

Phytochemical Analysis and Antibacterial Activity of Aqueous Extract of Citrus Aurantium (Bitter Orange) Leaves on Bacteria Isolated from Wound Infection

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Abstract- Wound infections remain a significant public health challenge due to the increasing prevalence of antibiotic-resistant bacteria and the limited effectiveness of some conventional antimicrobial agents. This study investigated the phytochemical constituents and antibacterial activity of the aqueous leaf extract of *Citrus aurantium* against bacteria isolated from wound infections. Fresh leaves of *C. aurantium* were collected, authenticated, air-dried, powdered, and extracted using distilled water. Phytochemical screening was carried out using standard qualitative methods, while bacterial isolates obtained from wound swabs were identified through cultural characteristics, Gram staining, and biochemical tests. The antibacterial activity of the extract was evaluated using the agar well diffusion method at concentrations of 100, 150, 200, 250, and 300 mg/ml, with tetracycline serving as the positive control. Phytochemical analysis revealed the presence of alkaloids, steroids, volatile oils, flavonoids, tannins, saponins, glycosides, saponin glycosides, and cardiac glycosides, while free anthraquinones were absent. Eleven bacterial genera were isolated, including *Staphylococcus*, *Pseudomonas*, *Klebsiella*, *Streptococcus*, *Enterococcus*, *Bacillus*, *Citrobacter*, *Serratia*, *Aeromonas*, *Propionibacterium*, and *Micrococcus* species. The aqueous extract exhibited varying degrees of antibacterial activity, with *Enterococcus* spp. showing the highest inhibition (16 mm at 250 mg/ml), while *Micrococcus* spp. showed no susceptibility. In general, antibacterial activity increased with extract concentration, although tetracycline demonstrated greater activity against most isolates. The findings indicate that *Citrus aurantium* leaves contain bioactive phytochemicals with appreciable antibacterial properties and may serve as a potential source of natural antimicrobial agents for managing wound infections.

Keywords- Phytochemical, *Citrus aurantium*, Wound, Infection, Bacteria.

I. INTRODUCTION

Wound infection remains a major public health concern worldwide because of its significant contribution to prolonged hospitalization, delayed wound healing, increased healthcare costs, and mortality. A wound is defined as a disruption in the normal anatomical integrity and function of the skin or underlying tissues caused by physical, chemical, thermal, or biological agents. Although the normal wound healing process involves a complex sequence of haemostasis, inflammation, proliferation, and tissue remodelling, microbial invasion can interrupt these stages, resulting in delayed healing and the development of chronic infections (Guo and DiPietro, 2010). The burden of wound infections is particularly pronounced in developing countries where poor hygiene, limited access to healthcare facilities, indiscriminate use of antibiotics, and inadequate infection control practices contribute significantly to disease prevalence (Akinjogunla et al., 2021). Wounds become infected when pathogenic microorganisms colonize damaged tissues, multiply rapidly, and overcome the host's immune defense mechanisms. The warm, moist, and nutrient-rich environment of an open wound provides favourable conditions for microbial growth. Common bacterial pathogens isolated from infected wounds include *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, and *Streptococcus pyogenes* (Akinjogunla et al., 2021; Alzand et al., 2024). Among these organisms, *Staphylococcus aureus* remains one of the most frequently isolated pathogens because of its ability to produce toxins, enzymes, and biofilms that facilitate

tissue invasion and resistance to host immune responses. Similarly, *Pseudomonas aeruginosa* has gained considerable attention owing to its intrinsic resistance to many antimicrobial agents and its ability to establish persistent infections through biofilm formation (Maksoud, et al., 2021). *Citrus aurantium* contains bioactive compounds known as phytochemicals, which can be used as an alternative antibacterial to reduce the spread of increasingly drug-resistant organisms (Oluwaseun and Israel, 2023). *Citrus aurantium* has a long history of usage and is believed to alleviate heart diseases and act as an anti-depressant and tonic among people living in the north of Iran (Ejaz et al., 2005). Information about possible bioactivities of this part of the plant is somewhat limited; therefore, an experiment was conducted to determine phytochemical constituents of the leaves aqueous extract and test the extract against bacteria isolated from wounds.

II. MATERIALS AND METHODS

Samples Collection

Fresh leaves of *Citrus aurantium* tree were collected from the garden of Sidi Mamman dairy farm, in Sokoto State. The plant was transported to the Herbarium section in the Biological Sciences Department of Usmanu Danfodiyo University, Sokoto where it was identified and then taken to Microbiology Laboratory, Usmanu Danfodiyo University, Sokoto for further analysis.

Pus from wounds were aseptically collected using sterile swab sticks from patients attending Kofar Kade Clinic, Sokoto. The swab samples were transported immediately to the Microbiology Laboratory of UDUS and each were soaked in 5mls of sterile nutrient broth and incubated for 24 hours.

Plant leaves Processing / Extraction

The plant leaves were air dried and powdered using clean pestle and mortar. A clean sieve was used to sieve the powdered sample. Fifty grams (50gms) of the powdered sample was suspended into 250mls of distilled water and stirred properly with a glass rod and was allow to stand for 24 hours. The soaked sample mixture was then stirred carefully by shaking the conical flask and then filtered through a clean

muslin cloth, the crude paste was discarded and the supernatant was then evaporated to dryness in hot air oven at 50°C. The residue was collected and stored in sterile containers for further analysis (Obafemi et al., 2006).

Phytochemical analysis

The following tests were conducted to determine phytochemical constituents of *C. aurantium* leaves aqueous extract:

i. Test for Alkaloids

About 2ml of the extract was stirred with 2ml of 10% aqueous Hydrochloric acid. 2–3 drops of Wagner's reagent were then added. The development of an orange-red precipitate (turbidity) was indicative of the presence of Alkaloids (Cuilel, 1994).

ii. Test for Cardiac Glycosides

2ml of 3.5% Ferric chloride solution was added to 2ml of the extract and allowed to stand for one minute. 1ml of concentrated H₂SO₄ was carefully poured down the wall of the test tube to form lower layer. A reddish-brown ring at interface indicates the presence of Cardiac Glycosides (Harborne, 1998).

iii. Test for Flavonoids

1ml of 10% NaOH solution was added to 3ml the extract. The development of yellow color indicates the presence of Flavonoid compounds (Harborne, 1998).

iv. Test for Free Anthraquinones

2ml of the extract was shaken with 2ml benzene, and 2ml of 10% ammonia solution was added. The mixture was shaken and presence of pink, red, or violet color in the ammonia (lower) phase indicates the presence of Anthraquinones (Harborne, 1998).

v. Test for Glycosides

2.5ml of 50% H₂SO₄ solution was added to 5cm³ of the extract in a test tube. The mixture was heated in boiling water for 15 minutes. The mixture was cooled and neutralized with 10% NaOH, 5ml of Fehling's solution was added and the mixture was boiled. A brick-red precipitate was observed which indicate the presence of Glycosides (Harborne, 1998).

vi. Test for Saponnin Glycosides

2.5ml of Fehling's solution A and B was added to 2.5ml of the extract. A bluish green precipitate shows the presence of Saponnin Glycosides (Harborne, 1998).

vii. Test for Saponnin

5ml of the extract was poured into a test tube containing 5ml of distilled water and the mixture was vigorously shaken. The whole tube was filled with froth that last for several minutes. This indicates the presence of Saponnin (Harborne, 1998).

viii. Test for Steroids

5ml of the extract was poured in 2ml of Chloroform. 2ml of Sulphuric acid was carefully added at the side of the test tube. A reddish-brown ring at interface indicates the presence of Steroids (Harborne, 1998).

ix. Test for Tannins

5% of Ferric chloride solution was added drop by drop into 2ml of the extract. The appearance of a dark green or blue-black color indicates the presence of Tannins (Harborne, 1998).

x. Test for Volatile oils

1ml of the extract was mixed with dilute HCl. A white precipitate was formed which indicated the presence of volatile oils (Evans, 2002).

Media Preparation

The media used were Nutrient Broth, Nutrient Agar, Blood Agar and Chocolate Agar and they were all prepared according to Manufacturer's instructions.

Nutrient Agar

Appropriate grams of Nutrient Agar powder were dissolved in the required milliliters of distilled water. The mixture was gently shaken and placed on a hot plate to attain homogeneity. The homogenous mixture was sterilized in an autoclave at 121°C for 15 minutes. After sterilization, the media was allowed to cool and was dispensed into petri plates under flaming before solidification.

Blood Agar

Blood agar was prepared by pouring 5% blood in 95% of Nutrient Agar at 50°C.

Chocolate Agar

Chocolate agar was prepared by heating blood agar on a hot plate until when Chocolate color was obtained.

Inoculation of bacterial cultures

The incubated nutrient broth suspensions (after 24 hours) were streaked onto blood agar and Chocolate agar plates under flaming and were incubated for 24 hours. After incubation, Bacterial colonies on blood agar plates were sub-cultured on Nutrient agar plates using streaking method to obtain pure cultures. The cultures obtained were sub-cultured in agar slant bottles and incubated for further use.

Identification of Bacteria

Microscopic examination and Biochemical tests were used for the identification of bacteria. These include Gram staining, Catalase test, Citrate utilization, Indole test, MR-VP (Methyl Red – Voges Proskauer) test, Urease test and Triple Sugar Iron Test (TSI Test).

Gram staining

A drop of distilled water was placed on a clean, grease free slide, an isolate was picked up with a heated wire loop (after allowing it to cool) and smear was made, it was then allowed to dry. The dried smear was stained with crystal violet solution (primary dye) for 60 seconds. It was then washed with distilled water. The excess water on the slide was drained off to avoid diluting the mordant. The smear was then flooded with iodine solution (mordant) for 30 seconds and then washed with distilled water. It was then decolorized with alcohol (Ethanol), adding it drop wise on the slide until all free color (blue) has been removed and then washed with distilled water. The smear on the slide was then flooded with safranin (counterstain) for 60 seconds; it was then washed with distilled water. It was then allowed to air dry and microscopic examination was carried out under ×100 objective lens (oil immersion) (Oyeleke and Manga, 2008).

Biochemical Tests

Catalase Test

A freshly isolated organism was put on a drop of hydrogen peroxide on a clean glass slide by using sterile wire loop. A characterizing bubbling of gas is

an indication of positive test, where as in negative reaction no gas bubbles was observed (Cheesbrough, 2003).

Citrate Utilization Test

The citrate broth was prepared and sterilized in an autoclave, after it has cooled to 37°C, the test organism was inoculated and then incubated for 24 hours at 37°C. Deep blue color appearance indicates positive test and no color change signify a negative test (Cheesbrough, 2003).

Indole Test

Each isolate was grown in 5ml of peptone water and incubated for 24 hours. After 24 hours of incubation, a kovac's reagent was used, usually three drops. A positive reaction was indicated by the development of a red color in the reagent layer above the broth within one minute. Absence of color change usually yellow indicates negative reaction (Oyeleke and Manga, 2008).

MR-VP (Methyl Red – Voges Proskauers)

This test was performed by inoculating a colony of test organism in 2mls of MRVP broth and incubated at 37°C for 48 hours. After the incubation period, 2–3 drops of methyl red was added. Red color indicates a positive test, while yellow color indicates negative test (Cheesbrough, 2003).

Urease test

Production of ammonium gas leads to the change in the pale-yellow color of the urease medium incorporated with about 0.3ml of urea bright pink color. Freshly isolated organism was taken from the nutrient agar using sterile wire loop and streaked on the slant surface of the urea medium. It was incubated for 24hrs at 37°C. The development of a pink color signifies urease positive, and the color remains unchanged (yellow) for negative result (Cheesbrough, 2003).

Triple Sugar Iron Test (TSI Test)

TSI media was prepared and sterilized in an autoclave. It was then slanted in test tubes to obtain a surface slope and allowed to solidify. Freshly isolated organism was picked with a sterile wire loop and streaked on the surface of the slope and then stabbed

to the bottom. It was then incubated at 37°C for 24hours. After the incubation period, several results were read. The production of H₂S can be detected by the presence of a black color in the media along the stabbed line. Gas production can be detected by the presence of gas bubbles or crack in the agar or complete disruption of the medium. Production of acid which change the color of indicator from red to yellow also observed for Glucose, Sucrose, and Lactose (Cheesbrough, 2003).

Sensitivity Testing

The antibacterial sensitivity testing of the plant was determined using agar-well diffusion method (Irobi et al., 1994). Slant stored cultures were further subculture to obtain fresh cultures of the identified bacteria. A loopful of each isolate from fresh cultures were diluted in separately. 5 ml of sterile water in test tubes to obtain a 0.5 McFarland standard. Sterile swab sticks were dipped into the suspensions of each isolate and were emulsified over Nutrient Agar plates. A sterile cork borer measuring 6 mm in diameter was used to create wells at appropriate intervals in the agar plates previously inoculated with the test organisms under aseptic conditions, after which the prepared extracts of varying concentrations (100, 150, 200, 250 and 300 mg/ml respectively) were introduced into the wells. Tetracycline was placed at the centre of the plates as a control. The agar plates were incubated at 37°C for 24 hours (Ogunnusi, 2006). After the incubation period, the plates were observed for zones of inhibition.

Table 1: Phytochemical Components of the Aqueous Extract of *Citrus aurantium* Leaves

S/No.	Metabolite	Concentration
1	Alkaloids	+++
2	Cardiac Glycosides	+
3	Flavonoids	++
4	Free Anthraquinones	-
5	Glycosides	+
6	Saponnin	+
7	Saponnin Glycosides	+
8	Steroids	+++

9	Tannins	+
10	Volatile Oils	+++

Key: +++ = Highly significant amount;
++ = Significant amount; + = Trace amount;
- = Absent

S/No.	Bacteria
1	Pseudomonas spp.
2	Citrobacter spp.
3	Streptococcus spp.
4	Staphylococcus spp.
5	Bacillus spp.
6	Serratia spp.
7	Enterococcus spp.
8	Klebsiella spp.
9	Aeromonas spp.
10	Propionibacterium spp.
11	Micrococcus spp.

Table 2: Bacteria Isolated from wound infections

Table 3: Diameter of zones of inhibition (mm+6) at varying concentrations (mg/ml) of the extract

S/No.	Isolates	100 mg/ml	150 mg/ml	200 mg/ml	250 mg/ml	300 mg/ml	Control
1	Aeromonas spp.	7	10	8	9	10	5
2	Bacillus spp.	9	11	11	11	11	18
3	Citrobacter spp.	Nz	Nz	Nz	Nz	10	9
4	Enterococcus spp.	8	8	11	16	11	13
5	Klebsiella spp.	Nz	Nz	Nz	9	Nz	14
6	Micrococcus spp.	Nz	Nz	Nz	Nz	Nz	10
7	Propionibacterium spp.	Nz	10	15	11	9	10
8	Pseudomonas spp.	7	9	10	13	9	21
9	Serratia spp.	9	10	11	11	10	4
10	Staphylococcus spp.	7	10	12	10	10	10
11	Streptococcus spp.	Nz	10	10	11	11	10

Key: Nz = No zone of inhibition

The present study evaluated the phytochemical constituents and antibacterial activity of the aqueous leaf extract of *Citrus aurantium* against bacterial isolates recovered from wound infections. The findings demonstrate that the aqueous extract contains several bioactive phytochemicals and possesses measurable antibacterial activity against a broad spectrum of wound-associated bacteria, although the degree of activity varied considerably among the isolates and extract concentrations. The phytochemical screening revealed the presence of alkaloids, steroids, volatile oils, flavonoids, tannins, saponins, saponin glycosides, glycosides, and cardiac

glycosides, while free anthraquinones were absent. Alkaloids, steroids, and volatile oils occurred in relatively high amounts (+++), whereas flavonoids were present in moderate concentrations (++). These findings are consistent with previous reports that *C. aurantium* leaves are rich sources of secondary metabolites with important pharmacological properties (Ejaz et al., 2005; Hamdan et al., 2011; Barreca et al., 2017). Variations in phytochemical composition among different studies are expected because the concentration of plant metabolites is influenced by several factors, including geographical location, climate, soil characteristics, plant maturity, season of harvest, extraction solvent, and extraction

technique (Pandey and Rizvi, 2009; Ncube et al., 2008). Since water was used as the extraction solvent in the present study, some less-polar phytochemicals may not have been extracted efficiently compared with organic solvents such as methanol or ethanol, which are generally reported to produce higher yields of phenolic compounds and flavonoids.

The high abundance of alkaloids observed in this study may explain part of the antibacterial activity demonstrated by the extract. Alkaloids are well known for interfering with bacterial DNA replication, protein synthesis, and cell division, thereby inhibiting bacterial growth (Cushnie et al., 2014). Likewise, flavonoids contribute significantly to antimicrobial activity by disrupting microbial membranes, inhibiting nucleic acid synthesis, reducing bacterial adhesion, and interfering with quorum sensing and biofilm formation (Xie et al., 2015; Farhadi et al., 2019). Tannins possess the ability to precipitate proteins and inhibit microbial enzymes, while saponins interact with membrane sterols, increasing membrane permeability and causing leakage of intracellular contents (Daglia, 2012). The presence of volatile oils is particularly important because essential oil constituents such as limonene, linalool, β -pinene, and linalyl acetate have been reported to exhibit antimicrobial, antioxidant, and anti-inflammatory properties that contribute to wound healing (Barreca et al., 2017; Chouhan et al., 2017). Steroids have also been associated with membrane stabilization and anti-inflammatory activities that may complement antimicrobial effects during tissue repair.

The bacterial isolates recovered from wound infections included *Pseudomonas* spp., *Citrobacter* spp., *Streptococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Serratia* spp., *Enterococcus* spp., *Klebsiella* spp., *Aeromonas* spp., *Propionibacterium* spp., and *Micrococcus* spp. These organisms represent both Gram-positive and Gram-negative bacteria commonly associated with wound infections worldwide. Similar bacterial profiles have been reported in numerous studies investigating wound microbiology, where *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, and members of the family

Enterobacteriaceae predominate (Akinjogunla et al., 2021; Alzand et al., 2024). The recovery of multiple bacterial genera reflects the polymicrobial nature of wound infections, particularly chronic wounds where several microorganisms coexist within biofilms. Biofilm formation substantially reduces antibiotic penetration and enhances bacterial tolerance to antimicrobial agents, making treatment increasingly difficult (Malone et al., 2017; Omar et al., 2017). The antibacterial susceptibility results demonstrated concentration-dependent activity of the aqueous extract against several isolates. In general, larger inhibition zones were observed as extract concentration increased, although some organisms exhibited irregular responses. *Bacillus* spp. showed consistent susceptibility across all tested concentrations, producing inhibition zones of 9–11 mm. This observation may be attributed to the relatively permeable cell wall structure of Gram-positive bacteria, which allows easier penetration of phytochemicals compared with Gram-negative bacteria possessing an outer membrane rich in lipopolysaccharides that restricts diffusion of antimicrobial compounds (Silhavy et al., 2010).

Enterococcus spp. exhibited the greatest susceptibility at 250 mg/ml with an inhibition zone of 16 mm before declining slightly at 300 mg/ml. Such fluctuations have been reported previously and may result from differences in phytochemical diffusion through agar media, interactions among bioactive compounds, or experimental variability associated with crude plant extracts (Balouiri et al., 2016). Similarly, *Propionibacterium* spp. demonstrated appreciable susceptibility, reaching 15 mm at 200 mg/ml, suggesting that the extract contains compounds capable of inhibiting anaerobic skin-associated bacteria involved in wound colonization. The activity against *Pseudomonas* spp. deserves particular attention because this organism is among the most difficult wound pathogens to eradicate due to its intrinsic resistance mechanisms, including multidrug efflux pumps, reduced membrane permeability, enzyme production, and extensive biofilm formation (Pang et al., 2019). Although inhibition zones ranging from 7 to 13 mm indicate only moderate activity, the findings suggest that the extract possesses compounds capable of partially

overcoming the organism's defense mechanisms. The moderate inhibition may be explained by synergistic interactions among flavonoids, terpenoids, and essential oil constituents present in *C. aurantium*. Previous investigations have similarly reported that citrus-derived essential oils and extracts inhibit *Pseudomonas* species, although higher activities are generally observed with essential oils or ethanolic extracts than aqueous preparations (Barreca et al., 2017).

Staphylococcus spp., another major wound pathogen, exhibited inhibition zones ranging from 7 to 12 mm. This finding supports previous reports that citrus phytochemicals possess antistaphylococcal activity through membrane disruption, enzyme inhibition, and oxidative stress induction (Xie et al., 2015). The comparable inhibition produced by the extract and tetracycline against *Staphylococcus* spp. in this study suggests that aqueous *C. aurantium* leaves may possess promising antibacterial potential deserving further investigation. The absence of inhibition against *Micrococcus* spp. at all tested concentrations and the limited activity against *Citrobacter* spp. and *Klebsiella* spp. indicate species-specific susceptibility. Gram-negative bacteria such as *Citrobacter* and *Klebsiella* possess an outer membrane containing lipopolysaccharides that acts as an effective permeability barrier against many hydrophilic antimicrobial compounds extracted by water (Silhavy et al., 2010). Furthermore, these organisms commonly produce β -lactamases, multidrug efflux pumps, and other resistance mechanisms that reduce susceptibility to both conventional antibiotics and plant-derived compounds (Murray et al., 2022). The lack of activity against *Micrococcus* spp. may reflect intrinsic resistance or insufficient concentrations of active phytochemicals in the aqueous extract.

Interestingly, tetracycline did not consistently outperform the plant extract against every isolate. While tetracycline produced larger inhibition zones against *Bacillus* spp., *Pseudomonas* spp., *Klebsiella* spp., and *Enterococcus* spp., the aqueous extract demonstrated comparable or superior activity against *Aeromonas*, *Serratia*, and *Staphylococcus* spp. Such observations support growing evidence that

medicinal plants may provide alternative antimicrobial agents capable of supplementing existing antibiotics, particularly as antimicrobial resistance continues to increase globally (World Health Organization, 2024). Nevertheless, the generally smaller inhibition zones produced by the extract compared with tetracycline indicate that crude aqueous extracts possess lower antibacterial potency than purified antibiotics. The variable antibacterial activity observed in this study also reflects the complexity of crude plant extracts. Unlike purified antimicrobial drugs, crude extracts contain numerous phytochemicals that may exhibit synergistic, additive, or antagonistic interactions. Synergistic effects among flavonoids, alkaloids, terpenoids, and phenolic compounds have been reported to enhance antibacterial efficacy by simultaneously targeting multiple bacterial pathways, thereby reducing the likelihood of resistance development (Farhadi et al., 2019). However, antagonistic interactions may also reduce the overall antimicrobial activity observed in crude preparations.

III. CONCLUSION

The findings of this study demonstrate that the aqueous leaf extract of *Citrus aurantium* contains several biologically active phytochemicals, including alkaloids, flavonoids, steroids, tannins, saponins, glycosides, cardiac glycosides, and volatile oils, which are known to possess antimicrobial properties. The extract exhibited varying degrees of antibacterial activity against several bacterial species isolated from wound infections, including *Staphylococcus* spp., *Pseudomonas* spp., *Enterococcus* spp., *Bacillus* spp., *Serratia* spp., *Aeromonas* spp., *Propionibacterium* spp., and *Streptococcus* spp. However, little or no activity was observed against *Micrococcus* spp. and some isolates of *Citrobacter* spp. and *Klebsiella* spp. at lower extract concentrations. Although the antibacterial activity of the aqueous extract was generally lower than that of tetracycline, the extract demonstrated appreciable inhibitory effects against several clinically important wound pathogens, suggesting that *Citrus aurantium* leaves possess promising antimicrobial potential. The antibacterial effects observed are likely attributable to the

synergistic action of the phytochemical constituents present in the leaves.

Overall, this study supports the traditional medicinal use of *Citrus aurantium* in the treatment of infections and highlights its potential as a natural source of antibacterial compounds. The plant may provide an alternative or complementary therapeutic option for the management of wound infections, particularly in the face of increasing antimicrobial resistance. Further studies involving solvent extraction, isolation and characterization of the active compounds, determination of minimum inhibitory and bactericidal concentrations, toxicity assessment, and in vivo evaluations are recommended to establish its safety, efficacy, and potential for pharmaceutical development.

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