Antibacterial activity of sweet orange (Citrus sinensis) juice extract on selected bacteria

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Plants have potentials to be developed into many new drugs yet to be discovered because of the countless chemical compositions in them. The investigation is targeted at the antibacterial activity of sweet orange juice extract on some bacteria using ethanol and ethyl ethanoate solvent to extract juice. Ditch method was used for the sensitivity testing against Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae and Neisseria gonorrheae with a dilution factor of $10^{-10}$ for inoculation from pure culture of each selected bacteria. Disc method was used to test streptomycin, ciprofloxacin, gentamycin and penicillin G against test organisms as positive controls. There was no significant difference in the effect of different concentrations of the same extract on test organisms. However, there was a significant difference in the ethyl ethanoate and alcohol extracts. The ethyl ethanoate extract showed minimum inhibitory concentration at 300 mg/ml on E. coli (31.5 ± 0.5 mm); N. gonorrheae (21 ± 0.0 mm) at 200 mg/ml; S. aureus (22 ± 0.0 mm) and K. pneumoniae (37 ± 3.0 mm) at 100 mg/ml; while ethanol extract at 100 mg/ml on E. coli (23.5 ± 1.5 mm) and K. pneumoniae (25 ± 5.0 mm); N. gonorrheae (13.5 ± 1.0 mm) and S. aureus (12.5 ± 2.5 mm) at 300 mg/ml and 200 mg/ml respectively. The zones of inhibition exhibited by streptomycin ranges from N. gonorrheae (14-24 mm) E. coli; ciprofloxacin varies from 15-21 mm on K. pneumoniae and S. aureus respectively. Gentamycin ranges from 14-20 mm on N. gonorrheae and S. aureus respectively; and penicillin G on N. gonorrheae (14 mm) and S. aureus (28 mm). It can be concluded that sweet orange juice of ethyl ethanoate extract was more effective than the ethanol extract and the positive control.

Key words: Antibacterial activities, ethanolic extract, ethanolic extract, sweet orange and microorganisms.

INTRODUCTION

Medicinal plants can be developed into many new drugs yet to be discovered because of the extraordinarily large chemical constituents found in them. The use of herbal medicine in Africa and Asia had been traced back to the time immemorial. The part of plants used as drug vary from the roots, barks, stems, leaves and seed as extracts and concoctions (Hassan et al., 2013). Many plants were used as antimicrobial agents because of various

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chemical constituents found in them. However, recently attention had been drawn towards extracts and biologically active compound from popular plant species. Plants have ability to synthesize aromatic substances such as phenolic, (for example phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, and tannins), nitrogen compounds (alkaloids, amines), vitamins, terpenoids (including carotenoids) and some other endogenous metabolites (Alo et al., 2012). These substances serve as plant defense mechanisms against predators like microorganism, insects and herbivores (Badar et al., 2008).

Orange is one of the most important commercial fruit cultivated on all continents of the world. The importance of the orange is attributed to its diversified use and cultivation worldwide and which probably stands first among the cultivated fruits. Citrus sinensis (sweet orange) is widely cultivated in Nigeria and many other tropical and subtropical regions (Piccinelli et al., 2008). Sweet orange commonly called orange is a member of the family Rutaceae and a main source of vitamins, especially vitamin C; but also has sufficient amount of folic acid, calcium, potassium, thiamine, niacin and magnesium (Angew, 2007). Sweet orange is the major source of vital phytochemical nutrients and for a long time have been valued for their wholesome nutrition and antioxidant properties. It has been scientifically established beyond reasonable doubt that oranges are very rich in vitamins and minerals that are beneficial to humans as nutrient and immune booster. According to Doughari and Manzara (2008), sweet orange juice can be used in the development of safe antibiotics for the treatment of bacterial infections. It was recently appreciated that other biologically active and non-nutrient compounds present in sweet orange juice such as antioxidants, as well as soluble and insoluble dietary fibers are reported to reduce the risk of cancers; while many chronic diseases such as arthritis, obesity and coronary heart diseases have been treated with sweet orange juice (Crowell, 1999).

Rehman et al. (2007) reported that essential oil of the citrus juice exhibits antifungal, antibacterial, antiviral and anti-parasitic properties. Recently, many microorganisms have developed resistance against many conventional antibiotics; because of acquisition and expression of resistant genes in them (Bakhru, 2001). Furthermore; conventional antibiotics had been associated with adverse health effects such as hypersensitivity, allergic reactions and immune suppressions (Ahmed and Beg, 2001). Hence, time had come to develop new antibiotics that are safe for the treatment of infectious disease. According to Bhardwaj and Laura (2009), fruits and plants possess secondary metabolites that can inhibit and kill most pathogens. The difference in the antibacterial activity of the various extracts showed that different extracts have varying antibacterial agents with different modes of action and bacteria susceptibility or that not all phytochemicals responsible for antibacterial activity are soluble in a single solvent (Kumar et al., 2011; Badar et al., 2008). Fruits are considered to have great potential therapeutic treatment for various microbial diseases and it is therefore necessary to carry out a study to validate the antibacterial activity of sweet orange on selected bacteria.

MATERIALS AND METHODS

Biological sample

Sample collection

Ten fresh sweet oranges (C. sinensis) free from insect infestation and other kinds of damage were plucked at early morning (7 am) from the Lagos State Polytechnic campus at Ikorodu area of Lagos State, Nigeria (Latitude 6.5945°N Longitude 3.3370°E).

Microorganisms

Pure cultures (clinical isolate) of test organisms were obtained from Nigeria Medical Research, Yaba (NIMER). The test organisms were purified by sub culturing and preserved on nutrient agar at 4°C before use. They included their reference numbers: Staphylococcus aureus (ATCC 25923) (gram positive bacteria), Escherichia coli (ATCC 25922), Klebsiella pneumoniae (ATCC 13883) and Neisseria gonorrhoeae (ATCC 49226) (gram negative bacteria).

Phytochemical screening of C. sinensis juice

The phytochemical analysis was carried out using the method described by Odebiyi and Sofowora (1978). The orange juices were screened for the presence of tannins, saponins, flavonoids, steroids, amino acid, terpenoids, carbohydrate, alkaloids as well as oil and fat.

Extraction of sweet orange juice

The sweet oranges were washed several times using clean water, peeled and sliced into halves and the juice were squeezed or squashed into the beaker. Orange juice (500 ml) was measured into two flasks each and 1000 ml of solvents (100% Ethyl ethanoate and 70% Ethanol) were added to the orange juice to make two different mixtures. The mixtures were left for two days to enable the solvents extract the active ingredients in the orange juice. The mixtures were filtered through Whatman filter paper (number 4) so as to obtain clean and clear filtrate from the residues of the extracts. The filtrates were concentrated in a vacuum using a rotary evaporator model (Buchi rotavapor R – 114) which ensures evaporation of the bulky solutions (filtrates) to the smaller volume concentrates (semi-solid) at temperature between 40 – 60°C. The resultant concentrates (extracts) were filter-sterilized using milipore filter 0.45 μm and they were ready for the antibacterial activities.

Preparation of extract concentration and the isolate

Six labeled beakers were separated into two groups. The first three breakers were used for the preparation of extract from ethyl ethanoate and the other three were used for the preparation of
Table 1. Phytochemical Constituents of C. sinensis juice.

| Active ingredients  | Quantitative analysis | Inférence         |
|---------------------|-----------------------|-------------------|
| Tannins             | ++                    | Moderate amount   |
| Saponins            | +++                   | High amounts      |
| Flavonoids          | ++                    | Moderate amount   |
| Steroids            | +++                   | High amounts      |
| Amino acid          | +                     | Slightly detected |
| Carbohydrate        | +                     | Slightly detected |
| Terpenoids          | +++                   | High amounts      |
| Alkaloids           | +++                   | High amounts      |
| Oil and fats        | +                     | Slightly detected |

\[
\sigma = \sqrt{\frac{\Sigma (x_i - \mu)^2}{N}}
\]

\( \sigma = \) plates standard deviation
\( \Sigma = \) summation
\( N = \) the size of the diameter of zone of inhibition
\( x_i = \) each value of diameter of zone of inhibition
\( \mu = \) mean value of the plates (diameter of zone of inhibition) in mm

The result from the above formulae (data) were then expressed as mean ± SEM (standard error mean) of duplicates and subjected to one-way analysis of variance (ANOVA), using the Statistical Analysis System (SAS 9.4 Version).

RESULTS AND DISCUSSION

Table 1 illustrates phytochemical constituents of sweet orange juice extract and the followings were present: tannins, saponin, flavonoid, terpenoid, steroid, amino acid, carbohydrate, alkaloid and oil and fat. These results were similar with the finding of Baba et al. (2018), except that amino acid was absent; while steroid and oil and fat were not analysed at all. Pytochemical analysis of the juice extracts showed that plant constituents such as alkaloids, saponins, terpenoid, tannins and flavonoid were present and that saponin in sweet orange juice was responsible for the antibacterial properties of the juice. According to Kumar et al. (2011) Citrus sinensis juice has large amount of saponin with haemolytic activity and cholesterol binding properties.

Table 2 shows that at 95% confidence level, there was a significant difference in the antibacterial activities (zones of inhibition) of extracts (ethyl ethanoate and ethanol respectively) on E. coli, K. pneumoniae, N. gonorrhoeae and S. aureus. Furthermore, according to the Duncan Post Hoc analysis of the ANOVA; there was no significant difference between the means of the zones of inhibition of the extracts (ethyl ethanoate and ethanol respectively) on N. gonorrhoeae and S. aureus and also there was no significant difference between the means of the zones of inhibition of the extracts (ethyl ethanoate and ethanol respectively) on E. coli and K. pneumoniae.
This means that the zones of inhibition of extracts on *E. coli* and *K. pneumoniae* were higher compared to the zones of inhibition of extracts on *N. gonorrheae* and *S. aureus*.

There was no significant difference in the effect of different concentrations of the same extract on *E. coli*, *K. pneumoniae*, *N. gonorrheae* and *S. aureus*. This means that the change in the concentration of the same extract does not affect or improve the potency of the antibacterial activities of the extract but there was a significant difference in the type of extract (ethyl ethanoate and ethanol extracts) that is to say ethyl ethanoate extract was more effective than the ethanol extract. Thus ethyl ethanoate extract showed a remarkable inhibition against *K. pneumoniae* (37±3.0 mm) and *E. coli* (29.5±0.5 mm) compared to ethanol extract on the same test organisms; which showed lower zones of inhibition. Gram negative bacteria have been reported to be more resistant to antibacterial agents due to the possession of an outer membrane permeability barrier that prevents the antimicrobial agents to reach inner part of the bacterial cell. The antibacterial activity against *E. coli* (gram negative) and *S. aureus* (gram positive) bacteria used in this study is an indication of its broad spectrum activity. This observation is in agreement with the report of Doughari and Manzara (2008) and Kumar et al. (2011). Ethyl ethanoate 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. This result concurs with the Kumar et al. (2011) findings.

Kumar et al. (2011) reported a maximum zone of inhibition (16 mm) against *E. coli* with ethyl ethanoate extract of the sweet orange juice. The variation in the antibacterial activity of the various extracts showed that different extracts have varying antibacterial agents with different modes of action and bacteria susceptibility or that not all phytochemicals responsible for antibacterial activity are soluble in a single solvent (Kumar et al., 2011 and Badar et al., 2008). Ethyl ethanoate extract was found to be a good solvent for the extraction of antibacterial agent in this study as it had shown the

| Organisms / Extract type | Concentration of extracts in (mg/ml)/ Means of zone of inhibition (mm) | Minimum inhibitory concentration (MIC)(mg/ml) |
|-------------------------|-------------------------------------------------|---------------------------------------------|
|                         | 100  | 200  | 300  |
| **Escherichia coli**    |      |      |      |
| Ethyl ethanoate extract | 29.5±0.5 | 29±1.0 | 31.5±0.5 | 300 |
| Ethanol extract         | 23.5±1.5 | 22.5±0.5 | 22±1.0 | 100 |
| **Klebsiella pneumoniae** |      |      |      |
| Ethyl ethanoate extract | 37±3.0 | 36±1.0 | 36±2.0 | 100 |
| Ethanol extract         | 25±5.0 | 21.5±3.5 | 19.5±0.5 | 100 |
| **Neisseria gonorrheae** |      |      |      |
| Ethyl ethanoate extract | 17.5±0.5 | 21±0.0 | 21±1.0 | 200 and 300 |
| Ethanol extract         | 0.0±0.0 | 6±6.0 | 13.5±1.0 | 300 |
| **Staphylococcus aureus** |      |      |      |
| Ethyl ethanoate extract | 19±2.0 | 21.5±0.5 | 22±0.0 | 300 |
| Ethanol extract         | 10±0.0 | 12.5±2.5 | 5.5±5.5 | 200 |

ANOVA of antibacterial activities of orange juice on different bacteria at different concentrations

| Source               | Type III sum of squares | Df | Mean square | F     | P value |
|----------------------|-------------------------|----|-------------|-------|---------|
| Model                | 12487.698*              | 7  | 1783.96     | 162.133 | 0 |
| Effect on organism   | 1148.95                 | 3  | 382.983     | 34.807 | 0 |
| Conc on organism     | 6.812                   | 2  | 3.406       | 0.31   | 0.738 |
| Type of the extract  | 810.844                 | 1  | 810.844     | 73.693 | 0 |
| Error                | 187.052                 | 17 | 11.003      |        |        |
| Total                | 12674.8                 | 24 |             |        |        |

*Neisseria gonorrheae* and *Staphylococcus aureus*; *Escherichia coli* and *Klebsiella pneumoniae*.
highest yield in the antibacterial activity of the sweet orange juice. However, none of the conventional antibiotics (positive control) as illustrated in Table 3 could match the inhibition zone of extract from ethyl ethanoate on the test organisms. According to Hassan et al. (2013), the ethanol extracts of Ocimum gratissimum (E. coli 17 mm; S. aureus 19 mm) and Vernonia amygdalina (E. coli 12mm; S. aureus 5mm) were the most effective on majority of test organisms among the water extracts of Ocimum gratissimum (E. coli nil, S. aureus 13 mm) and Vernonia amygdalina (E. coli nil, S. aureus nil) and the drugs; tetracycline (E. coli 16 mm, S. aureus 17mm) and flagy (E. coli nil, S. aureus 11 mm) used in their study. It can be concluded that K. pneumoniae and E. coli were more susceptible to the extracts (ethyl ethanoate and ethanol) compared to the zones of inhibition shown by N. gonorrhoeae and S. aureus. This finding concurs with Kumar et al. (2011) who asserted the highest zone of inhibition (16 mm) against E. coli with ethyl ethanoate extract of sweet orange juice.

Conclusion

The results obtained from this research proved that ethyl ethanoate and ethanol extracts of sweet orange juice have varying degree of antibacterial activity against the test organisms. This suggested that extracts of sweet orange juice can be useful in developing a new drug, which can be useful in treating bacterial infections caused by the test organisms in this study.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 3. Zone of inhibition of conventional antibiotics using disc method.

| Antibiotics     | Disc code | Neisseria gonorrhoeae | Staphylococcus aureus | Klebsiella pneumonia | Escherichia coli | Interpretation         |
|-----------------|-----------|-----------------------|-----------------------|---------------------|------------------|------------------------|
| Streptomycin    | S – 10    | 14                    | 23                    | 20                  | 24               | Susceptible            |
| Ciprofloxacine  | CIP – 5   | 18                    | 21                    | 15                  | 17               | Susceptible            |
| Gentamycin      | GM        | 14                    | 20                    | 17                  | 15               | Susceptible            |
| Penicillin G    | P         | 18                    | 28                    | 15                  | 14               | Susceptible            |

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