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Comparative Study On the Antimicrobial Activities of Extracts of Unripe and Ripe *Citrus Sinesis (*L. Osbeck) Peel

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ABSTRACT

Citrus sinesis is well known as one of the world's major fruit crop that is consumed fresh, juiced, and processed. The waste of *Citrus sinesis* processing industry can be used as a potential source of valuable products. A lot of work has been done on its essential oil which was tested for its natural antioxidant and anti-microbial properties but little work has been done on the antimicrobial property of the crude extract. This study is therefore designed to assess the antimicrobial properties of orange peel crude extract using pathogenic bacteria as target organisms. The main aim of the study is to evaluate the antibacterial activities of the ethanolic citrus peel extracts (bitter and sweet orange) on pathogenic bacteria following standard anti-bacterial activity procedures. The result of the antimicrobial assay of the orange peel extracts in this study showed characteristic zones of inhibition around the test organisms. These results suggest that the ethanolic extract of the peel of unripe orange exhibit higher antimicrobial potency against the test organisms compared to the ethanolic extract of the peel of ripe orange.

KEYWORDS: Antibacterial, Citrus, Orange peel, Hesperidin, Pathogenic microorganisms.

1. Introduction

Sweet orange *Citrus sinensis* (L. Osbeck) is widely grown in Nigeria and it is commonly called orange. It is a major source of vitamins, especially vitamin C, folacin, calcium, potassium, thiamine, niacin, magnesium, iron, chlorine, manganese, zinc, sodium, phosphorous, iodine, pectin, beta-carotene, amino acids and fibre (Kusuma *et al.,* 2019). Some important fruit of genus Citrus which sweet orange belong are *Citrus sinensis* (Orange), *Citrus paradise* (Grapefruit), *Citrus limon* (Lemon), *Citrus reticulata* (Tangerine), *Citrus grandis* (Shaddock), *Citrus aurantium* (Sour orange), *Citrus medica* (Citron), and *Citrus aurantifolia* (Lime) (Singh *et al.,* 2010). Sweet orange are well known as one of the world's major fruit crops that are consumed fresh, juiced, and processed. The waste of *C. sinensis* processing industry after juice extraction, such as peels, seeds and pulps, corresponding to about 50% of the raw processed fruit. These generated wastes can be used as a potential source of valuable by products (Victor *et al.,* 2020; Panwar *et al.,* 2021). Phytochemicals are present in edible fruits and vegetables and when eaten, potentially modulate human metabolism in a favorable manner,

thereby preventing chronic and degenerative diseases (Panche *et al.,* 2016). Antibacteria activities of the peel citrus fruit have been studied by many researchers (Edogbanya *et al.,* 2019). Sweet orange contains a variety of phytochemicals like hesperidin and naringenin. Naringenin has a bioactive effect on human health as antioxidant, free radical scavenger, anti-inflammatory, and immune system modulator (Zhang *et al.,* 2015). Hesperidin obtained from citrus cultures may have a potential therapeutically use as a mild anti-inflammatory agent, being also useful as a precursor of new flavonoids endowed with this activity (Da Silva *et al.,* 1994; Emim *et al.,* 1994). The emergence of multidrug resistant bacteria necessitate the need to look for natural sources of antibacterial over synthetic ones, which is not only safe with no side effects but relatively cheap.

A lot of work has been done on essential oil from citrus and it was found to be a rich source of bioactive compounds such as coummarin, flavonoids, carotenes, terpenes (Achilonu *et al.,* 2018; Edogbanya *et al.,* 2019). This study is therefore designed to assess the antimicrobial properties of orange peel crude extracts using pathogenic bacteria as target organisms. The main objective of the study is to evaluate the antibacterial activities of the crude ethanolic extract of ripe and unripe *C. sinensis* peel (ethanolic unripe and ripe orange peel extract) on pathogenic bacteria: *Lactobacillus spp, Escherichia coli, Streptococcus spp* and *Pseudomonas aeruginosa.*

E. coli has become a model organism for studying many life's essential processes partly due to its rapid growth rate and simple nutritional requirements. The major mode of transmission is fecal contamination of food and water. It is an almost universal inhabitant of the gut of humans and other warm-blooded animals where it can be an opportunistic pathogen causing a number of infections such as Gram-negative sepsis, urinary tract infections, pneumonia in immunosuppressed patients, and meningitis in neonates (Zhang *et al.,* 2015).

Pseudomonas aeruginosa is a common Gram-negative, shaped bacterium that can cause disease in plants and animals, including humans. Treatment of *P. aeruginosa* infections can be difficult due to its natural resistance to antibiotics. When more advanced antibiotic drug regimens are needed adverse effects may be severe (Zhang *et al.,* 2015). Infection from this organism can be transmitted from one patient to another via contact with fomites or by ingestion of contaminated food and water (Baron, 1996).

Lactobacillus is the most common probiotic found in food such as yogurt, and it is diverse in its application to maintain human well-being as it can help treat diarrhea, vaginal infections and skin disorders such as eczema (Zhang *et al.,* 2015). More recently, the *Pediococcus* species *P. dextrinicus* has been reclassified as a *Lactobacillus* species. According to metabolism, *Lactobacillus* species can be divided into three groups: Obligately homofermentative (group I) including: *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius.* Facultatively heterofermentative (group II) including: *L. casei*, *L. curvatus*, *L. plantarum*, *L. sakei* Obligately heterofermentative (group III) including: *L. brevis*, *L. buchneri*, *L. fermentum*, *L. reuteri.* (France *et al.,* 2016).

Streptococcus is a genus of coccus (spherical) gram-positive bacteria belonging to the phylum Firmicutes (*Ryan, 2004)* and the order Lactobacillales (lactic acid bacteria). Species of *Streptococcus* are classified based on their hemolytic properties (Patterson, 1996).

2. Materials and Methods

2.1 Collection of Plant Material and Extraction

The fruits of *Citrus sinesis* (Orange) ripe (sweet) and unripe (bitter/sour) were bought at Oja oba market, Owo, Ondo State and brought to the microbiology laboratory Achievers University, Owo, where the peels were carefully removed with sterilized table knives. The peels were washed under running tap water followed by sterile distilled water. These were air dried at room temperature for five (5) days, pulverized to a fine powder using a sterilized mixer grinder and stored in air-tight conical flask. Ethanolic extract 33.3% of the pulverized peel for both sweet and bitter orange was obtained and kept for five days. Each preparation was filtered through a sterilized *Whatman* No.1 filter paper into a sterile glass petri plates. The filtered extract was air dried and checked for sterility by exposure to UV rays for 24 h. The sterile air dried extract was stored at 4°C until further use.

2.2 Evaluation of Antibacterial activities

2.2.1 Test Organism for Antibacterial Activity

The bacterial strains used for this study were *Escherichia coli, Lactobacillus spp, streptococcus spp* and *Pseudomonas aeruginosa* were collected from the microbial culture collection bank of Department of Microbiology, Achievers University, Owo, Ondo-State. The bacterium was grown and maintained on Nutrient agar slants at 37°C. The organisms were sub-cultured once every 15 days.

2.2.2 Media Preparation / Maintenance of Culture

Nutrient agar (N.A) and Nutrient broth at pH (6.8) was prepared in 250 ml of distilled water for the evaluation of antibacterial activities of the ethanolic extract of the *Citrus* fruit peel.

2.2.2 Test for Sterility of Extract

The crude extracts and ethanolic extract obtained were tested to ensure their sterility by streaking them separately on to the sterile plates containing nutrient agar. The plates were
incubated at 37°C for 24 hours and then examined for possible growth of contaminants, the absence of which confirms the sterility of the test samples (Cheesbrough, 2000).

2.3 Methods for Testing Anti-Bacterial Activity.

2.3.1 Agar Well Diffusion Method (Pour Plate Method)

Agar well diffusion method elucidated by Ahmad and Beg, (2001) and Srinivasan *et al*., (2001) were followed. The antibacterial activities of ethanolic peel extract of *Citrus sinesis* against four pathogenic bacteria were evaluated by using this method. The nutrient agar plates were prepared by pouring 0.5ml of each organism, 15 ml of molten media into sterile petri-plates, allowed to gel. Wells or cups of 5 mm size were made with sterile cup borer into agar plates containing bacterial inoculums. Four holes were then filled with 0.5ml volume of the ethanolic extract of different concentrations 20mg/ml, 10mg/ml, 15mg/ml, 5mg/ml was poured into wells of inoculated plates was poured to challenge the organism and their reaction to the extracts. Antibiotics; septrin, Ampicillin, Ciprofloxacin, streptomycin was used as a positive control on various organism in comparison to the extract. Solvent ethanol was used as a negative control which would be introduced into the well. The plates thus prepared was kept at room temperature for ten minutes allowing the diffusion of the extracts into the agar (Rios *et al*., 1988). After incubation for 24 hours at 37^0C, the plates were observed Antibacterial activity were on the plates, were indicated by an inhibition zone surrounding the well containing the samples.
The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded if the radius of zone of inhibition was greater than 4 mm. The antibacterial activity results were considered as inactive if < 4.5 mm; 4.5-6 mm as partially active; while 6.5-9 mm as active and greater than 9 mm as very active.

2.3.2 Minimum Inhibitory Concentration (MIC) Assay

The minimum inhibitory concentration was determined using the tube dilution method in which 9 ml of sterile nutrient broth was dispensed into a test-tube and 1ml of the extract of varying concentration was added into the different tubes and 0.5ml of the standardize organism was inoculated and incubated for 24 hours at 37^0C the test tube with the least concentration of extract that showed no turbidity was taken as the minimum inhibitory concentration.

3 Results

3.1 Antibacterial Activity of Different Concentrations of Ethanolic Extract of *Citrus sinensis* (Unripe Fruit Peels)

In-vitro antimicrobial screening of ethanolic extracts of *Citrus sinensis* (unripe fruit peels) were carried out using different concentrations of 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml to monitor the extent of antimicrobial activity. Ciprofloxacin was used as positive control for this study. Lactob*acillus spp* shows the greatest zone of inhibition which increases with the concentration. This is followed by *Pseudomonas aeruginosa*, also the zone of inhibition increases with the concentration. *Streptomycetes spp* and *Escherichia coli* shows the poorest zone of inhibition and it has no zone of inhibition in the concentration of 5 mg/ml of *C. sinensis* ethanolic peel extract. Higher zone of inhibition was observed in the positive control (Ciprofloxacin). A significant difference ($p<0.05$) exist between the zone of inhibition of the positive control and the ethanolic extract even at higher concentration as shown in Table 1 below:

Table 1: Mean zone of inhibition of the ethanol extract on unripe orange fruit peels on some selected bacteria samples.

	Ciprofloxacin	Ethanol	Concentration of extracts (Mean ± S.E.M)			
Test bacteria	**+ve control**	**-ve control**	**5 mg/ ml**	**10 mg/ ml**	**15 mg/ ml**	**20 mg/ ml**
Escherichia coli	22.1 ± 1.1	NI	NA	3.0 ±0.1	5.5 ± 0.2	7.2 ±1.0
P. aeruginosa	18.4 ± 0.2	NI	2.0 ±0.4	4.0 ± 0.1	7.9 ± 0.2	10.0 ±1.0
Lactobacillus spp	23.5 ± 0.1	NI	2.5 ± 0.0	5.0 ± 1.0	8.0 ± 1.0	11.0 ±2.0
Streptomyces spp	21.2 ± 0.4	NI	NA	2.2 ± 0.1	6.0 ± 1.0	7.0 ±1.0

NI= No inhibition, NA= No activity
P= 0.032

3.2 Antibacterial Activity of Different Concentrations of Ethanolic Extract of *Citrus sinensis* (Ripe Fruit Peels)

The *in- vitro* antimicrobial screening of ethanolic extracts of *Citrus Sinensis* (ripe fruit peels) were carried out using different concentrations of 5 mg/ml, 10 mg/ml, 15 mg/ml and 20 mg/ml to monitor the extent of antimicrobial activity. Ciprofloxacin was used as positive control for this study. Lactob*acillus spp* shows the greatest zone of inhibition which increases with the concentration. This is followed by *Streptomycetes spp* whose zone of inhibition increases with concentration. *Escherichia coli and Pseudomonas aeruginosa* showed the poorest zone of inhibition and it has no zone of inhibition at lower concentration (5 mg/ml)

of *C. sinensis* ethanolic fruit peel extract. Higher zone of inhibition was observed in the positive control (Ciprofloxacin). A significant difference ($p<0.05$) exist between the zone of inhibition of the positive control and the ethanolic extract even at higher concentrations of the ripe orange fruit peel extract as shown in Table 2 below:

Table 2: Mean zone of inhibition of the ethanol extract of ripe orange fruit peels on selected bacteria samples

	Ciprofloxacin	Ethanol	Concentration of extracts (Mean ± S.E.M)			
Test bacteria	**+ve control**	**-ve control**	**5 mg/ ml**	**10 mg/ ml**	**15 mg/ ml**	**20 mg/ ml**
Escherichia coli	15.6 ± 0.2	NI	NA	NA	3.0 ± 0.2	5.1 ±0.0
P. aeruginosa	16.4 ± 0.1	NI	NA	2.2 ± 0.1	5.0 ± 1.0	6.0 ±1.0
Lactobacillus spp	22.5 ± 0.4	NI	2.2 ± 1.0	4.1 ± 0.1	6.0 ± 0.	9.1 ±0.4
Streptomyces spp	16.2 ± 0.1	NI	1.6 ± 0.1	2.8 ± 0.0	4.5 ± 0.1	6.4 ±1.0

Keys: NI=No Inhibition, NA= No Activity
P=0.038

3.3 Minimum Inhibitory Concentration (MIC) of the Ethanol Extract (Both Ripe and Unripe Orange) Fruit Peels on Bacteria Isolates Used.

The minimum inhibitory concentration (MIC) results showed that the plant extracts (both ripe and unripe) showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Lactobacillus spp* and *Streptomycetes spp* with MIC values ranging from 10.0 to 25 mg/ml. The tested extracts showed different levels of antimicrobial activity depending on bacteria species as shown in Table 3. The MIC of the ethanol extract of *Citrus sinensis* ranged from 5.00-20.0 mg/ml. The MIC value of the extract against *E.coli* was 20.0mg/ml showing resistant to lower concentration of the extract. The MIC value of the ripe fruit peel extract against *Pseudomonas aeruginosa* ranged from 15.0mg/ml to 20.0mg/ml, also the MIC value of *Lactobacillus spp* ranged from 10.0 mg/ml to 20.0mg/ml. Finally, the MIC of the extract against *Streptomycetes spp* ranged from 15.0 mg/ml to 20.0mg/ml respectively.

The lowest concentration of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism were recorded as the MIC value of the sample. Different concentrations ranged from 5mg/ml to 20/ml of unripe *Citrus sinensis* fruit peel extracts (ethanol) were prepared and the MIC values against different pathogenic bacteria (*Streptomycetes spp*, *Lactobacillus spp, P. aeruginosa* and *E. coli*) were observed and recorded The minimum inhibitory concentration of the ethanolic extract of the unripe oranges showed that the MIC for *Escherichia coli* was 15.0mg/ml to 20.0mg/ml, while the MIC for *Pseudomonas aeruginosa* was 10mg/ml and this continued through 15.0mg/ml to 20.0mg/ml respectively. The MIC for *Lactobacillus spp* was 10mg/ml and this also continued to 20.0mg/ml. The MIC value for *Streptomycetes spp* was 15mg/ml.

Table 3: Minimum inhibitory concentration (MIC) of the ethanol extract (both ripe and unripe orange) fruit peels on bacteria isolates used.

	MIC RIPE CONC				MIC UNRIPE CONC			
	5mg/ml	**10mg/ml**	**15mg/ml**	**20mg/ml**	**5mg/ml**	**10mg/ml**	**15mg/ml**	**20mg/ml**
Escherichia coli	+	+	+	MIC	+	+	MIC	-
P.aeruginosa	+	+	MIC	-	+	MIC	-	-
Lactobacillus spp	+	MIC	-	-	+	MIC	-	-
Streptomyces spp	+	+	MIC	-	+	+	MIC	-

Key: MIC= Minimum inhibitory concentration, +=growth observed, -= no growth observed

Table 5: Mean zone of inhibition of positive control antibiotics

Antibiotics Bacteria isolates	**Septrin (SXT)**	**Ampicillin (PN)**	**Streptomycin (S)**	**Ciprofloxacin (CPX)**
E. coli	NA	S	S	S
P. aeruginosa	NA	I	S	S
Lactobacillus spp	NA	S	I	NA
Streptomycetes spp	NA	NA	S	NA

Key: S= sensitive, I= Intermediate, NA= No activity

4 DISCUSSION

The result obtained from the antimicrobial assay of the orange peel extracts in this study showed characteristic zones of inhibition around the test organisms. These organisms include *Escherichia coli, and Pseudomonas aeruginosa, Lactobacillus spp and Streptomyces spp.* Amongst the isolates used in this experiment, *Escherichia coli is* the least susceptible to the extracts. The ethanol extract of the unripe orange fruit peels was active against *Escherichia coli* with zones of inhibition of 3.0 ±0.1, 5.5±0.2 and 7.2 ± 1.0 at concentration of 10mg/ml, 15mg/ml and 20mg/ml respectively (table 1). *Pseudomonas aeruginosa* was active at all concentrations with zones of inhibition of 2.0 ± 0.4, 4.0 ± 0.1, 7.9 ± 0.2 and 10.0 ± 1.0 for concentration of 5mg/ml, 10mg/ml, 15mg/ml and 20mg/ml respectively (Table 1). *Lactobacillus spp* has the highest zone of inhibition (11.0 ± 2.0) at a concentration of 20mg/ml. This result is similar to

the findings of Harry *et al.,* 2008; Saleem and Saeed, 2020 which reported antibacterial activity of *Citrus sinensis* on *S. aureus*, *Pseudomonas spp* and *Klebsiella spp* respectively.

The ethanol extracts of the ripe orange were active against Lactob*acillus spp, which* shows the greatest zone of inhibition (9.1±0.4) at a concentration 20mg/ml, this is followed by *Streptomycetes spp* (6.4±1.0) whose zone of inhibition increases at concentration 20mg/ml, *Escherichia coli and Pseudomonas aeruginosa* showed the poorest zone of inhibition and it has no zone of inhibition at lower concentration (5 mg/ml) of *C. sinensis* ethanolic fruit peel extract. Higher zone of inhibition was observed in the positive control (Ciprofloxacin). A significant difference ($p<0.05$) exist between the zone of inhibition of the positive control and the ethanolic extract even at higher concentrations of the ripe orange fruit peel extract (Table 2)

The results showed that the potency of the orange peel extracts on the test organisms had different hierarchy of susceptibility among the organisms. Generally, higher concentration of the extract showed a greater zone of inhibition; this result is in agreement with the report of (Bisno *et al*., 1996) which states that the higher the concentration of antibacterial substance, the higher it shows an appreciable zone of inhibition. (Israa and Ibrahim 2015) in their studies on the antibacterial activities of *Citrus paradisi* extracts on *S. aureus* and *E. coli* reported that the extracts had profound activities on the test organisms. (Semiz *et al.,* 2007), (Kumar *et al,* 2011), Amandeep and Ahmed (2009) and Nwankwo *et al,* (2014) all have also reported similar results for the various activities of citrus fruits extracts. Thus the present work is in agreement with theirs. Further studies are required to understand the nature of the antibacterial compounds present and also other biological importance of the stem extract and other parts of the orange plant.

The MIC of the ethanol extract of *Citrus sinensis* ranged from 5.00-20.0 mg/ml. The MIC value of the extract against *E. coli* was 20.0mg/ml showing resistant to lower concentration of the extract. The MIC value of the ripe fruit peel extract against *Pseudomonas aeruginosa* ranged from 15.0mg/ml to 20.0mg/ml, also the MIC value of *Lactobacillus spp* ranged from 10.0 mg/ml to 20.0mg/ml. Finally, the MIC of the extract against *Streptomycetes spp* ranged from 15.0 mg/ml to 20.0mg/ml respectively. The lowest concentration of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism was recorded as the MIC value of the sample (table 3). The minimum inhibitory concentration of the ethanolic extract of the unripe oranges showed that the MIC for *Escherichia coli* was 15.0mg/ml to 20.0mg/ml, while the MIC for *Pseudomonas aeruginosa* was 10mg/ml and this continued through 15.0mg/ml to 20.0mg/ml respectively. The MIC for *Lactobacillus spp* was 10mg/ml and this also continued to 20.0mg/ml. The MIC value for *Streptomycetes spp* was 15mg/ml (table 3)

There is hierarchy of susceptibility among the test organisms subjected to the ethanolic orange peel extracts. *Lactobacillus spp* has the highest zone of inhibition (11.0 ± 2.0) and (9.4 ± 0.4) for unripe and ripe orange peel extracts respectively. The MIC for all the test organisms was on the average of 15 mg/ml

As multidrug resistant strains of microorganisms are emerging and treatment of their infection is becoming difficult with time, infectious diseases are a global cause of increase in death rate. Present study confirms the potential of studied fruit peel waste to be used for therapeutic purpose to combat the multidrug resistant microorganism infection. This will also result in reduction of waste material, reusing it for beneficial purpose in an economical and environmental friendly manner.

5 Conclusions

This present work has shown that extracts from *Citrus sinesis* have activity against the test organisms used in this experiment. The rate at which pathogenic bacteria are developing resistance to common conventional antibiotics is alarming therefore it is heartwarming to note that we could find succor in abundantly available environmental waste like orange peels for the treatment of tropical diseases. It is hoped that therapeutics can be

developed from orange peels to which these organisms are yet to develop resistance. Therefore, the ethanol orange peel extract that has an antimicrobial property against these organisms may be harnessed as one of the highly needed drugs for treatment of bacterial infections in the developing world.

6 Recommendation

As a result of the positive antimicrobial activity of the ethanolic orange peel extracts used in this study against some of the test organisms, it is recommended that a more study should be conducted to especially harness the ethno-medical capacity of orange peel extracts. The enormous therapeutic potential of ethno-medicine can be harnessed to serve the purpose with lesser side effect and antibiotic resistance often associated with synthetic antimicrobials.

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