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Determination of Antimicrobial Activities of Crude Extract (Juice) of *Citrus Aurantifolia* (Lemun Tsami) on Some Selected Bacteria of Different Gram Reaction (*Staphylococcus aureus and Escherichia coli*).

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Abstract
Citrus aurantifolia is a nutrient-rich plant that is beneficial as an antibacterial agent as well as in the treatment of diverse ailments. In this study, the antimicrobial activity of the crude extract (juice) of *C. aurantifolia* was compared against Ciprofloxacin on two bacterial isolates (S. aureus and E. coli). The activity patterns of the extract and the standard antibiotics (Ciprofloxacin) on the isolates were determined using standard microbiological techniques. Highly significant activity (sensitivity) of the extract (juice) was observed on both isolates; with S. aureus recording a greater susceptibility; while E. coli responded moderately at the same concentrations (1/32, 1/16, 1/8, 1/4, 1/2 serial dilutions and undiluted form). The susceptibility of these organisms of different gram reactions to the extract is proof of the plant's potential to be used as a source of broad-spectrum drug against these bacteria. *C. aurantifolia* crude extract particularly the juice, therefore, may be employed as potential source of novel antibacterial agent to prevent the present antibiotic resistance perceived threat.

Keywords: Antimicrobial, Extract, *Citrus aurantifolia*, *S. aureus* and *E. coli*

Introduction

Plant resources have remained an integral part of human society throughout history. Besides fulfilling the primary needs like food and shelter, man has sought for a suitable remedy among plants for curing various diseases. Traditional medicine is defined as indigenous medicine that is used to maintain health and to prevent, diagnose, and treat physical and mental illnesses differently from allopathic medicine based on theories, beliefs, and experiences (Tilahun and Moa, 2018). Human health has been inextricably linked to the use of herbal medicines for millennia, making natural medicinal resources one of the oldest contributions of nature to human wellbeing (Hardy, 2019). According to reports, med-icinal plants have been the basis of treatment of various diseases in African tradition and other forms of treatment from diverse cultures of the world; both communicable and non-communicable diseases; as they can biosynthesize voluminous of bioactive phytochemicals (Kunene *et al*., 2020).

Results from several publications in the last two decades show that compounds (bioactive phytochemicals) derived from plants and their antimicrobial and antioxidant capacity tested both in vitro and in vivo gave positive results and are a viable alternative to the use of synthetic drugs or chemicals (Ncama *et al*., 2018). Hence, understanding the action mechanisms of these complex plant extracts and isolated plant-derived compounds is needed as they may help to pave the way towards combating life-threatening diseases (Li *et al.,* 2021).

Generally, citrus fruits (the family Rutaceae) exert health-beneficial effects by stimulating the immune, cardiovascular, and digestive systems. They also have anti-inflammatory, antibacterial, and anticancer properties (Rafiq *et al*., 2018). These properties result in part from the presence of numerous bioactive compounds, such as ascorbic acid, tocopherols, carotenoids, dietary fibre,

minerals, and a number of other compounds, such as flavonoids, phenolic acids, and tannins, which can and should be an integral part of a balanced diet (Czech, *et al*., 2020). *Citrus aurantifolia (C. aurantifolia*), also known as key lime, is one of the widely consumed citrus fruits in many cultural cuisines and juice production. *It* has widely been cultivated throughout the world and mostly used as a raw material for cosmetics, food flavoring, flavor enhancers in beverages, and as an ingredient in traditional medicine (Swandiny *et al*., 2021). The plant is loaded with various bioactive compounds rich in biological activities; including antimicrobial, antiviral, antifungal, anti-inflammatory, and analgesic, appetite stimulant, constipation remedy, etc. as a result of many Secondary metabolites found in *it* (Julaeha *et al*., 2022). Based on research, *C. aurantifolia* contains among other alkaloids, coumarins, flavonoids, carotenoids, phenolics, terpenes, limonoids, and essential oils (Ramadaini *et al*., 2020).

Materials and Methods

Plant Collection and Authentication.

Citrus aurantifolia (Lime) fruits were purchased from Gawon Nama market, Sokoto. The plant branch alongside fruits was identified and authenticated at the herbarium unit of the Department of Pharmacognosy and Ethnopharmacy, Usmanu Danfodiyo University, Sokoto. It was assigned a voucher number: PCG/UDUS/RUTA/0001, and ethical clearance for the work was also obtained from Sokoto state Ministry of Health with reference number: SMH/1580/V.IV.

Selection and Preparation of Plant Material

C. aurantifolia fruits that were free of decay and mold were taken and washed with distilled water severally to remove soil and other extraneous matter so that any dirt or bacteria residing on the surfaces would not be transferred to the interiors of the fruits and sterilized with 70 % ethanol. Then fruits were cut in halves with a sterilized knife. Juice was extracted using juice extractor and placed in a sterile bottle with screw cap (Bala and Kaushal, 2017).

Preliminary Screening for Phytochemicals Present in the Juice of *C. aurantiflia*.

The preliminary phytochemical screening (qualitative) of the fresh juice was carried out to determine the presence of different bioactive compounds (flavonoids, alkaloids, tannins, phenols, saponins, terpenoids, steroids).

Test for Flavonoids (Shibita's Test).

One milliliter (1 mL) of sodium hydroxide (NaOH) was mixed with 1mL of the extract (Juice) in a test tube. The formation of a yellowish coloration indicative of presence of flavonoids in the plant extract (Kumaresan *et al*., 2019).

Test for Saponins (Honeycomb or Frothing Test)

One milliliter (1 mL) of the juice was mixed with two milliliters (2ml) of distilled water in a test tube. The mixture was agitated vigorously for 5 min, and then allowed to stand for 25 minutes and observed for honeycomb froth, which indicated the presence of saponins (Ezeonu *et al,* 2016*)*.

Test for Alkaloids (Mayer's Test)

One milliliter (1 mL) of Mayer's reagents was added to a test tube containing one milliliter (1ml) of the juice. The formation of an orange red precipitate was an indicative of presence of alkaloids (Ezeonu *et al,* 2016*)*.

Test for Terpenoids (Sulphuric Test)

One (1ml) of the juice was added to 3 ml of chloroform; then 2 ml of concentrated sulphuric acid (H SO4) was added and heated for about 3 minutes. A reddish-brown color indicated the presence of terpenoids (Nata'ala *et al*., 2018).

Test for Tannins (Ferric Chloride Test)

A few drops of a 10% ferric chloride solution were added to 1 ml of the juice. The occurrence of blackish green coloration indicates the presence of tannins (Jain *et al*., 2020).

Test for Phenol (Ferric Chloride Test)

One milliliter (1ml) of the juice was added to 1ml of distilled water. Thereafter, 10% of ferric chloride solution was added in drops to the mixture. The presence of phenols is indicated by a formation of a bluish black color (Ayodele *et al*., 2020).

Test for Steroids (Test for Salkowski)

Two milliliters of the juice were added to 1ml of chloroform, then 1ml of sulfuric acid (H2 SO4) was carefully added to the mixture to form a

lower layer. The mixture was then observed for a reddish-brown color which indicates the presence of steroids (Khader *et al*., 2018).

Culture Media

Nutrient agar and nutrient broth were used and prepared according to the manufacturers' protocols.

Source of test organisms

Clinical Isolates of *Staphylococcus aureus* and *Escherichia coli* were obtained from the Medical Microbiology Laboratory unit of Usmanu Danfodiyo University Teaching Hospital, Sokoto. Following their isolation, the organisms were sub cultured on nutrient agar, incubated for 24 hours at 37°C; in order to obtain discrete colonies of the isolates. The colonies were identified by their cultural characteristics (colonial appearances), their gram and biochemical reactions (Amin *et al*., 2021).

Purification and Confirmation of Isolates

Gram Stain Procedure

Smears of the test organisms were aseptically made each, on clean grease free glass slides and allowed to air dry. The smears were heat fixed by passing over a Bunsen flame, smears were flooded with crystal violet dye, allowed to stain for one minute and rinsed with clean tap water; covered with Lugos's iodine for one minute and rinsed with water; briefly decolorized with acetone and rinsed with water. Smears were then counterstained with 0.5% aqueous solution of neutral red for one minute, rinsed with water and kept vertically to air dry. Smears were examined microscopically under oil immersion (X100) objective (Gharbi *et al*., 2019). Gram positive bacteria appeared purple, cocci and clustered; while gram negative bacteria appeared pinkish red and bacilli or rod shaped.

Catalase Test

A drop of 3% H_2O_2 was placed on a clean, grease-free and dried glass slide, using a sterile wooden stick, a small amount of colony of the test organisms was transferred to the H_2O_2 solution on the glass slide, emulsified and observed for evolution of oxygen bubbles, which was an indicative of the presence of catalase enzyme (Motse *et al*., 2019).

Coagulase Test

A drop of physiological saline was placed on each end of a glass slide, using a sterile wire loop, a portion of the test organism was transferred to each drop and emulsified. A drop of human plasma was added to one of the suspensions while the other served as control, it was mixed gently and observed for clumping. The presence of clumps (agglutination) showed that the organism was coagulase positive (Sakr *et al*., 2018).

Motility Test

A loopful of the broth culture of the test organism was placed on a clean glass slide, covered with a cover slip, viewed microscopically under X10 and X40 objectives and examined for a zigzag moving organisms which showed that the organism was motile (Amin *et al*., 2021).

Urease Utilization test

The entire slant surface was aseptically streaked with the test organism (while avoiding stabbing the butt as it served as a color control), tube was incubated at 37°C overnight; Slant was observed for reddish- pink color formation. Indicating positive Urease test while absence of reddish-pink coloration showed urease negative test (Nauclér *et al*., 2021).

Citrate Utilization Test

The medium (both butt and slant) was aseptically stabbed and streaked with a light inoculum of the test organism, incubated overnight at 37 C. It was observed for a color change from green to blue color. Formation of blue color was indicative of positive Citrate test while absence of color change showed Citrate negative test (Seifu *et al*., 2018).

Kligler's Iron Agar (KIA) Test

Using a sterile straight wire loop to inoculate the medium, first, the butt was stabbed to the bottom of the tube and then the slope was streaked in a zig-zag pattern, it was incubated at 37°C overnight with the tube tops loosely closed so that there will be an aeration in the medium. Lactose fermenter [Acid Slant/Acid Butt (yellow/yellow)]: Complete permanent acidification of both the deep and the slant of the tube by lactose-fermenting bacteria. Glucose and lactose both are fermented. This is characteristic of lactose-fermenting coliforms, such as

Escherichia coli and the *Klebsiella– enterobacter* species; and gas production within the space at the bottom of the tube and the split in the agar in the middle indicated positive KIA test while absence of acid/acid (yellow slant/yellow butts) showed a negative KIA test (Czajkowski *et al*., 2021).

Indole Test

Peptone broth was inoculated with the test organism, incubated at 37°C for 24h; 5 drops of Kovác's reagent were added and shook gently. The top layer was observed to form a red ring after some minutes. Formation of a red ring was an indicative of positive Indole test while absence of it indicated negative Indole test (Anger *et al*., 2019).

Preparation of the Inoculum

Direct colony suspensions of the test organisms were employed in the preparation of the working inocula as recommended by CLSI, (2019).

After overnight subculture, selected colonies of the isolates were aseptically picked with a sterile inoculating wire loop and suspended in 2mL sterile normal saline to make a suspension of the bacteria whose turbidity was adjusted to that of 0.5 McFarland standard (10^8 CFU/ml); against a white background under an illuminated surface. These were used within 30 minutes of preparation (CLSI, 2019).

Antimicrobial Sensitivity Testing (AST) of the Extract (Juice)

Agar well diffusion method was used to evaluate the antibacterial activity of the juice extract as described by Prinzi *et al*. (2022); in comparison with a standard broad-spectrum antibiotic, Ciprofloxacin which belongs to the class of drugs known as Fluoroquinolone.

Nutrient agar plates were prepared aseptically and allowed to set and dry. The plates were seeded with the standardized test inoculum by using sterile cotton swabs as follows: The swab sticks were dipped each in the separate bacterial suspension, excess fluids were drained from the swabs by pressing and rotating the swab sticks severally against the walls of the containers above the fluid levels; the swabs were streaked evenly over the entire surface of the medium while the plates were being rotated at approximately 60 degrees (CLSI, 2023). With the aid of standard cork borers (6mm in diameter), Six (6) wells were punched aseptically at different sites on the medium and labeled according to the concentration (dilution) of the extract, wells were aseptically filled each with a drop of the different concentrations (1/32, 1/16, 1/8, ¼, ½, and pure form) of the extract (Juice) while discs of the standard antibiotic, Ciprofloxacin (5ug/disc) was placed each at the centre of the media (positive controls).

The plates were allowed on a flat surface for about 30 minutes undisturbed; for pre-diffusion and then incubated at 37°C overnight. The resulting zones of inhibition (ZOI) were measured in millimeters using meter rule at every concentration of the agent against each bacterial isolate and recorded. Hence the susceptibility of each organism to the extract was identified by the zones of inhibition which were indicated by clear areas around the wells to which the different concentrations of the extract were added (Mostafa *et al*., 2018).

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibition concentration (MIC) gives the lowest concentration (highest dilution) of the extract that can inhibit the visible growth of the test organisms. This was determined by using the broth tube (11 test tubes) dilution method. 1ml of freshly prepared nutrient broth was aseptically dispensed into the sterile test tubes number 2 to 11; 1ml of the extract was put each in test tubes number 1, 2 and 9; and two-fold serial dilutions were carried out from number 2 to 8 test tubes. The content of tube 2 was evenly mixed and 1ml was transferred to tube number 3; this continued till the 8th test tube (i.e tubes were diluted 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128). 1ml of the bacterial suspension was then added to all the tubes except tubes 9 and 11; this was done for each test organism. Test tubes 9, 10 and 11 served as controls (extract, inoculum and broth).The tubes were thoroughly mixed and incubated at 37°C for 24 hours. Thereafter, tubes were visually observed for turbidity by comparing with the controls (Chikezie, 2017)

Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the extract was determined by sub culturing on freshly prepared nutrient agar (solid medium) a loopful from all the tubes that showed no visible growth in the MIC determination. These were inoculated at 37°C for 24h, the plates were observed for the growth of bacteria. The concentration of the extract that showed no growth was taken as the MBC (CLSI, 2019).

Results

The result of the preliminary screening for phytochemicals in the crude juice extract of *Citrus aurantifolia* (*C. aurantifolia*) is shown in Table 1. Alkaloids, Flavonoids, Saponins, Tannins, Seroids, Phenols and Terpenoids were present in the juice extract.

The antimicrobial susceptibility pattern of the isolates to the different dilution or concentration (1/32ml, 1/16ml, 1/8ml, ¼ml, ½ml, pure (v/v)) of the juice extract of *C. aurantifolia* is given in Table 2.

The antimicrobial activity (sensitivity) of Ciprofloxacin (5ug) on the test organisms is depicted in Table 3, with *S. aureus* recording sensitivity while *E. coli* was intermediate.

Table 4 shows the result of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract on the test organisms.

Table 1: Preliminary Screening for Phytochemicals in the Juice Extract of *C. aurantifolia*

Phytochemicals	Types of Test	Extract (*C. aurantifolia* Juice)
Alkaloids	Mayer's	Present
Saponins	Frothing (Honeycomb)	Present
Flavonoids	Shibita's (NaOH test)	Present
Phenols	Ferric Chloride	Present
Sterols	Salkowski's (Chloroform test)	Present
Tannins	Ferric Chloride	Present
Terpenoids	Sulfuric acid test	Present

Table 2: Antimicrobial activities (sensitivity) of Extract (*C. aurantifolia* juice) on the test organisms.

Test Organisms	Concentration of Extract (V/V) and Diameter of Zones of Inhibition (mm)					
	1/32	1/16	1/8	1/4	1/2	100
Staphylococcus. aureus	7	10	11	13	15	22
Escherichia. coli	9	11	12	14	15	20

Table 3: Antimicrobial activity (sensitivity) of Ciprofloxacin (5ug) on the isolates.

Isolates	**Zone of Inhibition** (Mm)	**Interpretation**
S. aureus	18	Sensitivity
E. coli	7.5	Intermediate

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extract on the test organisms

Test Organisms	**MIC (v/v)**	**MBC (v/v)**
Staphylococcus aureus	1/64 ml	1/2 ml
Escherichia coli	1/32 ml	1/2ml

Discussion

In recent years, herbal medicines have received considerable attention as an alternative way to compensate for perceived deficiencies in orthodox pharmacotherapy worldwide. Despite a lack of medical evidence to support their therapeutic efficacy and toxicological effects, the use of herbal medicine has increased considerably (Arika *et al*., 2015). According to the World Health Organization (WHO), up to 80% of the world's population in underdeveloped and developing countries rely on traditional medicine practices for their primary health care needs (WHO, 2002). Traditional medicines have been accorded greater acceptance in Africa because of the unavailability, unwanted side effects and high costs associated with orthodox medicines, inadequate health facilities and healthcare professionals, coupled with inadequate training of health workers (Piero *et al*., 2012). The therapeutic effects of medicinal plants can justifiably be attributed to, among others, the phytochemicals (bioactive compounds) in them especially the flavonoids, alkaloids, sterols, terpenoids, phenolic acids, lignans, tannins and saponins.

In this research, the preliminary phytochemical screening of the crude extract (juice) of *C. aurantifolia* revealed that the plant contains saponins, flavonoids, alkaloids, terpenoids, phenols, sterols and tannins, among others. These phytochemical compounds have been known to play roles in bioactivity of medicinal plants in general. For example, Flavonoids and some other phenolic components have been reported on their effective antioxidants, anticancer, antibacterial, cardioprotective agents, anti-inflammation, immune system promoting. Skin protection from UV radiation, and interesting candidate for pharmaceutical and medical application (Andreu *et al.,* 2018). Flavonoids are also known to improve cardiac function, decrease anginas and lowers cholesterol levels. These compounds act by regulation of inflammation mediators (Sánchez *et al*., 2008). While terpenoids, one of the abundant components besides flavonoids, have been reported to exert antimicrobial activities against both the antibiotic-susceptible and antibiotic-resistant bacteria, mainly via their abilities to promote cell rupture and inhibition of protein and DNA synthesis. In addition, terpenoids have been shown as one of secondary metabolites produced by aromatic and medicinal plants that played a key role in disease resistance. For example, monoterpenoids are antibacterial in nature, causing disruption in microbe multiplication and development, as well as interfering with their physiological and metabolic activities (Álvarez *et al*., 2021). Alkaloids are known to have blood glucose lowering activity. They have also been reported to demonstrate antioxidant activity responsible for various biological activities including anti-diabetic activity (Yang *et al*., 2001). Alkaloids are also known to be anti-arrhythmic effects, antihypertensive effects, anticancer and antimalarial activity (Abdirahman *et al*., 2015). Alkaloids are believed to have neuro-protective, memory and cognitive-enhancing effects, cholinergic and antioxidant activities in Alzheimer's disease (Speisky *et al*., 2006).

This study revealed a significant antibacterial activity of *C. aurantifolia* juice to the tested organisms at varying concentrations. A highly significant difference was observed between the inhibitory zone diameter of the extract compared with the standard antibiotic on both isolates. The susceptibility of these organisms of different gram reactions to the extract is a proof of the plant's potential to be used as a source of broad-spectrum drug against these test bacteria and other bacterial strains, which justifies the traditional use of the plant for therapeutic purposes (Castillo *et al*., 2000).

On the other hand, this in vitro study showed some level of biochemical resistance of these bacteria strains to Ciprofloxacin antibiotic; of which *E. coli* showed a greater resistance compared to *S. aureus* as observed from their inhibitory zone diameter supported by studies done by Ena *et a*l. (1995) and **Gilbert *et al.* (2001).** However, the diameter of zone of inhibition produced by the bacteria depends on several factors broadly classified as extrinsic and intrinsic parameters. Extrinsic parameters like pH of the medium, nutritional requirement, period and temperature of incubation, volume of well, size of inoculum etc. (Mata *et al*. (1994).

Hence, in vitro antimicrobial screening methods provide the required preliminary observation to select among the crude plants' products, those with potentially useful properties for further chemical and pharmacological studies. This study disclosed the need to use lime (*C. aurantifolia*) juice in reducing infections associated with *S. aureus* and *E. coli* as well as their disease risks.

Conclusion

The findings obtained from this research revealed that *Citrus aurantifolia* (lime) juice possesses phytochemicals of antibacterial significance. This antibacterial activity of *C, aurantifolia* was evident from the zones of inhibition (22mm and 20mm) recorded in both bacterial isolates compared to their susceptibilities to the standard antibiotic (Ciprofloxacin) as obtained from MIC and MBC results of the extract.

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