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ORIGINAL ARTICLE

Toxicity, antimicrobial and antioxidant activities of methyl salicylate dominated essential oils of *Laportea aestuans* (Gaud)

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2,2-Diphenyl-1-picrylhydrazyl;
Toxicity;
Antimicrobial;
Antioxidant;
Laportea aestuans;
Urticaceae

Abstract The colourless essential oils obtained by hydro-distillation from the whole plant of *Laportea aestuans* (Gaud) were analysed by GC and GC/MS. The major constituents in the oil were methyl salicylate (54.50%), fenchol (10.59%), 1, 2-cyclohexanedione dioxime (9.40%), 1, 4-octadiene (8.86%) and linalool (3.26%). The toxicity results obtained from brine shrimp lethality test gave LC_{50} value of 367.1805 μg/ml indicating that the oil is toxic. The oils showed appreciable antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Candida albicans, Rhizopus stolon, Aspergillus niger and Penicillium nonatum* at 200 mg/ml when compared to standards; gentamicin for bacteria and tioconazole for fungi. The oil was however very active against the fungi *R. stolon* and *A. niger* at 25–200 mg/ml. While the *in vitro* antioxidant activities of the oils determined by scavenging effect on 2, 2-diphenyl-1-picryl hydrazyl radical method showed that the oils have promising antioxidant activity as a free radical scavenger. At 0.1 and 0.2 mg/ml, the % inhibition of the essential oil (84.46% and 86.87%, respectively) was discovered to be higher than the % inhibition of α-tocopherol (15.4% and 12.4%).

1. Introduction

Increasing interest in herbal medicine is as a result of apprehension concerning the toxicity and safety of modern drugs and benefits of the complementary systems. The medicinal effect of plants results from the active constituents or secondary metabolites such as alkaloids, flavonoids, mucilage, bitters, glycosides, essential oils and terpenoids (Burkill, 1985; Trease and Evans, 1987; Newmann et al., 2000). Essential oil is any concentrated hydrophobe containing volatile aroma compounds from plants. Studies have revealed that essential oils serve as powerful antioxidants that produce adverse environment for damaging free radicals thus preventing mutations and oxidants in cells. They therefore function as scavengers for free radicals (Hanasaki et al., 1994; Potterat, 1997). Oxidation reactions can produce excess free radicals which in turn

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start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Gordon, 1996; Namiki, 1990; Mensor et al., 2001; Jovanovic et al., 1994).

Laportea aestuans, of the family Urticaceae is an herbaceous plant which appears as weed in new cultivations and fallows in West India, Africa and Asia. It is used traditionally as antimicrobial, anti-inflammatory, abortifacient, febrifuge, laxative, pain-killer, in pulmonary and stomach troubles amongst others (Boye and Ampofo, 1990; Nadine, 2004; Sofowora, 2008).

The aim of this research work is to determine the chemical composition of the essential oils of *L. aestuans* by carrying out gas chromatography and gas chromatography–mass spectrometry analysis, to determine the toxic level of the essential oils using Brine shrimp lethality test and subject the oil to antimicrobial and antioxidant screening. Brine shrimp lethality test is a bench top bioassay for the discovery and purification of bioactive natural products and they are an excellent choice for elementary toxicity investigations of consumer products (Dvorack et al., 1999; Meyer et al., 1982; Keddy et al., 1995). The antimicrobial effect is carried out in others to estimate the potency of the plant on certain micro-organisms as the plant is claimed to be an effective antimicrobial agent in traditional medicine practice. The activity is compared with antimicrobial standards, gentamicin and tioconazole. The antioxidant activity of *L. aestuans* oil was determined by *in vitro* assessment on DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. The main characteristic of an antioxidant is its ability to trap excess free radicals which can initiate degenerative disease (Bors and Saran, 1991; Arouma, 1996; Koppenol, 1993). The activity is compared with known antioxidant standards ascorbic acid, butylated hydroxylanisole and α-tocopherol.

2. Materials and methods

2.1. Materials

2.1.1. Plant materials

Fresh whole plant samples of *L. aestuans* were collected in June 2010 at the Botanical Gardens, University of Ibadan. Specimens were identified by Mr. E. Donatus at the Botany and Microbiology Department, University of Ibadan, Oyo State, Nigeria. The volatile oil was immediately collected from the fresh plant material by hydro-distillation using an all-glass scavenger apparatus.

2.1.2. Reagents

Hexane and methanol (BDH chemicals), butylated hydroxyanisole (BHA), α-tocopherol and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were obtained from Sigma Chemical Co. (Germany).

2.1.2.1. Test organisms. *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Candida albicans*, *Rhizopus stolon*, *Aspergillus niger* and *Penicillium nonatum* (micro-organisms were collected from the stock of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy of University of Ibadan). The test organisms were maintained on nutrient agar slopes and kept in a refrigerator at 4 °C. Hundred millilitres of aliquots of nutrient broth were inoculated with the culture of test micro-organisms using a loop and then incubated at 37 °C for 24 h.

2.1.2.2. Reference standards. Gentamicin at 5 mg/ml for bacteria and tioconazole (70%) for fungi (both for antimicrobial activity); ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol for antioxidant activity.

2.1.3. Major equipments used

UV–visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models), GC–mass spectrophotometer (Agilent Technologies), hydro distiller – Clavenger apparatus.

2.2. Method

2.2.1. Isolation of essential oils

The oil was obtained by hydro-distillation on a Clavenger type apparatus for 4 h in accordance with the British pharmacopoeia specifications (1980). The essential oil was collected and stored at 4 °C until analysis. The oil yield was calculated relative to the dry matter.

2.2.2. Analysis of the essential oils

2.2.2.1. Gas chromatography. GC–MS analysis of the essential oil was carried out on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091)-413:325 °C HP-5 column (30 m × 320 μm × 0.25 μm) was used with helium as the carrier gas at a flow rate of 3.3245 ml/min. The GC oven temperature was initially programmed at 50 °C (hold for 1 min) and finally at 300 °C (hold for 5 min) at a rate of 80 °C/min while the trial temperature was 37.25 °C. The column heater was set at 250 °C and was at split less mode while the pressure was 10.153 psi with an average velocity of 66.45 cm/s and a hold-up time of 0.75245 min was recorded. Mass spectrometry was run in the electron impact mode (EI) at 70 eV. The percentage compositions were obtained from electronic integration measurements using flame ionization detector (FID), set at 250 °C. The peak numbers and relative percentages of the characterized components are given in Table 1.

2.2.2.2. Gas chromatography–mass spectrometry. The essential oils were analysed by GC–MS on an Agilent Technologies 7890A GC system coupled to a 5975C VLMSD mass spectrometer with an injector 7683B series device. An Agilent (9091)-413:325 °C HP-5 column (30 m × 320 μm × 0.25 μm) was used with helium as the carrier gas at a flow rate of 3.3245 ml/min. GC oven temperature and conditions were as described above. The injector temperature was at 250 °C. Mass spectra were recorded at 70 eV. Mass range was from m/z 30 to 500.

2.2.2.3. Identification of components. The individual constituents of the oil were identified on the basis of their retention indices determined with a reference to a homologous series of *n*-alkanes and by comparison of their mass spectral fragmentation patterns (NIST 08.L database/chemstation data system) with data previously reported in literature (Adams, 1989; Joulain and Konig, 1998; Mclafferty and Stauffer, 1989).

Table 1 Chemical composition of essential oil of *L. aestuans* from GC–MS analysis.

S/N	RT (min)	Chemical composition	T.P. (%)
1	9.261	*N*-2-propynyl-2-Propyn-1amine	1.17
2	10.159	P-cymenene	1.27
3	10.554	Linalool	3.26
4	11.201	Fenchol	10.59
5	11.555	Trans-Rose oxide	2.65
6	11.796	2,4,6-Cycloheptatriene-1-one	2.53
7	13.198	Lavandulol	0.41
8	13.329	1,2-Cyclohexanedione dioxime	9.40
9	13.449	(E,E)-1,5-Cyclododecadiene	1.23
10	14.273	Methyl salicylate	54.50
11	14.451	Myrtenol	2.66
12	16.608	(E)-2-Octen-1-ol	0.88
13	14.748	1,4-Octadiene	8.86
14	15.678	*p*-Benzoquinone	0.59
Total			100.00

RT = Retention time, T.P. = Total percentage.

2.2.3. Brine shrimp lethality test

The toxicity level of the extracts was conducted according to Falope et al. (1993) and Oloyede et al. (2010). The brine shrimp lethality test (BST) was used to predict the presence, in the oils, of toxicity activity (Meyer et al., 1982). The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (10,000, 1000 and 100 ppm) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration for killing fifty percent of the larvae (LC_{50}) was determined using the Finney computer programme.

2.2.4. Antimicrobial analysis

Antimicrobial activities of the essential oils of *L. aestuans* were carried out using the agar well diffusion method. The 0.2 ml of an overnight broth culture of test micro-organisms was added to 20 ml of cooled molten agar.

2.2.5. Preparation of essential oil sample for antimicrobial analysis

A stock solution of the oil sample (100%) was prepared in hexane, 1 ml was taken to prepare 50% of the sample's concentration and this was done until 3.125% concentration was obtained.

2.2.5.1. Agar diffusion: pour plate method for bacteria. An overnight culture of each organism *S. aureus*, *E. coli*, *B. subtilis*, *P. aeruginosa*, *S. typhi*, and *K. pneumoniae* was prepared. The 0.1 ml of each of the organism was taken into 9.9 ml of sterile distilled water (SDW) to give 10 ml of 1:100 (10^2) dilution. The 0.2 ml was taken into the prepared molten nutrient agar (NA) at 45 °C and this was aseptically poured into the sterile plates and allowed to set on the bench for about 45 min. The stock was maintained on nutrient agar slant and sub-cultured in nutrient broth for incubation at 37 °C prior to each antimicrobial testing. Inoculation of the test organisms on nutrient agar-prepared plates was achieved by flaming a wire loop on a spirit lamp, cooling the wire loop (air cooling) and fetching the test organisms. The discs were prepared using a Grade No. 1 Whatman filter paper. One hundred discs were obtained by punching and putting in vial bottles and sterilizing in an oven at 150 °C for 15 min. Thereafter the cups (9 mm diameter) were aseptically bored into the solid nutrient agar using a sterile cork-borer. A sterile cork-borer was used to create wells (or holes) inside the set plates. The test solutions of oils (50 µl) at a concentration of 40 g/ml were then introduced into each of the designated cups on each plate ensuring that no spillage occurred. The same amount of the standard antimicrobial agent and solvents was introduced using syringes into the remaining cups on each plate to act as positive and negative controls, respectively. The plates were left at room temperature for 2 h, allowed to diffuse into the medium, turned upside-down and thereafter incubated at 37 °C for 24 h in an incubator. Clear zones of inhibition were observed. Activity or inactivity of the oil was tested in triplicate and the diameters of zones of inhibition were measured in millimetre (mm) using a transparent well-calibrated ruler. The positive control for bacteria is gentamicin at the concentration of 5 mg/ml (Rojas et al., 2003; Cushine and Lamb, 2005; Duraipandiyan et al., 2006).

2.2.5.2. Agar diffusion: pour plate method for fungi. Molten sterile Sabouraud dextrose agar (SDA) was poured aseptically into the sterile plates and allowed to cool down for 45 min. The 0.2 ml of 1:100 dilutions of the organisms *C. albicans*, *R. stolon*, *A. niger* and *P. nonatum* was spread on the surface using a sterile spreader. Then, a sterile cork-borer was used to create wells inside the plates. The same procedure described for the above anti-bacterial activity was followed from this stage. The positive control for the fungi is 70% tioconazole. All the plates for the fungi were incubated at 28 °C for 48 h unlike bacteria that was incubated at 37 °C for 24 h. The clear zones of inhibition were observed and recorded using the same method as described in the case of bacteria (Hadecek and Greger, 2000; Bayer et al., 1986).

Table 2 Brine shrimp lethality test of *L. aestuans* essential oils[a].

	1000 ppm	Concentration 1000 ppm	100 ppm	Control
Survivor	0	0	0	30
Dead	30	30	28	0
LC_{50} (μg/ml)	367.1805			

[a] LC_{50} < 1000 = Toxic, LC_{50} > 1000 = Not toxic, upper confidence limit 1339.4570, lower confidence limit 21.1602.

2.2.6. Free radical scavenging activity

2.2.6.1. Scavenging effect on DPPH. A 0.5 mM of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) solution in methanol was prepared and 3 ml of this solution was mixed with the oil sample in methanol. The free radical scavenging activity of the oil at various concentrations 0.1 and 0.2 mg/ml was then determined. The decrease in absorption at 517 nm of DPPH was measured after 10 min of incubation. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on ascorbic acid, butylatedhydroxylanisole (BHA) and α-tocopherol which are known antioxidant agents. All tests and analyses were run in triplicates and the result obtained was averaged (Koleva et al., 2002; Oloyede and Farombi, 2010). The activities were determined as a function of their % inhibition which was calculated using the formula:

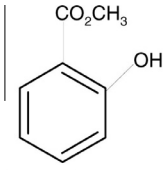
$$\%I = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

3. Results and discussion

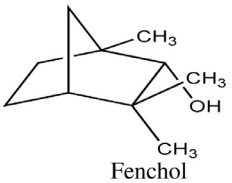
3.1. GC–MS analysis of the essential oils

Essential oils from 300 g of freshly collected whole plant samples of *L. aestuans*, obtained by means of hydro-distillation gave 0.60% (w/w). The essential oils, colourless with characteristic smell were analysed by GC and GC/MS systems using a polar column. 14 constituents representing 100% of the total essential oils were identified in the plant. The result of the analysis is presented in Table 1.

The following compounds *N*-2-propynyl-2-propyn-1amine, P-cymenene, linalool, fenchol, trans-rose oxide, 2,4,6-cycloheptatriene-1-one, lavandulol, 1,2-cyclohexanedionedioxime, (E,E)-1,5-cyclododecadiene, methyl salicylate, myrtenol, (E)-2-octen-1-ol, 1,4-octadiene, and *p*-benzoquinone, were obtained in order of the retention time in the hydro distilled oil.



Methylsalycilate



Fenchol

The major constituents in the oil were methyl salicylate (54.50%), fenchol (10.59%), 1,2-cyclohexanedione dioxime (9.40%), 1,4-octadiene (8.86%) and linalool (3.26%). The presence of methyl salicylate in this plant confirms the ethno medicinal importance of *L. aestuans*. Methyl salicylate is used in perfumery and as a flavouring material. Fenchol or 1,3,3-trimethyl-2-norbornanol is a terpene and an isomer of borneol and is used also in perfumery.

3.2. Brine shrimp toxicity test

The toxicity result of the essential oils of *L. aestuans* showed that it is toxic to brine shrimp larvae (Table 2). The essential oil with LC_{50} of 367.1805 μg/ml corroborated the presence in the oil of medicinally active compounds as it has been observed that medicinally active natural products are at most times toxic to *Artemia silina* nauplii (Oloyede et al., 2010; Onocha and Ali, 2010). The high toxicity of the oil can be beneficial in the therapy of some ailments involving cell or tumour growth.

3.3. Antimicrobial screening

The essential oils showed promising inhibitory potential especially against *Klebsiella pneumoniae, Salmonella typhi, Candida*

Table 3 Antimicrobial screening of the essential oils of *L. aestuans*.

Concentration	Zones of inhibition (mm)									
	S.a	*E.coli*	*B.sub*	*Ps.a*	*Kleb*	*Sal.*	*C.a*	*Rhiz*	*A.n*	*Pen*
1	12	10	10	10	14	16	14	16	16	12
2	10	–	–	–	12	14	12	14	14	10
3	–	–	–	–	10	12	10	12	12	–
4	–	–	–	–	–	–	–	10	10	–
5	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–
−ve	–	–	–	–	–	–	–	–	–	–
+ve	36	34	36	34	36	36	24	24	26	26

Integers 1–6 represent essential oil at various concentrations viz: (1) 200 mg/ml, (2) 100 mg/ml, (3) 50 mg/ml, (4) 25 mg/ml, (5) 12.5 mg/ml, (6) 6.25 mg/ml. −ve = negative control (hexane), +ve = positive control {gentamicin at 5 mg/ml for bacteria or tioconazole (70%) for fungi} = no inhibition, *S.a = Staphylococcus aureus*, *E. coli = Escherichia coli*, *B. sub = Bacillus subtilis*, *Ps.a = Pseudomonas aeruginosa*, *Kleb = Klebsiella pneumoniae*, *Sal. = Salmonella typhi*, *C.a = Candida albicans*, *Phiz = Rhizopus stolon*, *A.n = Aspergillus niger*, *Pen = Penicillium nonatum*.

Table 4 Scavenging effect of *L. aestuans* essential oils on DPPH.

Conc. (mg/ml)	Oil sample	Ascorbic acid	BHA	α-Tocopherol
0.1	0.15355 ± 0.07	0.0843 ± 0.010	0.0370 ± 0.006	0.6800 ± 0.029
0.2	0.12975 ± 0.01	0.2893 ± 0.128	0.0460 ± 0.008	0.7040 ± 0.003

Absorbance of essential oil, ascorbic acid, BHA and α-tocopherol at 517 nm.

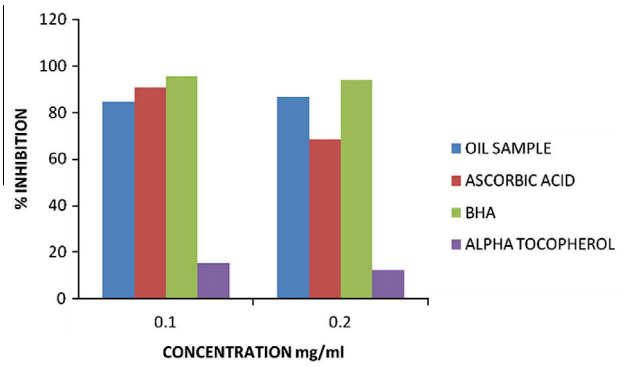


Figure 1 % Inhibition of DPPH free radical scavenging activity of essential oils of *L. aestuans*[*]. [*]% Inhibition of oil and reference standards.

albicans, Rhizopus stolon, and Aspergillus niger used in our analysis (Table 3) at 50–200 mg/ml but no activity was observed at lower concentrations for some of the organisms. The standards however showed better activity against the organisms than the essential oil sample.

3.4. Antioxidant activity

The essential oil sample was screened using 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). It was observed that the essential oil of *L. aestuans* scavenged free radical generated by DPPH as there was decrease in absorbance at 517 nm, 0.15355 ± 0.07 at 0.1 mg/ml and 0.12975 ± 0.01 at 0.2 mg/ml, respectively (Table 4).

The % inhibition of the essential oils increased with the increase in the concentration of the oils (Fig. 1). At 0.1 and 0.2 mg/ml, the % inhibition of the essential oil (84.46% and 86.87%, respectively) was discovered to be higher than the % inhibition of α-tocopherol (15.4% and 12.4%) but lower than that of ascorbic acid (90.9% and 68.7%) and BHA (95.4% and 94.3%).

The % inhibition of oil increased with concentration while that of the standards decreased with concentration. Overall, *L. aestuans* has free radical scavenging activity in the reaction involving DPPH radical.

4. Conclusion

This study has shown that the essential oils from *L. aestuans* have *in vitro* anti oxidant and antimicrobial activities. The oil contains 14 compounds highly dominated by methyl salicylate from the GC–MS analysis. Brine shrimp lethality test showed that the essential oil is toxic. The oil showed inhibitory potential against the 10 micro-organisms used in the study especially at 200 mg/ml. Photometric determination of antioxidant activity of the essential oils of *L. aestuans* revealed that the oil showed very good activity as a radical scavenger in the experiment involving 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH).

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