cancer cell lines in addition to its antimicrobial efficacy [18]. The plant also demonstrated bioactive activities for cold, fever, sinusitis, sore throats, asthma and bronchitis [8]. Plants can contribute to the advancement of novel chemo-preventive agents as they have been proven essential in forming potentially useful structures. The initial steps to this achievement is performing antibacterial activities in this study, the phytochemical screening indicated that the extracts (Ethanol and methanol) of *Citrus* (Zest) contained *alkaloids, saponins, tannins, glycosides, phenol,* and carbohydrates, the phytochemicals were recorded in all Zest parts. Out of the 52 isolates of *E.coli* tested against the citrus extract, 40 isolates (76.9%) were susceptible to the citrus extracts, while 12 isolates (23.0%) were resistant ; Out of 60 isolates of *Staphylococcus aureus* tested against the extracts, 45 isolates (75%) were susceptible to the citrus extract while 15 (25%) were resistant. The 40 susceptible isolates of *E.coli* were 10(55.55%) isolates from *Citrus sinensis* in methanol extract ,8(44.44%) isolates from *Citrus sinensis* in ethanol extract. 6 (50%) isolates from *Citrus aurantifolia* in methanol extract, 6(50%) isolates from *Citrus aurantifolia* in ethanol extract. 6(60%) isolates,from *Citrus limon* in methanol extract, 4(40%) isolates from *Citrus limon* in ethanol extract. The 45 susceptible isolates of *Staphylococcus aureus* were 12(60%) isolates from *Citrus sinensis* in methanol extract ,8(40%) isolates from *Citrus sinensis* in ethanol extract. 8(53.33%) isolates from *Citrus aurantifolia* in methanol extract, 7(46.66%) isolates from *Citrus aurantifolia* in ethanol extract. 5(50%) isolates,from *Citrus limon* in methanol extract, 5(50%) isolates from *Citrus limon* in ethanol extract. *Citrus sinensis* showed the highest inhibition followed by *Citrus aurantifolia* and then *Citrus limon*. Considerable antimicrobial activities of the plant extract were recorded in this work and these could be as result of the presence of the bioactive compounds in Citrus. Methanol and Ethanol extracts had the highest inhibitory activities against test organisms while the aqueous extract had little effect on the test organisms. This is probably because the plant bioactive compounds are more soluble in organic solvents. The growth of all the pathogens tested in this was inhibited by the plant extracts. That the seed ethanol methanol extract recorded microbial inhibition even at 15.6mg/ml on *S. aureus* suggests a strong possibility of using Citrus in the production of chemotherapeutic agent which could be use in the treatment of pharyngitis, impetigo, rheymatic fever, glomerulonephrities etc.

CONCLUSION

The result obtained in this present study deduced that the zest extract of Citrus spp tested have antibacterial against *E.coli* and *S.aureus* tested.

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APPENDIX

Citrus sinensis (Sweet orange)

Citrus limon (Lemon)

Citrus aurantifolia (Lime)