

Full Length Research Paper

Chemical analysis and antimicrobial activity of the essential oil of *Syzigium aromaticum* (clove)

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Steam distillation of the dry flower buds of *Syzigium aromaticum* (clove) yielded 7% (w/w) of the pure light yellow oil. Gas chromatography-mass spectrometry (GC-MS) analysis of the oil revealed that the components were eugenol, caryophyllene, eugenol acetate and alpha-humulene, with eugenol being the main component. The antimicrobial sensitivity of the volatile oil against some Gram-negative bacteria (*Escherichia coli* ATCC 35218, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Citrobacter* spp. and *Enterobacter cloacae*), a Gram-positive bacterium (*Staphylococcus aureus* ATCC 25923), and a fungus (*Candida albicans*) showed a broad spectrum of activity. The minimum inhibitory concentration (MIC) was determined for each organism as 2.4, 1.6, 0.27, 0.016, 0.23, 1.63, 0.73 and 0.067 mg/ml for *S. aureus* ATCC 25923, *E. cloacae*, *S. paratyphi*, *K. pneumoniae*, *E. coli* ATCC 35218, *E. coli*, *Citrobacter* spp. and *C. albicans*, respectively. Antioxidant screening of clove oil with 2,2-diphenyl-picryl-hydrazyl radical (DPPH) was positive, indicating the presence of free radical scavenging molecules which can be attributed to the presence of eugenol, a phenolic compound.

Key words: *Syzigium aromaticum*, clove oil, antimicrobial, antifungal, Gram-negative bacteria, Gram-positive bacteria, Gas chromatography, mass spectrometry.

INTRODUCTION

Infectious diseases remain an important cause of morbidity and mortality in developing and developed nations (Lewis et al., 2006). They account for approximately one-half of all deaths in tropical countries of which bacterial infections seems to be the most prevalent (Iwu et al., 1999). The discovery and development of antibiotics have led to a dramatic improvement in the ability to treat infectious diseases and is among the major advances of the 20th century. Unfortunately, development of effective antibacterial agents has been accompanied by the emergence of drug-resistant organisms due to the irrational and overuse of antibiotics, failure to complete a course of treatment, genetic versatility of microbes and horizontal transfer of resistant genes among bacterial species. All the mentioned factors diminish the clinical effectiveness of antibiotics (Amit et al., 2006; Aibinu, 2007). There is therefore a need for continuous search for new, effective and affordable antimicrobial agents (Cowan, 1999). In recent times, there has been renewed interest on plants

as sources of antimicrobial agents due to their use historically and the fact that a good portion of the world's population, particularly in developing countries, rely on plants for the treatment of infectious and non-infectious diseases. Antiseptic properties of plant volatile oils have been recognised since antiquity (Dorman et al., 1999). Indeed, clove oil is commonly used as an anaesthetic in the relieve of toothache in dentistry. It is also used as a carminative, rubefacient and serves as a preservative in herbal recipes, signifying possible antimicrobial properties (Odugbemi, 2006).

Clove oil can be obtained from the flower buds of *Syzigium aromaticum*, family Myrtaceae. We herein report the extraction and analysis of the chemical constituents of clove oil (*S. aromaticum*) with the elucidation of its major component attributed to its acclaimed antimicrobial activities.

MATERIALS AND METHODS

Collection and identification of plant materials

The flower buds of clove plant used in this study were purchased

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from Mushin market in Lagos, Nigeria. The flower buds were identified and authenticated by Mr T.I. Adeleke at the Department of Pharmacognosy, Faculty of Pharmacy, University of Lagos.

Extraction of volatile oil by steam distillation

The dried flower buds were used in obtaining the clove oil by steam distillation, using the Clavenger apparatus (Pyrex), as described by Harbone (1998). In brief, the dried flower buds were grinded and 120 g weighed out, placed in a round bottom flask and water was added to approximately three-quarter full. The distillation apparatus was then 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 (w/v).

Test organisms

The organisms used comprised of five Gram-negative organisms (*Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Salmonella paratyphi* and *Citrobacter* spp.), a Gram-positive bacterium (*Staphylococcus aureus* ATCC 25923) and a fungus (*Candida albicans*). *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 were used as control strains in the assays. The test organisms were obtained from the Antimicrobial Resistance and Medicinal Plants as Antimicrobials Laboratory of the Department of Medical Microbiology and Parasitology, College of Medicine, University of Lagos.

Standardisation of inoculum

The test organisms were subcultured onto fresh plates of Mueller-Hinton agar (Oxoid, UK) for 24 h and Saboraud dextrose agar (Oxoid, UK) for 5 - 7 days at 37°C for bacteria and fungi, respectively. Colonies from these plates were suspended in Mueller-Hinton broth and Saboraud broth (Oxoid, UK) to a turbidity matching 0.5 McFarland standard (10^8 colony forming units (cfu)/ml).

Antimicrobial assay

The media used for antimicrobial assays were Mueller-Hinton agar for bacteria and Saboraud agar (Oxoid, UK) for fungi. All were incubated appropriately as specified for each organism by Aibinu et al. (2007). Labelled media plates were uniformly seeded with the different test microorganisms, by means of a sterile swab rolled in the suspension and streaked onto the agar's surfaces. Wells of 10 mm in diameter were punched using a sterile cork borer. Into each well, 100 µl of the oil extract was dropped. Ciprofloxacin (Fidson Healthcare Ltd, Nigeria), diluted to a concentration of 0.005% (w/v) and methanol 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, 3.48, 6.95, 13.91, 27.81, 55.61, 111.25, 445.0 mg/ml. Dilutions were made using methanol as solvent. Each plate was kept in the refrigerator at 4°C for 1 h to allow for diffusion of extract, before incubating at 37°C for 24 h as described by Pretorius et al. (2003). The diameter of the zone of inhibition around each well was measured in millimeter for each concentration of the extract used and used as positive bio-

activity. The zone diameter thus obtained was plotted against the log concentration of the extract used for each organism and the MIC was obtained from the graph as described by Lamikanra (1999). A representative plot is shown in Figure 1. Briefly, the straight line obtained from the graph was extrapolated to a point equivalent to the diameter of the well punched into the agar using the sterile cork borer (10 mm). The antilog of the concentration corresponding to a zone diameter of 10 mm was taken as the MIC. Determinations were carried out for each test organism.

Analysis of clove oil extracts using gas chromatography-mass spectrometry (GC-MS)

The chromatographic procedure was carried out using a Shimadzu QP2010-GC-MS with autosampler. The sample was diluted 25 times with acetone and 1 µl was injected into the column. A fused silica capillary column HP5-MS (30 m x 0.32 mm, film thickness 0.25 µm) was used. Helium was the carrier gas, and a split ratio of 1/100 was used. The oven temperature used was maintained at 60°C for 8 min. The temperature was then gradually raised at a rate of 3°C per min to 180°C and maintained at 180°C for 5 min. The temperature at the injection port was 250°C. The components of the test solution were identified by comparing the spectra with those of known compounds stored in the NIST library (2005). The chromatographic effluent was used as a sample inlet for the mass spectrometer. The ion source temperature was set at 200°C. The fragmented ions were separated by the analyzer, according to their various mass-to-charge ratios (Jayaprakasha et al., 2002).

Screening for free radical scavenging activity

The methanolic solution of the clove oil extract was spotted on a silica gel plate and 0.2% of a methanolic 2,2-diphenyl-picryl-hydrazyl (DPPH) solution was sprayed onto the spot. A change in the colour of the DPPH spray solution from deep violet to yellow was considered positive.

RESULTS

Extraction of essential oil and antimicrobial assay

The oil obtained was yellow in colour with a density of 1.06 mg/ml and a refractive index value of 1.526. The methanolic solutions of the extract were found to have potent antimicrobial activity against all the Gram-positive and Gram-negative organisms tested. The MICs (indicated in brackets) recorded for the test organisms were as follow: *S. aureus* ATCC25923 (2.4 mg/ml), *Enterococcus cloacae* (1.6 mg/ml), *Salmonella paratyphi* (0.27 mg/ml), *Klebsiella pneumoniae* (0.016 mg/ml), *E. coli* ATCC35218 (0.23 mg/ml), *E. coli* (1.63 mg/ml), *Citrobacter* spp. (0.73 mg/ml), and *Candida albicans* (0.067 mg/ml). Zones of inhibitions recorded for Ciprofloxacin (0.005% [w/v]) were 32 mm for *S. aureus* ATCC 25923, 10 mm for *E. cloacae* and the *E. coli* isolate, 33 mm for *E. coli* ATCC 35218 and 22 mm for *Citrobacter* spp., *S. paratyphi* and *K. pneumoniae*. Ciprofloxacin did not inhibit the growth of *C. albicans* (Figure 1 and Table 1).

Gas chromatography-mass spectrometry (GC-MS)

Three peaks were identified from the GC-MS data. These

Table 1. Zones of inhibition (mm) showing the antimicrobial activity of clove oil extract at various concentrations.

Test Organisms	COE	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	MIC mg/ml	Cip	Met
Gram +ve Bacteria														
concentrations.	890	445	222.5	111.3	55.61	27.81	13.91	6.95	3.48	1.74	0.87		0.005%	98%
<i>Staphylococcus aureus</i> ATCC25923	20	23	23	22	21	19	17	15	14	14	12	0.38	32	10
Gram -ve Bacteria														
<i>Enterobacter cloacae</i>	18	22	20	19	19	18	17	11	11	10	10	1.6	10	10
<i>Escherichia coli</i> isolate	18	23	23	20	20	17	15	13	12	10	10	1.63	10	10
<i>Escherichia coli</i> ATCC35218	18	21	20	20	20	18	16	14	14	13	13	0.23	33	12
<i>Citrobacter</i> spp.	18	24	19	18	17	17	14	14	14	12	11	0.73	22	12
<i>Salmonella paratyphi</i>	18	21	21	21	17	17	17	14	14	14	12	0.27	22	13
<i>Klebsiella pneumoniae</i>	20	21	19	19	18	18	17	16	16	15	14	0.02	22	13
Fungus														
<i>Candida albicans</i>	21	35	35	35	30	30	28	28	23	23	13	0.07	N	12

Key: Clove Oil Extract (COE), Extract Concentration in mg/ml (C1-C10), Methanol (Met), Ciprofloxacin (Cip), No zone of inhibition (N).

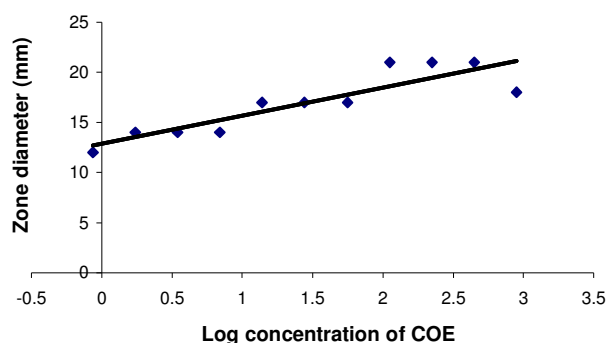


Figure 1. Graph of zone diameter against log concentration of clove oil for *S. paratyphi*. A representative plot showing how MIC was determined. The linear regression equation of the line for *S. paratyphi* was $y = 3.5636x + 12.025$. Log concentration was determined for the zone diameter of 10 mm (diameter of well). The concentration thus obtained was taken as the MIC.

major peaks were identified as eugenol, eugenol acetate and caryophyllene from the GC-MS database (Figures 2 and 3). The retention times (RT) were as follows: eugenol (12.5 min), caryophyllene (13.3 min) and eugenol acetate (14.7 min). Mass spectrometry data were as follows: m/z (eugenol): 164 M^+ , 149 ($M-CH_3^+$), 131($M-(CH_3+H_2O)^+$), 103, 91 ($C_7H_7^+$); m/z (eugenol acetate): 206 M^+ , 164($M+H-CH_3CO^+$), 149 ($M+H-(CH_3CO^+ + CH_3^+)$), 91($C_7H_7^+$), 65($C_5H_5^+$), 43 (CH_3CO^+); and m/z (Caryophyllene): 204 M^+ , 147 ($M-C_4H_9^+$), 121 ($M-C_6H_{11}^+$), 93 ($C_7H_9^+$), 80($C_5H_{10}^+$).

Free radical scavenging activity

The deep violet colour of DPPH vanished and turned yellow on spraying the silica gel containing the clove oil extract indicating the presence of a free radical scavengers.

DISCUSSION

The methanolic solutions of the extracts were found to have potent antimicrobial activity against all the Gram-positive and Gram-negative organisms tested. Clove oil exhibited a broad spectrum antimicrobial activity with a minimum zone diameter of 10 mm for *E. cloacae* and *E. coli* isolate and a maximum zone diameter of 35 mm for *C. albicans*. Ciprofloxacin (0.005% [w/v]) was more active against *S. aureus* ATCC25923, *E. coli* ATCC 35218, *Citrobacter* spp., *S. paratyphi* and *K. pneumoniae* at all concentrations of the extract. However, the clove oil extract was more active against *E. cloacae* and an *E. coli* isolate at concentrations of 3.48 mg/ml and above. Ciprofloxacin was inactive against *Candida albicans*, but the extract appeared to have very potent activity against *C. albicans* (Table 1). Indeed, clove oil is traditionally used in the treatment of oral candidiasis and athlete's feet.

The GC-MS data showed eugenol as the major constituent of clove oil, followed by eugenol acetate. The mass spectral data for eugenol showed the molecular ion at 164 which corresponds to the relative molecular mass of

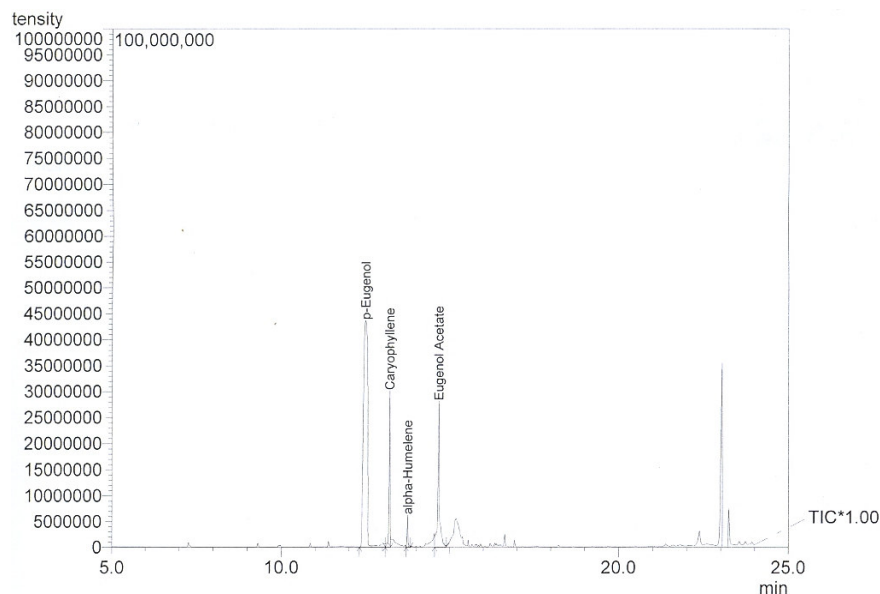


Figure 2. Gas chromatogram of clove oil extract showing eugenol, eugenol acetate and caryophyllene as the major constituents.

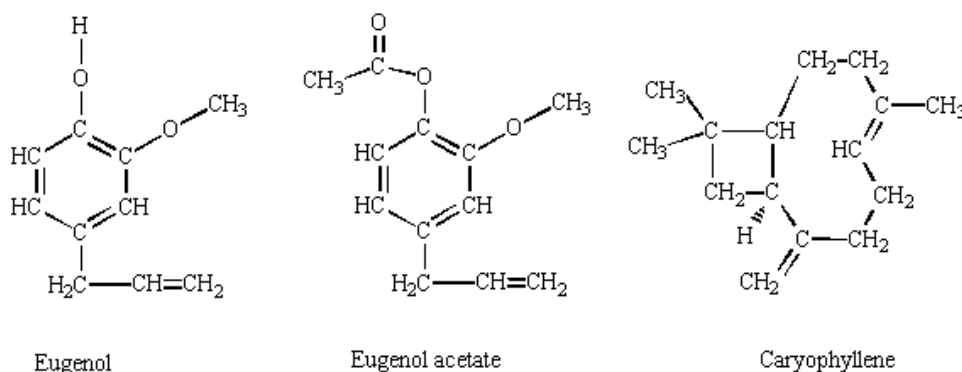


Figure 3. Chemical structures of the components of clove oil (eugenol, eugenol acetate and caryophyllene).

of eugenol, the fragment at 149 corresponds to loss of the methyl group from the side chain, peak 91 is typical of a tropylium ion signifying the presence of a benzyl group in the molecule. The mass spectral data for eugenol acetate showed the molecular ion peak at 206 corresponding to the relative molecular mass of eugenol acetate, the peak at 164 signifies the loss of the acetate, peak 91 (tropylium ion peak) also indicates the presence of a benzyl group. The peak at m/z 65 is due to loss of an acetylene group from the tropylium ion. The mass spectral data for caryophyllene shows the molecular ion peak at 206 which corresponds to the RMM of caryophyllene. The peaks signify stepwise loss of hydrocarbons from the parent molecule. Eugenol is a phenolic compound (Figure 3). Phenols are known to have anti-septic properties (Pelczar et al., 1998), which is con-

sistent with the antimicrobial data obtained for these compounds. Caryophyllene (Figure 3) has also been shown to possess antimicrobial properties, though not as potent as eugenol (Dorman et al., 2000).

The DPPH test provided information on the reactivity of the test compound with a free radical, Methanolic solution of 2,2-diphenyl-picryl-hydrazyl radical (DPPH) has a deep violet colour, which vanishes in the presence of a free radical scavenger. Phenolic compounds are known to have antioxidant and antimicrobial properties (Pelczar et al., 1998). The effect observed may be due to eugenol, which is a phenol. Free radical damage is implicated in a number of disease processes, including asthma, cancer, cardiovascular diseases, diabetes and inflammatory diseases. Pharmacological action of clove oil against some of these diseases can be investigated, as various

clinical and laboratory research has shown that other antioxidants can be used in the prevention and treatment of some of these diseases (Miller, 1996).

In conclusion, the volatile oil of *S. aromaticum* was active against all the microorganisms tested with a minimum zone diameter of 10 mm for *E. cloacae* and *E. coli* isolate and a maximum zone diameter of 35 mm for *C. albicans* and a maximum of 35 mm for *C. albicans*. GC-MS analysis of the oil extract showed eugenol, eugenol acetate, caryo-phyllene as the major constituent. Eugenol and caryo-phyllene are known to possess antibacterial and antifungal properties. Hence the antibacterial and anti-fungal properties demonstrated by the clove oil extract can be attributed to the compounds identified.

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