African Journal of Biotechnology Vol. 7 (13), pp. 2227-2231, 4 July, 2008
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB08.391
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Evaluation of the chemical constituents and the antimicrobial activity of the volatile oil of *Citrus reticulata* fruit (Tangerine fruit peel) from South West Nigeria

G.A. Ayoola[1]*, O. O. Johnson[1], T. Adelowotan[2], I. E. Aibinu[2], E. Adenipekun[2], A.A. Adepoju-Bello[1], H. A. B. Coker[1] and T.O. Odugbemi[2]

[1]Department of Pharmaceutical Chemistry, University of Lagos, CMUL campus, Lagos, Nigeria.
[2]Depatment of Medical Microbiology and Parasitology, College of Medicine, University of Lagos, Lagos, Nigeria.

Accepted 16 June, 2008

The volatile oil of tangerine fruit (*Citrus reticulata)* was extracted by steam distillation and assessed for antibacterial and antioxidant activity. The volatile oil was tested against some Gram-negative organisms (*Escherichia coli* ATCC 35218, *E. coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella paratyphi, Proteus mirabilis* and *Citrobacter spp*); Gram-positive organisms such *as Staphylococcus aureus* ATCC 25923, *S. aureus, Enterococcus faecalis* and a fungus (*Candida albicans*). The minimum inhibitory concentration (MIC) was determined with concentrations of oil extract ranging from 0.87 to 445 mg/ml. Result of the study showed that the oil has a broad spectrum antibacterial activity. MIC recorded were *S. aureus* (0.74 mg/ml), *S. aureus* ATTC 25923 (2.46 mg/ml), *E. faecalis* (1.26 mg/ml), *S. typhi* (2.07 mg/ml), *K. pneumoniae* (0.56 mg/ml), *E. coli* ATTC 35218 (0.19 mg/ml), *E. coli* (1.95 mg/ml), *P. aeruginosa* (0.97 mg/ml), *C. albicans* (0.68 mg/ml). Antioxidant screening with 2,2-diphenyl-1-picrylhydrazyl (DPPH) was negative. Analysis of the chemical constituent by GC-MS showed the presence of D-limonene as the major constituent. Other constituents found were α-pinene and β-pinene.

Key words: *Citrus reticulata,* antimicrobial, antifungal, gas chromatography, mass spectrometry.

INTRODUCTION

In recent years the general public has shown an increased interest in the use of herbal medicines in preference to synthetic drugs. This is based on the belief that natural products are intrinsically less dangerous and can be obtained at a lower cost. However, most natural products contain a complex mixture of components of which only one or a few are active. Identification of the active component can lead to the synthesis of more potent analogues that can be readily formulated into more traditional dosage forms.

Volatile oils, also known as essential oils are lipophilic compounds containing volatile aroma compounds. They are generally isolated form non-woody plant materials by distillation methods, solvent extraction or cold expression. They are used in perfumes, cosmetics, flavouring food and drinks and for scenting. Medicinal uses proposed by sellers of essential oils vary from skin treatments to remedies for cancer. These are often based on historical uses of these oils. These claims are now subject to regulations in most countries and there is a need to ascertain some of these traditional uses scientifically (Iwu et al., 1999).

Tangerine oil is obtained from the fruit peel of *Citrus reticulate*, family Rutaceae. It is traditionally used as an antiseptic, antispasmodic, stomachic, sedative, diuretic and to improve circulation (Burkill, 1984; Odugbemi, 2006). The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity (Martindale, 1910; Hoffmann and Evans, 1911). Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile oils have regained

*Corresponding author. E-mail: oyetayo68@yahoo.com. Tel: +234-8055465428, +23418771985.

momentum. Increase in the emergence of new bacterial strains that are multi-resistant coupled with the non-availability and the high cost of new generation antibiotics have resulted in increase morbidity and mortality (Aibinu et al., 2003; Amit and Shailendra, 2006: Lewis and Ausubel, 2006). Hence the need to look for potent antimicrobials from other sources. The aim of this study is to investigate the antiseptic property of tangerine oil and to identify the constituents that may be responsible for its antimicrobial property.

MATERIALS AND METHODS

Collection and identification of plant materials

Tangerine fruits were purchased from Kuto market, Abeokuta in Ogun state, Nigeria. The fruits were identified at the Forestry Research Institute of Nigeria, Ibadan (FRIN) and given a voucher specimen number (FH107930).

Extraction of volatile oil by steam distillation

Peels from 100 tangerine fruits were used in obtaining the tangerine oil by steam distillation (Harbone, 1998) using the Clavenger apparatus (Pyrex). The fresh tangerine peels were placed in the round bottom flask and filled with water to about three quarter full. The distillation apparatus was connected to the flask. The trap arm was filled with water to allow the oil to condense on the water layer. Heat was applied from the heating mantle 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. Distillation was continued until there was no more difference in successive readings of the oil volume. The oil was drained off and dried over anhydrous sodium sulphate (BDH). The density of the oil was determined according to the weight : volume ratio.

Test organisms

The organisms used comprise six gram-negative organisms (*Eschericha coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella paratyphi Proteus mirabilis and Citrobacter spp)*, two gram-positive organisms *Staphyloccocus aureus, Enterococcus faecalis* and a fungus (*Candida albicans*). The test organisms were obtained form the research laboratory of Medical Microbiology and Parasitology of the College of Medicine, University of Lagos.

Control organisms

Control strains of *E. coli* ATCC 35218 and *S. aureus* ATCC 25923 (Remel Ltd, UK) were used and tested along with the organisms.

Standardisation of inoculum

The test organisms were subcultured onto fresh plates of Mueller-Hinton agar (Oxoid, UK) for 24 h and Saboraud dextrose agar for 5 - 7 days at 37˚C for bacteria and fungi, respectively. Colonies from these plates were suspended in Mueller-Hinton broth (Oxoid, UK) and Saboraud broth (Oxoid, UK) to a turbidity matching 0.5 McFarland standard (10^8 cfu/ml). The media used for antimicrobial assays were Mueller-Hinton agar (Oxoid, UK) for bacteria and Saboraud agar (Oxoid, UK) for fungi. All were incubated appropriately as specified for each organism (Aibinu et al., 2007).

Antimicrobial assay

Labeled media plates were uniformly seeded with the different test microorganisms, by means of a sterile swab rolled in the suspendsion and streaked on the plate's surfaces. Wells of 10 mm in diameter were punched in the sterile cork borer. The density of the oil was determined by measuring the weight: volume ratio and found to be 0.89 mg/ml. 100 µl of the extract was dropped into each well. Ciprofloxacin antibiotic suspension (0.005%) and neat solvents were dropped into each well to a volume of 100 µL. This was repeated using different concentrations of the oil extract. Concentrations of extract used were 0.87, 1.74, 6.95, 27.81, 55.63, 111.25, 445.0 mg/ml. Dilutions were made using methanol as solvent. Each plate was kept in the refrigerator at 4^{o}C for 1 h to allow for diffusion of extract, before incubating at 37^{o}C for 24 h.

Determination of minimum inhibitory concentration (MIC)

The diameter of the zone of inhibition around the well, measured in millimeter, is used as positive bioactivity. MIC is defined as the lowest concentrations able to inhibit any visible bacterial growth on the culture plates. This was determined graphically, by plotting zone diameter (in mm) against the log concentration. The straight line obtained is extrapolated to a point equivalent to the diameter of the cup. The antilog of the corresponding concentration was taken as the MIC (Lamikanra, 1999).

Gas chromatography-mass spectrometry

The chromatographic procedure was carried out using Shimadzu QP2010-GC-MS with autosampler. A fused silica capillary column HP5-MS (30 m x 0.32 mm, film thickness of 0.25µm) was used. Helium was the carrier gas, a flame ionization detector and a split ratio of 1/100 were used. The temperature of the column used was maintained at 60^{o}C for 8 min. The temperature was then gradually raised at a rate of 3^{o}C per minute to 180^{o}C and maintained at 180^{o}C for 5 min, while maintaining the temperature at the injection port at 250^{o}C. 1.0 µl of the substance to be examined was injected. The components of the test solution were identified by comparing the spectra with spectra of known compounds stored in NIST library 2005. The chromatographic effluent was used as a sample inlet for the mass spectrometer. The ion source temperature was set at 200^{o}C. The fragmented ions were separated by the analyzer, according to their various mass-to-charge (m/z) ratios.

Rapid thin layer chromatography (TLC) screening for antioxidant activity

The methanolic solution of the clove oil extract was spotted on a silica gel plate. 0.2% methanolic DPPH solution was sprayed on the spot. A change in the colour of the DPPH spray solution from deep violet to yellow was considered positive (Oke and Hamburger, 2002).

RESULTS

Extraction of the volatile oil

A light yellow oil was obtained with a low yield of 0.005%w/w and a density of 0.89 mg/ml.

Table 1. Zones of inhibition (mm) of organisms to tangerine oil.

Test organisms	Concentration (mg/ml)												Cip	Met
	TOE	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	MIC		
	890	445	222.5	111.25	55.61	27.81	13.91	6.95	3.48	1.74	0.87	mg/ml	0.005%	98%
Gram positive bacteria														
Staphylococcus aureus ATCC 25923	23	18	17	15	13	15	14	10	10	9	9	2.46	25	10
Staphylococcus isolate	13	22	22	19	16	14	16	14	12	12	11	0.74	27	10
Enterococcus faecalis	>30	>30	>30	>30	>30	>30	12	12	11	12	11	1.26	18	10
Gram negative bacteria														
Salmonella paratyphi	11	18	16	15	14	11	11	11	11	19	19	2.07	24	10
Klebsiella pneumoniae	12	16	17	14	13	13	13	13	13	12	11	0.19	26	12
Escherichia coli ATCC 35218	11	17	14	13	14	13	13	13	13	12	11	0.19	26	12
Escherichia coli isolate	12	18	15	13	13	13	12	11	10	10	10	1.95	11	10
Proteus mirabilis	N	N	N	N	N	N	N	N	N	N	N		30	N
Pseudomonas aeruginosa	14	15	15	15	13	12	11	10	11	11	11	0.91	19	10
Fungus														
Candida albicans	>30	>30	>30	>30	>30	>30	19	18	13	12	11	0.68	0	12

TOE = Tangerine Oil Extract; C1 - C10 = Extract Concentration; Met = Methanol; Cip = Ciprofloxacin

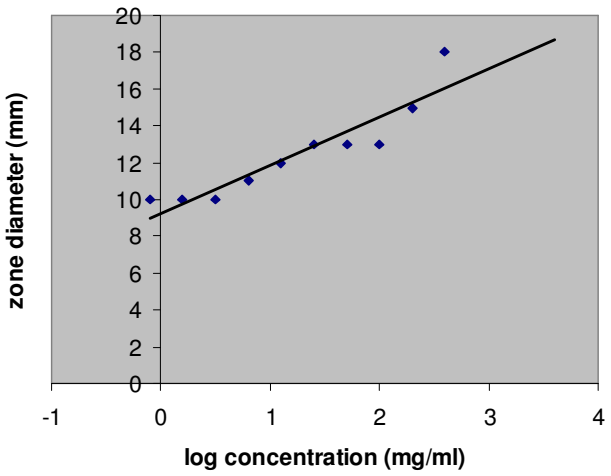

Figure 1. Graph of zone diameter against log concentration of tangerine oil for *E. coli* isolate.

Antimicrobial assay

The methanolic solution of the extract was active against all the Gram negative and Gram positive organisms tested except *Proteus mirabilis*. It also showed potent antimicriobial activity against *C. albicans*. MICs were as follows: *S.aureus* (0.74 mg/ml), *S. aureus* ATTC 25923 (2.46 mg/ml), *E. faecalis* (1.26 mg/ml), *S. typhi* (2.07 mg/ml), *K. pneumoniae* (0.56 mg/ml), *E. coli* ATTC35218 (0.19 mg/ml), *E. coli* (1.95 mg/ml), *P. aeruginosa* (0.97 mg/ml), *C. albicans* (0.68 mg/ml). A minimum zone diameter of 11 mm for *K. pneumonia* and *E. coli* ATCC 35218 isolate and a maximum >30 mm for *E. faecalis* and *C. albicans* were recorded for the undiluted oil extract (Table 1 and Figure 1)

Gas chromatography-mass spectrometry

Three peaks were identified from the gas chromatograph (Figure 2). These peaks were identified as D-limonene, α-pinene and β-pinene from the GC-MS database. Mass spectrometry data were as follows: m/z (D-Limonene): 136 M^+, 121 $(M+H-CH_3)^+$, 107 $(M+H-C_2H_5)^+$, 93 $(M+H-C_3H_7)^+$, 79 $(M+H-C_4H_9)$, 68 $\{C_5H_8 - (M+H-C_5H_8)^+\}$, 41 $(C_3H_5)^+$, 27 $C_2H_4^+$. m/z (α-Pinene): 136 M^+, 121 $(M+H-CH_3)^+$, 93 $(M+H-C_3H_7)^+$, 77 $(M-H-C_4H_{10})^+$, 41 $(C_3H_5)^+$, 27 $C_2H_4^+$. m/z (β-Pinene): 136 M^+, 121 $(M+H-CH_3)^+$, 107 $(M+H-C_2H_5)^+$, 93 $(M+H-C_3H_7)^+$, 77 $(M-H-C_4H_{10})^+$, 41 $(C_3H_5)^+$, 27 $C_2H_4^+$.

Antioxidant activity

The tangerine oil extract did not change the colour of the DPPH spray

DISCUSSION

The oil obtained was yellow in colour with a density of 0.89 mg/ml. The methanolic solutions of the extracts were found to have a broad spectrum activity against all the gram positive and gram negative organisms tested except *P. mirabilis*. The undiluted extract, however, only had potent activity against the gram positive bacteria but no significant activity against the gram negative bacteria

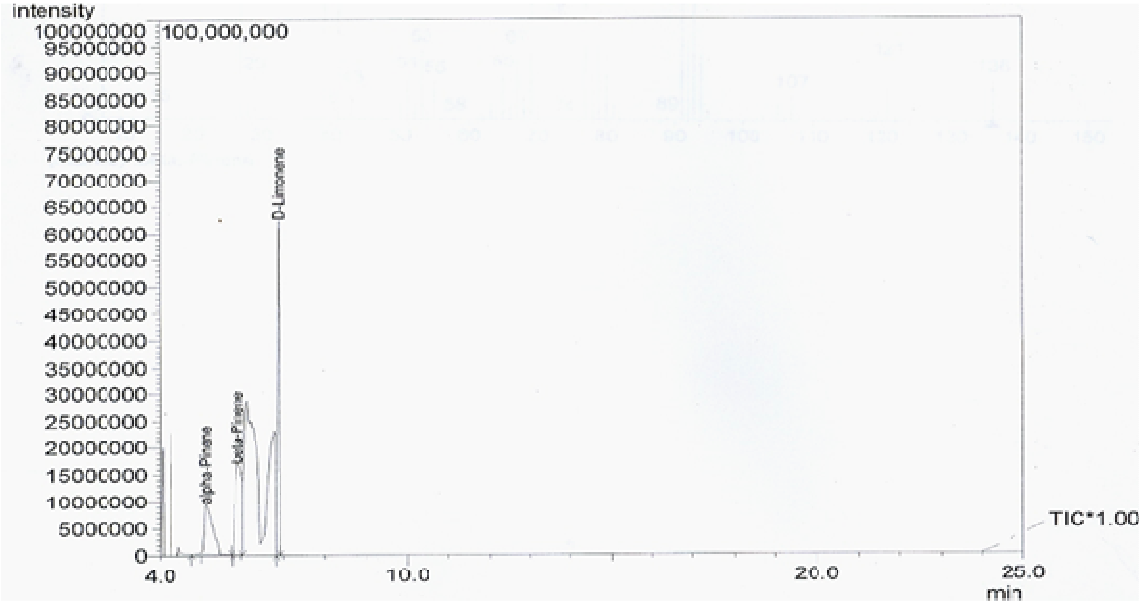

Figure 2. Gas chromatogram of tangerine oil extract showing D-limonene, α-pinene and β-pinene as the constituents.

as the zone diameter measured were similar to those measured for methanol (the diluting solvent – Table 1). The undiluted extract and diluted extracts had potent activity against *E. faecalis* and *C. albicans*. The difference in antimicrobial activity between the undiluted oil extracted and the methanolic oil solution can be attributed to the vehicle (i.e. methanol). The methanol may be allowing easy diffusion of the oil extract through the agar into the bacteria cell. The extra coating on the Gram negative organism may pose an extra bacteria hence the reduced activity against Gram negative bacteria. Ciprofloxacin was more potent as an antibacterial agent when compared to the neat extract and the diluted extracts. It was about ten times more potent and 14 times more potent against *E. coli* isolate and *E. faecalis*. Ciprofloxacin was active against *P. mirabilis* but the oil extract was not active against this organism.

The GC-MS showed the presence of α-pinene, β-pinene and limonene, limonene being the major constituent in the oil extract. Analysis of the MS data showed that these compounds are hydrocarbons. Fragmentation patterns show stepwise cleavage of the alkyl groups. The structures showed that they are terpenes with an empirical formula of C_5H_8. The presence of a molecular ion at m/z = 68 further supports the presence of the isoprene molecule which is the building block for terpenes. The three compounds have the same molecular weight of 136 but they were resolved on the chromatogram. Furthermore, the fragmentation pattern on the mass spectrum of each compound is different. α and β-pinene are both stereoisomers. The activity of the oil is expected to be related to the respective composition of plant volatile oils. D-Limonene, α and β-pinene have been shown to have antimicrobial properties, and so it is not surprising that the oil extract had potent antimicrobial properties against a wide range of organisms (Dorman and Deans, 2000).

Conclusion

The methanol solution of the volatile oil of *C. reticulata* was active against all the gram positive and gram negative organisms and against *C. albicans* tested except *P. mirabilis,* with a minimum zone diameter of 9 mm for *S. aureus* ATTC 25923 and a maximum of >30 mm for *E. faecalis* and *C. albicans*. The undiluted oil extract was more active against the gram positive organisms tested with a minimum zone diameter of 11 mm for *S. paratyphi* and *E. coli* ATCC 35218 and a maximum of >30 mm for *E. faecalis* and *C. albicans.* It was found to be less potent compared to Ciprofloxacin on the bacteria tested. It, however, demonstrated potent activity against the fungus, *C. albicans*. Analysis of the oil extract showed D-limonene as the major constituent. Other constituents identified were α-pinene and β-pinene. These compounds are known to possess antibacterial and antifungal properties (Pelczar et al., 1998). Hence the antibacterial and antifungal properties demonstrated by the tangerine oil extract can be exploited further with a view to generate new potent antimicrobial agents.

ACKNOWLEDGEMENTS

We would like to appreciate Mr. B. A. Benjamin of the Pharmacognosy Department, Mrs Y.A Bashorun, Mr I.O Olatunji and Mr. M. Olajide of Pharmaceutical Chemistry Department and the technical staff of the Department of Medical Microbiology and Parasitology, University of La-

gos, for excellent technical support. We also thank Ms J. O. Ashamu for excellent assistance in the preparation of this manuscript.

REFERENCES

Aibinu I, Adenipekun T, Adelowotan T, Ogunsanya T, Odugbemi T (2007). Evaluation of the antimicrobial properties of different parts of Citrus aurantifolia (Lime fruit) as used locally. Afr. J. Trad. CAM. 4: 185-190.

Aibinu I, Odugbemi, T, Mee BJ (2003). Extended-Spectrum Beta-Lactamases in Isolated of *Klebsiella spp* and *Eschericha coli* from Lagos, Nigeria. Niger. J. Health. Biomed. Sci. 2(2): 53-60.

Amit R, Shailendra S (2006). Ethnomedicinal approach in biological and chemical investigation of phytochemicals as antimicrobials. Available at http://www.phamainfo.net.

Burkill HM (1984). The useful plants of West Tropical Africa. Royal Botanical Garden Kew. 2nd edition, Families M-R, 1: 647.

Dorman HJD, Deans SG (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88: 308-316.

Harbone JB (1998). Essential oils, In Phytochemical Methods: A guide to modern techniques in plant analysis, 3rd ed. Chapman & Hall, PA, USA. pp. 110-124.

Hoffman C, Evans AC (1911). The uses of spices as preservatives. J. Indian Eng. Chem. 3: 835-838.

Iwu WM, Duncan AR, Okunji CO (1999). New antimicrobials of plant origin In Janick J (ed.). Perspectives on new crops and new uses. ASHS Press, Alexandria, VA. pp. 457-462.

Lewis K, Ausubel F (2006). Focus on antibacterials. Nature Biotech. 24(12): 1453-1602.

Lamikanra A (1999). Essential microbiology. 2nd edn. AMKRA, Lagos, Nigeria, pp. 128-129.

Martindale WH (1910). Essential oils in relation to their antiseptic powers as determined by their carbolic coefficients. Perfumery Essential Oil Res. 1: 266-296.

Odugbemi TO (2006). Outlines and Pictures of Medicinal Plants from Nigeria, University of Lagos Press, Lagos, Nigeria, p. 91.

Oke JM, Hamburger MO (2002). Screening of some Nigerian medicinal plants for antioxidant activity using 2,2,-Diphenyl-Picryl-Hydrazyl radical. Afr. J. Biomed. Res. 5: 77-79.

Pelczar MJ, Chan ECS, Krieg NR (1998). Control of microorganisms, the control of microorganisms by physical agents. In Microbiology. New York: McGraw-Hill International. pp. 469-509.