**The International Journal of Biotechnology**
*2013 Vol.2, No.6, pp.113-122*
*ISSN(e): 2306-6148*
*ISSN(p): 2306-9864*


# ANTIMICROBIAL ACTIVITY OF ORANGE OIL ON SELECTED PATHOGENS

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## ABSTRACT

*The antimicrobial activity of orange oil extracted by steam distillation from peels of orange fruits (Citrus sinensis) was screened against some medically important microorganisms. Gram-positive bacteria (Staphyloccocus aureus 001, S. aureus ATCC 25923, Enteroccocus feacalis 002, E. feacalis ATCC 295212); Gram-negative bacteria (Pseudomonas aeruginosa 003, Escherichia. Coli 004, E. coli ATCC 29522) and fungi (Candida albicans 010, C. albicans ATCC 90028) were used. The minimum inhibitory concentration (MIC) of orange oil was estimated using disc diffusion method at concentrations ranging from (1.65-422 mg/ml). Orange oil exhibited inhibitory effects against all the organisms. The MICs observed for the isolates were: S. aureus 001 (1.65 mg/ml), S. aureus ATCC 25923 (0.69 mg/ml), Enterococcus feacalis 002 (0.09 mg/ml), E. feacalis ATCC 295212 (0.05 mg/ml), P. aeruginosa 003 (1.85 mg/ml), E. coli 004 (1.37 mg/ml), E. coli ATCC 29522 (0.82 mg/ml), C. albicans 010 (0.02 mg/ml), C. albicans ATCC 90028 (0.01 mg/ml). The GC-MS revealed that orange oil contained mainly cyclohexane, 1-methylene-4-(1-methylethenyl; bicyclo [4.1.0] hept-2-ene, 3,7,7-trimethyl; D-limonene; 1, 6-octadien-3-ol, 3,7-dimethy-l,2-aminobenzoate; 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl); 2-cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl); 2-methoxy-4-vinylphenol; 3- furanacetic acid, 4-hexyl-2, 5-dihydro-2,5-dioxo; naphthalene, 1,2,3,5,6, 8a-hexahydro-4,7-dimethyl- 1-(1-methylethyl) -, (1S-cis) and n-hexadecanoic acid. The result indicated that orange oils have antimicrobial properties and may be applied in local therapies in the treatment of diseases caused by the microorganisms tested. Further research is needed to achieve appropriate formulation.*

**Keywords:** Orange oil, Limonene, Antimicrobial, *Citrus sinensis.*

## 1. INTRODUCTION

The prevalence of pathogenic microorganisms has greatly necessitated the introduction of broad-spectrum antibiotics, immunosuppressive corticosteroids, and antitumor agents (Pfaller *et al.*, 1994; De Brito Costa *et al.*, 2003). *Candida albicans* is the most common fungal opportunistic

 

pathogen in humans which causes either septicaemia or mucosal infection (Odds and Bernaerts, 1994). Pathogenic *C. albicans* strains isolated from different clinical sources are increasingly responsible for hospital outbreaks (Berber and Ekin, 2008). Human diseases due directly or indirectly to these infectious agents which infect man have become a primary constraint to productivity and is now severely impacting both economic and socio-economic development in many developing countries of the world (Obasohan *et al*., 2010). Essential oils are basically extracts which have been concentrated and obtained from different parts of plants. They differ in their active ingredients and are used for various purposes based on the composition of their active ingredients. Some oils are used to promote physical healing, for example to treat swelling or fungal infections (Mercier and Knevitt, 2005). In the past 6,000 years, essential oils have been applied in therapeutics. They have also been extensively used in cosmetics, drugs and perfumes by the ancient Chinese, Indians, Egyptians, Greeks and Romans. Essential oils are commonly used for spiritual, therapeutic, hygienic, and ritualistic purposes (Patricia, 2004). Some others are used for their usefulness in enhancing relaxation or as deodorants. Orange blossom oil, for example, contains a large amount of an active ingredient that is thought to be calming (Mercier and Knevitt, 2005). Citrus plant which is a native of tropical Asia is easily available and has shown a wide range of uses in treatment of various diseases (Pellati *et al*., 2002). Orange oil therefore, has a lot of therapeutic functions such as anti-inflammatory, antiseptic, anti-depressant, tonic, carminative, antispasmodic, diuretic and as a sedative. Limonene, which is found in the oil of citrus peels, is a by-product of the orange juice industry. It is used as a less toxic substitute for xylene in histopathology and microscopy (Wynnchuk, 1994; Kierman, 2008). Limonene aids digestion and detoxifies system, eases constipation, relieves water retention, promotes circulation, increases absorption of vitamin C, supports immunity to fight colds and flu, strengthens and rejuvenates skin. In this study, we analyzed constituents of orange oil by employing GC-MS. The antimicrobial activity of the essential oil was also evaluated.

## 2. MATERIALS AND METHODOLOGY

### 2.1. Sample Collection

Ripe orange fruits (*Citrus sinensis*) were purchased from Mushin market, Lagos-state, Nigeria. The fruits were identified, confirmed, and authenticated in the herbarium at the Department of Botany, University of Lagos, Nigeria. They were given herbarium voucher specimen number (LUH5017).

### 2.2. Extraction of Orange oil Using Steam Distillation

Orange oil was separated from orange peels by the process of steam distillation (Harbone, 1998) using the Clavenger apparatus (Pyrex UK). The peels were placed in the round bottom flask and filled with water to about three quarter full. The distillation apparatus was set up and connected to the flask. Water was filled into the trap arm to allow the oil condense on the water layer. The heating mantle supplied the needed heat and as the water in the flask boiled, steam

carrying the volatile oil rose through the neck of the flask condensing on the surface of the condenser onto the water on the graduated trap arm. The distillation process was continued until there was no more difference in successive readings of the oil volume. This was followed by draining off of the oil which was subsequently dried over anhydrous sodium sulphate (BDH). The density of the oil was determined according to the weight: volume ratio (Ayoola *et al*., 2008).

### 2.3. Source of Microbial Cultures

Cultures were from clinical sources. They were obtained from the Medical Microbiology and Parasitology Research Laboratory of the College of Medicine, University of Lagos, Nigeria. They include: two strains of Gram-positive bacteria (*Staphylococcus aureus 001*, *Enterococcus faecalis 002*); two strains of Gram-negative bacteria (*Escherichia coli 004*, *Pseudomonas aeruginosa 003*) and a fungus (*Candida albicans 010*). Control strains of *Staphylococcus aureus* (ATCC 25923), *Enterococcus feacalis* (ATCC 295212), *Escherichia coli* (ATCC 29522), and *Candida albicans* (ATCC 90028) were used and tested along with the organisms. The bacterial cultures were maintained in their respective agar slants at 4°C throughout the course of the study and used as stock cultures.

### 2.4. Inoculum Standardization

The test organisms were subcultured unto fresh plates of Mueller-Hinton medium and incubated for 24 h at 37$^0$C for bacteria and fungus respectively. The agar plates were stored at 4$^0$C until required. Overnight cultures from these plates were suspended into Mueller-Hinton broth (Oxoid, UK) for antimicrobial assays for both bacteria and fungus respectively. They were adjusted to a turbidity matching the 0.5 McFarland standard.

### 2.5. Screening of Orange Oil for Antimicrobial Activity

Screening of orange oil for antimicrobial activity was done by the agar well diffusion method (Thitilertdecha *et al*., 2008) using microorganism cell suspension at 0.5 McFarland standards. Each labelled medium plate was uniformly seeded with a test organism by means of sterile swab rolled in suspension and streaked on the plate surface. Wells of 5 mm in diameter and 2 cm apart were punched on the culture medium. The oil extract was used as a stock (neat extract) and the methanol (99%) was used for diluting the oil and as control. Ciprofloxacin antibiotic suspension (0.5 mg/ml) and neat solvent (methanol 99%) were placed in wells on each plate along with the test extract as reference controls. Approximately 100 µl, of the various concentrations (422.0, 211.0, 105.5, 52.75, 26.38, 13.19, 6.59, 3.29 and 1.65 mg/ml) were dropped into each well to fullness (Deans and Dorman, 1999). Each plate was kept in the refrigerator at 6$^0$C for 1 h before incubating at 37$^0$C for 18-24 h. Zones of inhibition around the wells, measured (in mm) were used as positive bioactivity.

### 2.6. Determination of Minimum Inhibitory Concentrations (MICs)

After incubation of the plates, the diameter of the zone of inhibition around the well, measured in millimetre, was used as positive bioactivity. This was determined graphically, by plotting zone diameter (in mm) against log of concentration. The straight line obtained was extrapolated to a point equivalent to the diameter of the cup (5 mm). The MIC was taken as antilog of the corresponding concentration obtained (Lamikanra, 1999).

### 2.7. Gas Chromatography- Mass Spectrophotometry (GC-MS) Technique

Gas Chromatography-Mass Spectrophotometry (GC-MS) was employed in the identification of the oil constituents. This method supersedes the juice extraction, refractometer and polarimetry methods (Trevor, 2001) of analysis. The exudates obtained from steam distillation was therefore analysed chromatographically. The chromatographic procedure was carried out using a 7890A GC system (Agilent Technologies) equipped with a mass selective detector (MSD) 5975C (Agilent Technologies), injector series model 7683B and HP-5MS capillary column (30 m x 0.320 mm, 0.25 µm film thickness). The temperature of the column was maintained at $35^0$C for 1 min. It was then raised at the rate of $10^0$C per min for a hold time of 3 min. Finally, the temperature of the injection port was maintained at $220^0$C and that of the detector at $250^0$C for 3 min hold time. This was adapted in order to prevent excess long chain fatty acids from accumulating on the GC column. Helium was the carrier gas. The following parameters were maintained: Pressure= 112.0 kPa, Total flow= 32.7 ml/min, Column flow =1.90 ml/min, Linear velocity =50 cm/sec. The chromatographic effluent was then analyzed by the MSD.

## 3. RESULTS

### 3.1. Extraction Procedure

The steam distillation experiment resulted in orange oil with 0.46% w/w yield and a density of 0.85 g/ml.

### 3.2. GC and MS Analysis

The essential oil constituents was analyzed by GC-MS and resulted in the identification of ten compounds. The peaks were identified as D-limonene; cyclohexane, 1-methylene-4-(1-methylethenyl; bicyclo [4.1.0] hept-2-ene, 3,7,7-trimethyl; 1, 6-octadien-3-ol, 3,7-dimethy-l,2-aminobenzoate; 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl); 2-cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl); 2-methoxy-4-vinylphenol; 3- furanacetic acid, 4-hexyl-2, 5-dihydro-2,5-dioxo; naphthalene, 1,2,3,5,6, 8a-hexahydro-4,7-dimethyl- 1-(1-methylethyl) -, (1S-cis) and n-hexadecanoic acid (Fig.1). However, the most abundant compound was 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) with 5.66% w/v (Table 1) which has been found to be a derivative of limonene (Olivera *et al.*, 2001). Limonene is well known for its antimicrobial and antiseptic activities (Magwa *et al.*, 2006). It has been implicated in nutrition and health applications as well as in cosmetic products. Limonene is rapidly absorbed in the gastrointestinal tract in humans

(Magiatis *et al*., 1999; Martins *et al*., 2000; Filipowicz *et al*., 2003). After, absorption, d-limonene is rapidly distributed to the various tissues of the body and is readily metabolized (Martins *et al*., 2000). D-limonene is also used in food manufacturing and medicines, e.g. as a flavoring to mask the bitter taste of alkaloids, and as a fragrant in perfumery; it is also used as botanical insecticide (Venkatsen *et al*., 2005; Chen *et al*., 2006; Erazo *et al*., 2006).

### 3.3. Antimicrobial Activity

The volatile oil exhibited considerable inhibitory effects against *Candida albicans 010* in particular. Table 2 shows the zones of inhibition of the organisms to orange oil at various concentrations. The Gram-positive bacteria were susceptible to the extract with inhibitory concentration ranging from 0.05 mg/ml to 1.65 mg/ml. The MICs recorded were: *S. aureus 001* (1.65), *S. aureus* ATCC (0.69), *Enterococcus faecalis 002* (0.09), *Enterococcus faecalis* ATCC (0.05), *Escherichia coli 004* (1.37), *Escherichia coli* (0.82), *Pseudomonas aeruginosa 003* (1.85), *Candida albicans 010* (0.01), *Candida albicans* ATCC (0.02). The Gram-negative bacteria have MIC ranging from 0.82 mg/ml 1.85 mg/ml (Table 2). The activity against the fungus proved that the oil extract was potent for *Candida albicans 010* with MIC of 0.02 mg/ml. Generally, the ciprofloxacin proved to be more potent than the orange oil extract.

**Table-1.** Major Identified Chemical Constituents of the Essential Oil of *Citrus sinensis*.

| S/N | Compound | RT (min) | Relative Percentage (%) |
|---|---|---|---|
| 1. | D-Limonene | 3.136 | 0.46 |
| 2. | Cyclohexane, 1-methylene-4-(1-methylethenyl | 3.341 | 1.86 |
| 3. | Bicyclo [4.1.0] hept-2-ene, 3,7,7-trimethyl | 3.670 | 1.15 |
| 4. | 1, 6-Octadien-3-ol, 3,7-dimethyl,2-aminobenzoate | 3.755 | 4.36 |
| 5 | 3-Cyclohexen-1-ol, 4methyl-1-(1-methylethyl) | 4.386 | 5.66 |
| 6 | 2-Cyclohexen-1-ol, 2-methyl-5-(1-methylethenyl) | 4.884 | 2.01 |
| 7 | 2-Methoxy-4-vinylphenol | 6.094 | 3.90 |
| 8 | 3- Furanacetic acid, 4-hexyl-2, 5-dihydro-2,5-dioxo | 8.419 | 1.19 |
| 9 | Naphthalene, 1,2,3,5,6, 8a-hexahydro-4,7-dimethyl | 8.751 | 2.98 |
| 10 | -1-(1-methylethyl) -, (1S-cis) | | |
| 11 | n-Hexadecanoic acid | 14.971 | 1.79 |

**Fig-1.** Gas chromatogram of orange oil extract showing the various constituents.

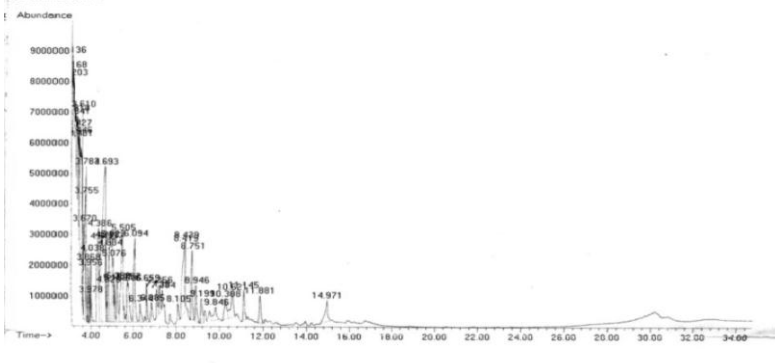

**Table-2.** Zones of inhibition (nm) of organisms to orange oil at various Concentrations

| Test organism | Concentration (mg/ml). | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | OOE | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | Met | Cip | MIC |
| | 844 | 422 | 211 | 105.5 | 52.75 | 26.38 | 13.19 | 6.59 | 3.30 | 1.65 | | 0.005% | Mg/ml |
| **Gram positive bacteria** | | | | | | | | | | | | | |
| Staphylococcus aureus 001 | 20 | 15 | 13 | 10 | 10 | 9 | 8 | 7 | 6 | 6 | 6 | 18 | 1.65 |
| Staphylococcus aureus ATCC 25923 | 7 | 15 | 15 | 10 | 10 | 8 | 8 | 9 | 8 | 7 | 5 | 17 | 0.69 |
| Enterococcus feacalis 002 | 30 | 28 | 25 | 25 | 25 | 25 | 15 | 13 | 15 | 15 | 7 | 30 | 0.09 |
| **Gram negative bacteria.** | | | | | | | | | | | | | |
| Escherichia coli 004 | 30 | 20 | 16 | 10 | 8 | 7 | 7 | 9 | 8 | 8 | 7 | 30 | 1.37 |
| Escherichia coli ATCC 29522 | 7 | 19 | 10 | 9 | 9 | 8 | 9 | 8 | 8 | 7 | 5 | 30 | 0.82 |
| Pseudomonas aeruginosa 003 | 9 | 10 | 10 | 10 | 8 | 7 | 6 | 5 | 6 | 6 | 6 | 15 | 1.85 |
| **Fungi** | | | | | | | | | | | | | |
| Candida albicans 010 | 30 | 30 | 25 | 20 | 22 | 18 | 20 | 20 | 20 | 20 | 6 | 5 | 0.01 |
| Candida albicans ATCC 90028 | 30 | 30 | 30 | 30 | 30 | 30 | 20 | 20 | 18 | 18 | 6 | 6 | 0.02 |

OOE = Orange oil extract, D1-D9 = Extract concentration, Met = Methanol,
Cip = Ciprofloxacin, MIC = minimum inhibitory concentration.

## 4. DISCUSSION

It was depicted that orange oil had a more consistent antimicrobial activity when used along with organic solvent (methanol) than the neat extract (orange oil). This is probably because methanol had the ability to aid the diffusion of the extract in the media used for the assay. This also corroborates the fact that water is almost universally the solvent used to prepare herbs traditionally Lamikanra (1999).

In addition, *Staphylococcus aureus 001* had a zone diameter of 13 mm for neat extract and 15 mm for Dilution 1(Table 2). Also, *Staphylococcus aureus* ATCC 25923 with 7 mm (extract) zone diameter and 15 mm (dilution 1) zone diameter. This also shows the importance of methanol in the dilution of the extracts. Exception in this case was *Enterococcus faecalis 002*, where the orange oil was more potent as neat extract than the methanolic extract. This is in line with the report of Jigna *et al.* (2005) which states that some traditional medicines are more effective when used alone. Jafari *et al.* (2011) showed the antimicrobial activity of essential oil of *Citrus aurantifolia* against specific pathogens. According to this study, essential oil of *C. aurantifolia* can inhibit spoilage bacteria and consequently, reduce the risk of diseases associated with consumption of contaminated products. Previous studies have shown the various constituents of orange oil. Veriotti and Sacks (2002) carried out a high speed characterization and analysis of peels of five species of orange oil. The work of Subba *et al.* (2006) also showed inhibitory effect of essential oils

of orange and lemon oil tested on bacteria and fungi in nutrient media. Orange oil was found to be more effective antimicrobial agent than lemon oil. All other conditions being identical, 2, 000 ppm of orange oil had effect on all the Gram-positive cultures tested, including the spores of *Bacillus subtilis*. Subba *et al.* (2006) showed that, orange oil was the only additive that showed inhibition of growth of the Gram-negative cultures, excepting *Serratia marcescens*. The workers found that yeasts, in general, were more sensitive than bacteria to orange oil, the effective dose being 500 ppm, at a cell concentration of 1 million/ml. This was a definite advantage over the antibiotics, which had no action on fungi. Orange oil therefore can be said to have potential antimicrobial activities. The antibacterial activity of natural spices on multi drug resistant *Escherichia coli* carried out by Rahman *et al.* (2010) also revealed that all bacteria tested were susceptible to undiluted lime juice. This confirms the fact that natural species might have anti bacterial activity against enteric pathogens and could be used for prevention of diarrheal diseases.

## 5. CONCLUSION

This study demonstrates the use of GC/MS in the analysis of *Citrus sinensis* peels using a simple, inexpensive chromatographic technique. The novel, sensitive, accurate, and precise GC-MS assay which supersedes the refractometric and polarimetric methods was useful in determination of the orange oil constituents. 3-cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) which was observed to be the most abundant constituent is a derivative of limonene. Limonene has been implicated to be an effective antimicrobial agent. The antimicrobial activity of orange oil may therefore, be harnessed against medically important pathogens that are implied in various infections.

## 6. ACKNOWLEDGEMENTS

The authors would like to appreciate Mrs. Nike Aladeshokan of the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos for procurement of organisms. Thanks are also due to the staff of the Pharmaceutical Chemistry Department, CMUL campus, University of Lagos, Nigeria for allowing us the use of their laboratory facilities for an aspect of the study.

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