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# Phytochemical constituents of methanolic and chloroform extracts of *citrus sinensis* peels and their antimicrobial effects on some human pathogens

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**Abstract**

The study investigated the phytochemical constituents and antimicrobial effects of chloroform fractions and residual methanolic extracts of *Citrus sinensis* peels dried at different environmental conditions on *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aspergillus niger* (test pathogens) using the Agar - Well diffusion method. The results of the analysis showed that the phytochemical constituents of *Citrus sinensis* peels were not affected by the different drying conditions of the peels. The presence of tannins, alkaloids, cardiac glycosides, flavonoids and phenols was observed in both residual methanolic extracts (A and B) while no saponins and anthroquinones were found in the extracts. Only cardiac glycoside was present in the chloroform fractions (X and Y). Significant inhibitory effects of all the extracts on some of the organisms were recorded. The minimum inhibitory concentration (MIC) values for the extracts ranged between 12.5 to 50 mg/ml. Extract A indicated the best MIC value of 12.5 mg/ml for *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. Also, the minimum bactericidal concentration (MBC) values of the extracts, ranged between 25 to 100 mg/ml. An extended inhibitory activity was observed for extract B against *Escherichia coli* possibly due to the drying condition of the peels, however, with a higher MIC value of 50 mg/ml and a bacteriostatic effect. None of the extracts showed antifungal activity. The moderate antibacterial activity demonstrated by the extracts, depicts the medicinal potentials of *Citrus sinensis* peels thus, justifying its use in ethnomedicine against bacterial pathogens.

**Keywords**: Antimicrobial, *Citrus sinensis*, pathogens, peels, phytochemical.

## 1. Introduction

Plants have been a valuable source of natural products with interesting therapeutic effects for decades. The therapeutic properties of plants are mainly due to the secondary metabolites they synthesize and store in various parts within. Infectious diseases due to microorganisms have continually threatened the existence of man and animals. They account for one-half of all deaths in the tropics [1, 2]. Also, some of these microorganisms are becoming multi-drug resistant to many of the available commercial antimicrobial drugs. Hence, the a need for continuous research into plants with medicinal potentials with the view of finding new and better alternative treatment agents that will help manage and alleviate the menace caused by the pathogens.

Citrus fruits belong to the Rutaceae family. It is the largest and most important fruit crop in the world [3-5]. Global production has rapidly and strongly grown beyond 100 million metric tons per year in the last decade [6, 7]. In 2011, it had an estimated global production of approximately 123 million tons [3, 4]. Citrus is an excellent source of vitamin C, Ca, K, thiamin, niacin, Mn and a sufficient amount of folacin [8]. The plants have been used traditionally to treat constipation, cramps, colic diarrhoea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression, and stress management among others [9]. Similarly, *Citrus sinensis* extracts have been deployed to treat and prevent issues of vitamin deficiencies, flu, and viral and bacterial infections among others [10]. Phytochemicals and bioactive compounds in Citrus fruits such as the phenolic compounds, carotenoids, vitamins, fibre, malingering and hesperidin, have been seen to aid their antibacterial activity [11-14].

The Orange accounts for 60 percent of Citrus fruit's global production [5] and the peel forms about 45 percent of the total bulk [15]. As such, a huge amount of orange peels is usually available as a product and can be turned into assets if properly harnessed.

There is limited empirical research available on the waste peel of *Citrus sinensis* (sweet orange) also known as Oloma, Lemu and Oloso among the Igbo, Hausa and Yoruba major ethnic tribes of Nigeria respectively. Ethyl acetate extract of the peel contains complex compounds such as limonoids, alkaloids, flavonoids, steroids, triterpenoids, phenolics, saponins and coumarins [16]. Lemonoids, the active component of insecticides have been observed to exhibit a broad

spectrum larvicidal activity against mosquitoes [17, 18]. Ethyl acetate extract of the peel against some gram-positive and gram-negative microorganisms showed great inhibitory activities at all the test concentrations [19]. Essential oil, water and ethanol extracts of peels of *Citrus sinensis* have also been observed to have a positive effect on *Escherichia coli* and Vibrio species [20, 21].

Attempt to contribute to continuous efforts to find new antimicrobial agents prompted this research. The current study is focused on the phytochemical constituents of the chloroform fractions and residual methanolic extracts of peels of *Citrus sinensis* dried at different conditions and their antimicrobial effects on some pathogenic microorganisms.

## 2. Materials and methods

### *2.1. Plant materials*

*Citrus sinensis* peels were obtained from a fruit vendor in Sabon gari Zaria local government area, Kaduna state Nigeria in January 2022. The peels were properly washed with cold tap water to remove sand and soil particles and then divided into two portions labeled, A and B. Portion A was dried outside in an open space under the sun and portion B was dried in an enclosed space at room temperature. The dried peels were separately milled using a wooden mortar and pestle and the milled samples were stored in separate bags and kept for later use.

### *2.2 Extracts preparations*

The powdered plant materials 60 g each were soaked with 250 ml of methanol in an aspirator and agitated at intervals for 48 h. The extracts formed were filtered using number 1 Wattman filter paper and the filtrate was concentrated in a rotary at 40°C, and further air dried to constant weights to give crude methanolic extracts A and B. The dried crude extracts formed were separately soaked in 150 mL of chloroform in an air-tight glass container and were kept for extraction with regular agitation for a period of 48 h after which, the extracts were percolated using number 1 Wattman filter paper, leaving behind the residual methanolic extracts A and B, and the filtrates concentrated using a rotary at 40°C and further air dried to constant weights to give chloroform fractions X and Y respectively.

### *2.3 Phytochemical screening*

This was carried out on each of the extracts for the presence or absence of tannins, alkaloids, cardiac glycosides, saponins, flavonoids, phenols and anthraquinone using the standard method described by Kumar *et al.* and Nwachukwu *et al.* [22, 23].

### *2.4 Antimicrobial screening*

This was determined for all the extracts *in vitro* using some human pathogens of *ococStaphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeroginosa, Candida albicans* and *Aspergillus niger* by the Agar- Well diffusion method as reported by Madumelu *et al.* [24, 25, 26]. The organisms were obtained from the medical microbiology unit, at Ahmadu Bello University Teaching Hospital Zaria, Nigeria. The antimicrobial effect of the extracts on the organisms was compared with those of Ciprofloxacin and Econazole drugs used as positive controls for the bacteria and fungi organisms respectively in the study.

### *2.5 Media used*

Mueller–Hinton Agar (MHA) and Potato Dextrose Agar (PDA) were used as the growth medium for both the bacteria and fungi organisms respectively.

### *2.6 Sensitivity test*

The antimicrobial growth media were prepared according to the manufacturer's instructions and sterilized at 121°C for 15 min. The initial concentration of each extract used for the Zones of Inhibition (ZOI) analysis was 100 mg/ml. Two-fold serial dilution was made to obtain other concentrations of 50, 25 and 12.5 mg/ml. These were the concentrations of the extracts used for the ZOI analysis, and the results were measured and recorded in millimetres (mm).

### *2.7 Minimum inhibitory concentration (MIC)*

The MIC analysis was conducted to know the minimum concentration at which each of the extracts will inhibit the growth of the organisms. This was done using the broth dilution method. Mueller-Hinton broth was used as the diluent and was prepared according to the manufacturer's instructions. MC Farland's standard turbidity scale number 0.5 was equally prepared to give turbid solution which was used as the test criteria. The test organisms (0.1 ml) in normal saline were then respectively inoculated into different test tubes of different prepared concentrations of each extract that showed positive results of the ZOI test. The respective contents were incubated at $37^0$ C for 24 h after which each tube was then observed for the presence or absence of growth. The lowest concentration in each series with no notable sign of growth (Absence of turbidity) was recorded as the MIC.

### *2.8 Minimum bactericidal concentration (MBC)*

The MBC analysis was done to ascertain the effect of the antimicrobial agents on the organisms, whether bacteriostatic or bactericidal. The MIC results were used to determine the MBC for each extract. A wire loop was sterilized and then dipped into each test tube containing the MIC content for a loopful. This was streaked on sterile nutrient agar plates. The incubation of each plate was done at $37^0$ C for 24 h and was examined for the presence or absence of growth. The lowest concentration of the extracts without growth was recorded as the MBC.

## 3. Result and Discussion

**Table 3.1:** Weight of extracts produced (g)

| A | B | X | Y |
|---|---|---|---|
| 3.2 | 4.8 | 1.2 | 0.9 |

**Table 3.2:** Result of phytochemical screening

| **Phytochemicals** | **A** | **B** | **X** | **Y** |
|---|---|---|---|---|
| Tannins | + | + | - | - |
| Alkaloids | + | + | - | - |
| Cardiac glycosides | + | + | + | + |
| Saponins | - | - | - | - |
| Flavanoids | + | + | - | - |
| Phenols | + | + | - | - |
| Anthraquinone | - | - | - | - |

KEY: + = Present - = Absent

**Table 3.3:** Antimicrobial result of extract A

| | Concentration of extract (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Test organisms | 100 | 50 | 25 | 12.5 | Controls | MIC | MBC |
| *Staphylococcus aureus* | 16 | 13 | 11 | 0 | 16 | 12.5 | 25 |
| *Bacillus subtilis* | 16 | 14 | 10 | 0 | 32 | 12.5 | 25 |
| *Escherichia coli* | 0 | 0 | 0 | 0 | 30 | ND | ND |
| *Pseudomonas aeruginosa* | 14 | 11 | 10 | 0 | 40 | 12.5 | 25 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 26 | ND | ND |
| *Aspergillus niger* | 0 | 0 | 0 | 0 | 32 | ND | ND |

KEY: ND = Not Determined

**Table 3.4:** Antimicrobial result of extract B

| | Concentration of extract (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Test organisms | 100 | 50 | 25 | 12.5 | Controls | MIC | MBC |
| *Staphylococcus aureus* | 15 | 12 | 10 | 0 | 16 | 12.5 | 25 |
| *Bacillus subtilis* | 14 | 12 | 0 | 0 | 32 | 50 | 100 |
| *Escherichia coli* | 14 | 12 | 0 | 0 | 30 | 50 | NIL |
| *Pseudomonas aeruginosa* | 16 | 13 | 10 | 0 | 40 | 12.5 | 25 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 26 | ND | ND |
| *Aspergillus niger* | 0 | 0 | 0 | 0 | 32 | ND | ND |

KEY: ND = Not Determined, NIL = Bacteriostatic

**Table 3.5:** Antimicrobial result of extract X

| | Concentration of extract (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Test organisms | 100 | 50 | 25 | 12.5 | Controls | MIC | MBC |
| *Staphylococcus aureus* | 16 | 14 | 12 | 0 | 16 | 12.5 | 25 |
| *Bacillus subtilis* | 18 | 15 | 12 | 0 | 32 | 12.5 | 50 |
| *Escherichia coli* | 0 | 0 | 0 | 0 | 30 | ND | ND |
| *Pseudomonas aeruginosa* | 16 | 13 | 10 | 0 | 40 | 25 | 50 |
| *Candida albicans* | 0 | 0 | 0 | 0 | 26 | ND | ND |
| *Aspergillus niger* | 0 | 0 | 0 | 0 | 32 | ND | ND |

KEY: ND = Not Determined

**Table 3.6:** Antimicrobial result of extract Y

| | Concentration of extract (mg/ml) | | | | | | |
|---|---|---|---|---|---|---|---|
| Test organisms | 100 | 50 | 25 | 12.5 | Controls | MIC | MBC |
| *Staphylococcus aureus* | 20 | 18 | 16 | 0 | 16 | 25 | 50 |
| *Bacillus subtilis* | 16 | 13 | 10 | 0 | 32 | 12.5 | 25 |
| *Escherichia coli* | 0 | 0 | 0 | 0 | 30 | ND | ND |
| *Pseudomonas aeruginosa* | 0 | 0 | 0 | 0 | 40 | ND | ND |
| *Candida albicans* | 0 | 0 | 0 | 0 | 26 | ND | ND |
| *Aspergillus niger* | 0 | 0 | 0 | 0 | 32 | ND | ND |

KEY: ND = Not Determined

The results of the phytochemical analysis (Table 3.2) showed that the phytochemical constituents of *Citrus sinensis* peels were not affected by the different drying conditions of the plant materials. The result revealed the presence of tannins, alkaloids, cardiac glycosides, flavonoids and phenols in both residual methanolic extracts (A and B) and the absence of saponins and anthraquinones in the extracts. Also, only cardiac glycoside was seen to be present in the chloroform fractions (X and Y). The difference in polarity of the solvents could be the reason for this outcome, as more

of the metabolites tend to be solubilized by the more polar methanol solvent.

The results of ZOI, MIC and MBC of the extracts on the test organisms were summarized in Tables 3.3 to 3.6. Each of the four extracts was effective against at least two or more of the organisms in a concentration-dependent manner. Their ZOI ranged between 10 to 20 mm. Extract A was effective on *Staphylococcus aureus, Bacillus subtilis*, and *Pseudomonas aeruginosa*, and had ZOI values of 16, 16, and 14 mm respectively (Table 3.3). Also, extract B, showed the ZOI values of 15, 14, 14, and 16 mm on *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* respectively (Table 3.4). Furthermore, the ZOI values of extract X were recorded as 16, 18, and 16 mm for *Staphylococcus aureus, Bacillus subtilis*, and *Pseudomonas aeruginosa* respectively (Table 3.5). Lastly, extract Y inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis* by 20 and 16 mm respectively (Table 3.6). All the extracts were insensitive to the fungi organisms in all the test concentrations. Furthermore, extract B showed a relatively wider spectrum activity in that, only the extract was sensitive to *Escherichia coli* (Table 3.4). The observed wider spectrum activity of the extract could be due to the different environmental conditions at which the plant materials were dried as well as the solvent used for its extraction. The antibacterial effects of the extracts on the test organisms in the current study were found to be less favourable than those of the positive control drugs.

The MIC values of the extracts ranged between 12.5 to 50 mg/ml and were most effective against *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* with MIC values of 12.5 mg/ml for extract A (Table 3.3). Also, extract X, showed a similar MIC result as extract A except that the value is slightly higher for *Pseudomonas aeroginosa* (MIC = 25 mg/ml) (Table 3.5). Furthermore, extract B had almost a similar MIC result with A and X, but with a higher MIC value of 50 mg/ml for *Bacillus subtilis* and *Escherichia coli* (Table 3.4). Lastly, extract Y showed MIC values of 25 and 12.5 mg/ml for *Staphylococcus aureus* and *Bacillus subtilis* respectively (Table 3.6).

The concentrations at which the antimicrobial agents could kill the test organisms (MBC values) ranged between 25 to 100 mg/ml and were observed for *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa* for the extracts except for extract Y which was bactericidal on only *Staphylococcus aureus* and *Bacillus subtilis* (Table 3.6). Extract B demonstrated a bacteriostatic effect on *Escherichia coli* (Table 3.4). The results of the current antimicrobial studies are consistent with the previous reports of antimicrobial activities of *Citrus sinensis* peels on similar organisms [27, 28]. The antibacterial potency of extracts of *Citrus sinensis* peels on many enteric pathogens was reported [29]. The extracts of *Citrus sinensis* peels were found to be effective against *Klebsiella pneumonia* [30]. Phenolics contents and the antimicrobial properties of fresh and dry *Citrus sinensis* peel extracts were recorded with the fresh peel extract having a better effect on most of the studied pathogens except for *Aspergillus niger* and *Penicillium notatum* which were inhibited more by the dry peel extract [31]. The antibacterial effect of *Citrus sinensis* peel extracts against dental caries pathogens of *Streptococcus mutans* and *Lactobacillus acidophilus* that cause tooth decay was reported [32]. The observed significant antimicrobial effects demonstrated by the extracts on the organisms in the current study may be strongly related to the presence of the secondary metabolites, and the bioactive compounds synthesized by the plant and stored in the peels.

## 4. Conclusion

The current study revealed that the various extracts of *Citrus sinensis* peels, dried at different environmental conditions have antibacterial potentials, particularly on *Staphylococcus aureus, Bacillus subtilis* and *Pseudomonas aeruginosa*. None of the extracts showed antifungal activity. The antibacterial properties of the peels diminished when dried under the sun, but the phytochemical constituents were not affected. The observed moderate antibacterial activities demonstrated by the extracts, depict the medicinal potentials of the peels of *Citrus sinensis* thus, confirming the use of the plant in ethnomedicine for the treatment of many ailments of bacteria origin.

## 5. Recommendation

There is a need for more studies towards isolation, identification and characterization of the bioactive agents from the various extracts and fractions of the peels, and their activity test with a wider spectrum of organisms conducted for better insight into the medicinal potentials of the plant material. Also, additional *in vivo* pharmacological studies are recommended to validate the results of the *in vitro* studies.

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