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MOLECULAR DETECTION AND ANTIBACTERIAL ACTIVITY OF *CITRUS SINENSIS* PEEL EXTRACTS AGAINST *STAPHYLOCOCCUS AUREUS* AND *KLEBSIELLA PNEUMONIA* ISOLATED FROM WOUNDS

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ABSTRACT

The global rise of resistant bacteria threatens the effectiveness of antibiotics, recommending that herbs' natural chemical constituents could provide alternative antimicrobial and insecticidal properties. The study evaluated *Citrus sinensis* peel extracts' impact on bacterial species isolated from wound samples collected from six hospitals. Quantitative Phytochemical analysis was conducted on the *Citrus sinensis* peel extracts, Isolates were tested biochemically and molecularly for detection and confirmation of *Staphylococcus aureus* and *Klebsiella pneumonia*. The study assessed the antibacterial activity of *Citrus sinensis* peel extracts using agar disc diffusion method, revealing significant bioactive compounds such as alkaloids, flavonoids, saponins, steroids, glycosides, and tannins. The extracts effectivity against *Staphylococcus aureus* and *Klebsiella pneumonia* comes from its bioactive phytochemical components. The result from the morphological and biochemical tests revealed the presence of *Staphylococcus aureus, Klebsiella pneumonia, Staphylococcus epidermis, Pseudomonas aeruginosa, Escherichia coli* and *Enterobacter spp* but only *Staphylococcus aureus* and *Klebsiella pneumonia* were confirmed by molecular method with a sequence identity of 99.35% and 97.41% respectively when analyzed using the Basic Local Alignment Search Tool (BLAST) of the NCBI package. The study revealed that *Citrus sinensis* peel extracts showed antibacterial activity against *Staphylococcus aureus* and *Klebsiella pneumonia* at different concentrations and Ciprofloxacin as a control. The ethanolic extract showed high activity, while the aqueous extracts showed significant activity. The study suggests *Citrus sinensis* peel has potential as an antibacterial agent for wound infection treatment, suggesting the need for its phytochemical composition quantification and purification.

Keywords: Antibacterial, Wound isolates, *Citrus sinensis* peel, *Staphylococcus aureus*, *Klebsiella pneumonia*, Molecular detection

INTRODUCTION

Antibiotic resistance is a critical global health issue with significant implications for public health. The increasing prevalence of antibiotic-resistant bacteria poses a significant challenge to healthcare systems worldwide. A resistance mechanism's gene can be directly transferred through conjugation, transformation, or transduction, or they can undergo mutation leading to resistance mechanism (Sabtu *et al*., 2015). Bacteria (such *Staphylococcus, Escherichia coli, Klebsiella pneumoniae* etc.) can easily exchange genetic material, including genes that confer resistance to antibiotics (Sabtu *et al*., 2015).

The severity of common diseases and the rates of morbidity and mortality among patients can both be exacerbated by the lack of efficient antibiotics. Antibiotic resistance causes more than 500,000 fatalities worldwide annually, of which 40% are newborn deaths (Kaprou *et al*., 2015). Moreover, antibiotic resistance has substantial negative consequences on healthcare expenses due to delayed treatment, longer hospital stays, and a higher chance of infection spread. According to recent research by Centre for Disease Control, treating infections brought on by six multidrug-resistant organisms alone might cost more than $4.6 billion a year in the USA (Nelson *et al*., 2021).

Resistance to specific antibiotics has been reported in different parts of Nigeria. Ghebremedhin *et al*., 2009, reported the emergence of a community-associated methicillin-resistant *Staphylococcus aureus* and *Klebsiella pneumoniae* in southwest Nigeria while Lamikanra *et al*., 2011, reported the rapid evolution of flouroquinolone-resistant *Escherichia coli* in a Nigerian community. The rate of antibiotic resistance among common Gram-positive and Gram-negative isolates from various clinical specimens in a tertiary hospital in Nigeria was reported to be at the high (Okesola and Oni, 2009).

The plants of Citrus are the most widely planted. Citrus fruits are more abundant sources of bioactive substances that are beneficial to human health. These include carotenoids, vitamin C, limonoids, acridone alkaloids, flavonoids, essential oils, vitamin B complex and minerals (Parashar *et al.*, 2014). Other Citrus fruits included in the group are *Citrus medica, Citrus reticulata, Citrus limonum, Citrus vitis* and *Citrus aurantifolia* (Okwi and Emenike, 2006). Due to their high content of vitamin, mineral, and antioxidant content, including flavonoids, citrus fruits are frequently ingested. A family of phenolic chemicals known as flavonoids has numerous biological features, such as Antibacterial, antiviral, antithrombotic, hepatoprotective, and anticancer properties (Nisha *et al*., 2013).

This research seeks to explore the potential of *Citrus sinensis* peel extracts as a source of novel antimicrobial agents in the context of combating antibiotic-resistant microorganisms and for the molecular detection of clinical infections employing the conventional polymerase chain reaction (PCR) method and 16sRNA.

MATERIALS AND METHODS

Sample Collection

Wounds swabs were collected aseptically using sterile swab sticks from six (6) hospitals in Kaduna North Local

Government Area. 72 samples were collected and labeled appropriately and transported in ice container to the Biochemistry laboratory, Kaduna State University for processing.

Raw material
Fresh *Citrus sinensis* was obtained from fruit sellers at Kawo market, Kawo, Kaduna state. *Citrus sinensis* was washed thoroughly and peeled, the orange peel was shade-dried at 30°C for seven days before being ground into powder with a mortar and pestle and placed into clean vials for further analysis.

Preparation of plant extracts
The powered peel material was subjected to soxhlet extraction with ethanol and distilled water. About 150ml of ethanol and distilled water was added to 50g of dry powder separately, which was extracted for 72 hours. The obtained extracts were filtered using Whatman filter paper and then dried using a rotary evaporator and water bath at 40°C to 50°C. Separately, these were gathered and kept at 4°C in sealed containers (Varghese *et al*., 2013). This was carried out in triplicate.

Phytochemical Evaluation of *Citrus Sinensis* Peel
Citrus sinensis was screened for phytochemicals in accordance with the protocol of (Sofowora, 1993; and Evans, 2002) in the Department of Microbiology, Faculty Science, Kaduna State University (KASU) to ascertain the following phytochemical constitution;

Test for alkaloids
One ml of each extract was treated with few drops of Dragendorff's reagent. Orange brown precipitate indicated the presence of alkaloids

Test for flavonoid
One ml of NaOH was added to 3 ml of each extract. A yellow colouration indicated a positive test for flavonoids.

Test for saponins
Two ml of each extract was added to 5ml of distilled water and the solution was shaken vigorously for 30 seconds, stable persistent frothing indicated the presence of saponins.

Test for tannins
Two ml of each extract was added to 1ml of ferric chloride (FeCl3) and blue-black or greenish –black precipitate indicated the presence of tannins.

Test for steroids
Five drops of concentrated H2S04 was added to 1 ml of each extract. A red colouration indicated the presence of steroids.

Test for terpenoids
Two milliliters of chloroform and 3 ml of concentrated H2SO4 was carefully added to 5 ml of each extract to form a layer. A reddish-brown color at the interface indicated the presence of terpenoids.

Test for cardiac glycosides
Five ml of each extract was treated with 2 ml of glacial acetic acid and 1 drop of ferric chloride solution. 1 ml of concentrated H2SO4 will then be added. A brown ring at the interface indicated the presence of a deoxy sugar characteristic of cardenolides.

Test for reducing sugars
A little amount of Fehling's reagent was added to the both extracts, and the mixture was boiled for 2 minutes. A brick red colour indicated the presence of glycosides.

Test for carbohydrates
Molisch's reagent was added to 2 ml of both extracts. A little amount of concentrated sulphuric acid was added to it and allowed to form a layer. The mixture was shaken well, and allowed to stand for few more minutes, which will then be diluted by adding 5 ml of distilled water. Purple precipitate ring showed the presence of carbohydrates.

Isolation of test microorganisms
The samples (wound swab) were inoculated on freshly prepared MacConkey agar plate for the isolation of *Klebsiella pneumonia* and Mannitol salt for the isolation of *Staphylococcus aureus*. Suspected *Staphylococcus aureus* colonies appeared yellow on the plate after incubation for 24hours at 37°C and *Klebsiella pneumonia* appeared as large, pink, mucoid and elevated colonies with smooth surface on the agar plate. The isolates were then gram stained (Cheesbrough, 2006).

Anti-bacterial activity of plant extracts: The antibacterial activity of *Citrus sinensis* peel's ethanolic and aqueous extracts was assessed using the disc diffusion method. Different concentrations (100, 200, and 300 mg/ml) of extracts were prepared, and *Staphylococcus aureus* and *Klebsiella pneumonia* were seeded into the appropriate medium. Filter paper discs soaked with the extracts were placed on test organism-seeded plates, and ciprofloxacin (10 g/ml) was used as a standard antibiotic. The antibacterial assay plates were incubated at 37°C for 24 hours, and the diameter of inhibition zones was measured using a transparent ruler.

Genomic DNA Extraction
The DNA of isolates from pure culture samples was extracted using the phenol/chloroform method. The samples were pulverized, and 1.5 ml of the isolate was added to a micro-centrifuge tube. The tube was then re-suspended in buffer and SDS. The tubes were filled with 50 µl of Rnase, incubated at 370 ºC for 15 minutes, and Protinase K was diluted and heated at 500 ºC until full lysis occurred. The same volume of phenol/chloroform was added and centrifuged for 5 minutes at 10000 rpm and 40 ºC. The aqueous DNA layer was pipetted, precipitated using sodium acetate and isopropanol, and washed with 70% ethanol. The DNA was dried and dissolved in Tris-EDTA buffer. The concentration of DNA was measured using absorbance at 260 nm, and the purity of the DNA preparation was measured by the ratio of absorbance at 280 nm to 260 nm to 260 nm.

PCR amplification of 16sRNA gene
The study utilized DNA Taq polymerase for polymerase chain reaction (PCR) amplification, using a set of oligonucleotide primers specifically designed for annealing to the 16S rRNA sequence of the isolates. The primers were dissolved in distilled water and stored at -20 ºC. The PCR reaction involved a 50 µl reaction with 1-100ng DNA templates, 50 pmoles of each primer, and dNTPs at a final concentration of 0.25 mM each. The PCR was performed at 95°C for 2 minutes, followed by annealing and extension at 55-62°C for 30 seconds and 72°C for 1 to 4 minutes. Precision specifications for annealing temperature and extension time were established based on primer melting temperature and

fragment length. Using universal 16S rDNA primers (Forward; GGACTACAGGGTATCTAAT) and (Reverse; AGAGTTTGATCCTGG).

Gel electrophoresis of DNA

DNA fragments were separated using agarose gel electrophoresis using 1% w/v agarose in Tris acetate-EDTA solution. A loading dye was applied to the sample, containing bromophenol blue, sodium dodecyl sulphate, EDTA, and glycerol. Ethidium bromide was added to visualize DNA under ultraviolet light. A 1-kilogram base ladder was used to measure light fragment size.

DNA sequencing and analysis

Automated DNA sequencing was conducted. Sequences obtained in FASTA format was compared to other sequences in the databases (GenBank) using the Basic Local Alignment Search Tool (BLAST) package at http://www.ncbi.nlm.nih.gov/blast.

Statistical analysis

The statistical significance of the experiments was analyzed by one-way analysis of variance (ANOVA). The comparism of means.

RESULTS AND DISCUSSION

Table 1: Phytochemical composition of the peel extract of *Citrus sinensis*

Constituent	Orange peel (aqueous)	Orange peel (ethanol)
Alkaloid	+	+
Flavonoid	+	+
Saponins	+	-
Tannins	+	+
Steroids	+	+
Triterpenoids	-	+
Carbohydrates	+	-
Anthraquinones	-	-
Cardiac Glycosides	+	-

Key: (+) present; (-) absent

Table 2: Cultural Identification and gram properties of bacteria isolated

Test organism	Media	Morphological characteristics	Microscopy
Staphylococcus aureus	Mannitol Salt Agar	Yellow, Large, mucoid	Gram positive, Cocci in cluster
Staphylococcus epidermis	Mannitol Salt Agar	Red colonies with zones	Gram positive, Cocci in cluster
Klebsiella pneumonia	MacConkey Agar	Cream, mucoid	Gram negative, Rod in cluster
Pseudomonas aeruginosa	MacConkey Agar	Colourless, flat and smooth colonies	Gram negative, Rod in cluster
Escherichia coli	MacConkey Agar	Pink, non-mucoid, dry colonies	Gram negative, Rod in cluster
Enterobacter spp	MacConkey Agar	pink, mucoid, small colonies	Gram negative, Rod in cluster

Table 3: Biochemical characterization; characterization of *S. aureus* and *K. pneumonia*

Basic characteristic	Properties	
	S. aureus	***K. pneumonia***
Catalase	+	+
Coagulase test	+	+
Citrate	+	+
Indole test	_	_
Methyl red test	+	_
Voges- proskaeur test	+	+
Glucose	+	+
Sucrose	+	+
Lactose	+	+
Sugar fermentation test	_	+
H2S	_	_
Motility test	_	_

Key: (+) present; (-) absent

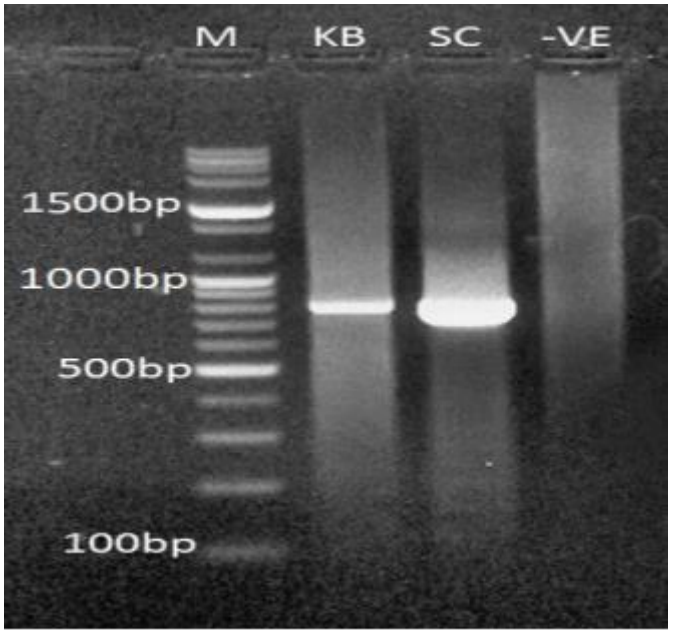

Plate 1: Gel picture of PCR product: The 16s rRNA amplicons of *Staphylococcus aureus* and *Klebsiella pneumonia* isolates shown on GelDoc. Key: lane 1= DNA Ladder (M); lane 2= *Klebsiella pneumonia* (KB); lane 3= *Staphylococcus aureus* (SC); lane 4= Negative control.

Table 4: Antibacterial activity of *Citrus sinensis* peel extracts against *Staphylococcus aureus* and *Klebsiella pneumonia*

Zone of inhibition in (mm) test bacteria

Concentration of extracts (mg/ml) *Staphylococcus aureus* and *Klebsiella pneumonia*

	Concentration of extracts (mg/ml)	***S. aureus (mm)***	***K. pneumonia (mm)***
Ethanol	300	18.75 ± 0.35	15.50 ± 0.70
	200	15.25 ± 0.35	11.15 ± 0.21
	100	10.12 ± 0.17	7.75 ± 0.35
Ciprofloxacin control		24.65 ± 0.49	20.50 ± 0.70
Aqueous	300	20.0 ± 0.00	16.40 ± 0.56
	200	16.62 ± 0.53	12.85 ± 0.21
	100	14. 87 ± 0.17	9.25 ± 0.35
Ciprofloxacin control		27.46 ± 0.84	20.50 ± 0.70

Table 5: The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of bacteria showing significant sensitivity to the extract

Extract/ Test bacteria	**MIC (mg/ml)**	**MBC (mg/ml)**
Ethanol Extract		
Staphylococcus aureus	100mg	200mg
Klebsiella pneumonia	100mg	100mg
Aqueous Extract		
Staphylococcus aureus	100mg	300mg
Klebsiella pneumonia	100mg	300mg

Discussion

The phytochemical composition and effect of the aqueous and ethanolic extracts of the *Citrus sinensis* peel was assessed on *Klebsiella pneumoniae* and *Staphylococcus aureus* to establish the scientific basis of for its usage. The results of the phytochemical composition of *Citrus sinensis* peel performed on the ethanol and aqueous extracts of the *Citrus sinensis* peel revealed the absence of anthraquinones in both the aqueous and ethanol extracts, along with the presence of some significant bioactive compounds like alkaloids, flavonoids, steroids, glycosides, and tannins. The phytochemical compositions of *Citrus sinensis* peel from this study is similar and comparable to a study by El-Desoukey *et al*., (2018) who reported the aqueous and ethanolic extracts of the *Citrus sinensis* peel contains alkaloids, flavonoids, tannins and saponin compounds while glycosides were not found. It also agrees with findings of Baba *et al.*, (2018) who investigated the phytochemical compositions of the *Citrus sinensis* and its effect on some clinical Bacteria species isolated from wounds. Several of these phytoconstituents are known to contain a wide spectrum of antimicrobial properties (Owoseni and Ajayi, 2010; Dhiman *et al*., 2012).

The result from the cultural identification and biochemical tests in table 2 and 3 revealed the presence of *Staphylococcus aureus, Klebsiella pneumonia, Staphylococcus epidermis, Pseudomonas aeruginosa, Escherichia coli* and *Enterobacter*

spp. Certain biochemical components such as Catalase, Coagulase, Citrate and Voges- proskaeur test etc were analyzed on *Staphylococcus aureus* and *Klebsiella pneumonia.* This finding corresponds with the report of Mitchell *et al.,* (2012) which indicated that *Staphylococcus aureus* as a Gram-positive bacterium, forming irregular clusters of cocci and appears to ferment the Mannitol in the MSA media and produce acid which result to the appearance of yellow colonies. This result corresponds with a report by Baron (1996), who reported *Staphylococcus epidermis* grows on MSA media, but does not ferment mannitol. *Klebsiella pneumonia* on MacConkey Agar media appeared as Gram negative bacteria with a morphology of creamy, mucoid texture.

The result of the molecular detection carried out in plate 1 shows clear detection of the two test organisms on the 1.2 % agarose gel, Staphylococcus aureus binds at 769bp while Klebsiella pneumoniae binds at 720bp. The partial 16S sequences of *Staphylococcus aureus* and *Klebsiella pneumoniae* obtained in FASTA format shows a promising result of 99.35% and 97.41% for *Staphylococcus aureus* and *Klebsiella pneumoniae* respectively after analyzing the sequences utilizing NCBI's Basic Local Alignment Search Tool (BLAST) package.

The result of the antibacterial activities presented in table 4, of both the ethanolic and aqueous extract of the *Citrus sinensis* peel using disc diffusion method at various concentrations (100, 200 and 300 mg/ml) were used against *Staphylococcus aureus* and *Klebsiella pneumoniae* from wound infection of patients and Ciprofloxacin (10µg/ml) was employed as standard Antibiotic. The results indicated that the *Citrus sinensis* peel shows favorable action against the isolated microorganisms. The maximum activity for the ethanolic and aqueous extracts against *Staphylococcus aureus* was 18.75 ± 0.35mm at a concentration of 300 mg/ml and 18.15 ± 0.49mm at the same concentration, respectively. Activity on *K. pneumonia* shows appreciable inhibitory effect of 15.50 ± 0.70mm at 300mg using ethanol extract and 16.40 ± 0.56mm at 300mg using aqueous extract. The results of (Motamedi *et al.,* 2009) who found that *Citrus sinensis* peels were efficient against a variety of bacteria are consistent with this observation. The inhibitory activity (mm) from this study was similar and comparable to a report by Hassan et al., 2021 which indicates *Citrus sinensis* Ethyl ethanoate extract at various concentrations (mg/ml) demonstrated the highest antibacterial activity against *K. pneumoniae* (37 ± 3.0 mm), *E. coli* (29.5 ± 0.5 mm), *S. aureus* (22 ± 0.0 mm) and *N. gonorrheae* with the minimum zone of inhibition (21 ± 0.0 mm) at 100 mg/ml, 100 mg/ml, 300 mg/ml and 200 mg/ml respectively. The results of (Nada and Zainab, 2013) found that the aqueous extracts of peel and juice from fresh and dried Citrus and sweet lemon reported antimicrobial action against six Gram-positive and eight Gram-negative bacterial and one yeast isolates.

The results of table 5; minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests shows that the ethanolic and aqueous extracts of the *Citrus sinensis* peel are both very effective against the bacteria isolates. It was also discovered that the extracts have both bacteriostatic and bacteriocidal effects on the test bacteria, with the exception of the aqueous extract, which is less effective.

This is study corroborates with the findings by Musa *et al*., (2019) who reported a significant activity of aqueous, methanol, and soxhlet extracts of the *Citrus sinensis* peel against *S. aureus* and *E. coli* on MIC and MBC. This outcome is consistent with research by Camarda *et al*. (2007) who found that extracts with increased antibacterial ingredient concentrations could exhibit meaningful inhibitions. It also agrees with findings by Oikeh et al., (2020) who demonstrated significant MIC values ranging from 12.5 to 100 µg/m for Fresh peel extract and Dry peel extract of the *Citrus sinensis* against *S. aureus, E. faecalis* and *P. aeruginosa* with the MBC demonstrating higher values obtained from 25 µg/mL to 200 µg/mL for Fresh peel extract and Dry peel extract of the *Citrus sinensis* against *S. aureus, E. faecalis* and *P. aeruginosa and E. coli.* A low degree of activity at a low extract concentration can indicate that the extracts' active ingredient concentrations are too low to have any discernible antibacterial effects (Ashebir and Ashenati, 1999).

CONCLUSION

This study investigated the antibacterial properties of the *Citrus sinensis* peel extracts (aqueous and ethanol) against *Staphylococcus aureus* and *Klebsiella pneumoniae* isolated from wounds. The findings verify the presence of various bioactive compounds in the peels, similar to previous studies. Both extracts exhibited promising antibacterial activity against the tested bacteria, with zones of inhibition comparable to existing research. The results also indicate bacteriostatic and bactericidal effects, suggesting the extracts can inhibit and kill bacterial growth. These findings support the traditional use of *Citrus sinensis* peels for their potential antibacterial properties and encourage further exploration of their development as natural antimicrobial agents.

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