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American Journal of Essential Oils and Natural Products

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American
Journal of
Essential
Oils and
Natural
Products

ISSN: 2321 9114
AJEONP 2016; 4(1): 01-06
© 2016 AkiNik Publications
Received: 15-11-2015
Accepted: 18-12-2015

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Essential oil constituents, anticancer and antimicrobial activity of *Ficus mucoso* and *Casuarina equisetifolia* leaves

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Abstract

Essential oils were obtained by hydrodistillation of *Ficus mucoso* Welw. and *Casuarina equisetifolia* L. leaves and analyzed for their constituents by means of gas chromatography-mass spectrometry (GC-MS). The major volatile components of *F. mucoso* leaves were α -phellandrene (13.0%), *p*-cymene (11.3%), germacrene D (10.5%), β -caryophyllene (9.7%), 1,8-cineole (9.5%) and α -copaene (8.7%). High levels of α -phellandrene (40.6%), *p*-cymene (15.7%), 1,8-cineole (14.1%) and terpinolene (8.4%) characterized *C. equisetifolia* oil. *Ficus mucoso* oil demonstrated strong inhibitory activity against Hs578T human breast tumor cells (98.18%) relative to PC-3 human prostate carcinoma cells (47.17%); *F. mucoso* and *C. equisetifolia* essential oils also exhibited significant antimicrobial activity (39-625 μ g/mL) on pathogens employed in the assays.

Keywords: *Ficus mucoso*, *Casuarina equisetifolia*, essential oils composition, cytotoxicity, antimicrobial activity

1. Introduction

Aromatic plants are frequently used in traditional medicine because of their essential oils. In the last few years, there has been an increase in the use of aromatic medicinal plants and their essential oils in scientific research and industrial applications including nutritional, pharmaceutical, and cosmetic uses [1-4]. At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, cosmetic, and perfume industry [5]. Moreover, various potent biological activities including antimicrobial, antioxidant, anti-inflammatory, and anticancer are attributed to essential oils [6-7].

Ficus mucoso Welw. is a large tree 30-40 m high with a spreading open crown, in the rain forest, often on river banks. It belongs to the family Moraceae [8]. The young leaves and fruits are edible and have a pleasing aroma. The plant is used in the treatment of insanity, generalized edemas and leprosy. The bark and leaf decoction is taken for diarrhea and dysmenorrhea [9]. In several parts of the world, *Ficus* species are used in traditional medicines. *Ficus thonningii*, *F. natalensis*, *F. exasperata*, *F. lepriurii* and *F. asperifolia* have been implicated for use in venereal diseases, urinary tract infections, viral infections and as antiseptic [10]. There is a paucity of information on the phytochemistry of *F. mucoso* and biological activity of the essential oil, however a number of researchers have reported on the leaf volatile constituents of some *Ficus* species [11-16].

Casuarina equisetifolia L. belongs to the plant family Casuarinaceae. It is a rapid-growing tree to 25 m high, native of Australia and occurring commonly in the West African region. A number of forms exist, some with drooping, some with horizontal to erect branches [17]. The leaf is used in the treatment of toothache and dysentery [10]. The plant is a source of biologically active compounds such as catechin, ellagic acid, gallic acid, quercetin and lupeol, which are antioxidants [18], coumaroyl triterpenes [19] and d-gallicocatechin (casuarina) [20]. The plant is also known to store tannins [21] and proline [22]. *Casuarina equisetifolia* has been reputed to demonstrate antimicrobial activity [23]; the seed and fruit contain essential oils [14].

There have been sustained increases in the incidences of breast and prostate cancer in developing countries in recent years and intensive scientific research on this subject matter is imperative. Sixty percent of currently used anticancer drugs are derived one way or the other from natural sources [24-25].

As a part of our phytochemical and biological studies on natural essential oils, the aim of this work was to investigate the chemical composition of the leaf essential oils of *F. mucoso* and *C. equisetifolia* and their potential antimicrobial and antiproliferative activities on target pathogens and human breast and prostate cancer cell lines, respectively. This is the first report on the biological activity of the essential oils from these plants.

2. Materials and methods

2.1 Plants collection and authentication

The mature fresh leaves of *F. mucoso* and *C. equisetifolia* were collected in the month of July within the University of Ibadan, Nigeria. Plant samples were authenticated by Mr. F. Usang of the Herbarium Headquarters, Forest Research Institute of Nigeria (FRIN), Ibadan, Nigeria, where voucher specimens (FHI 407412 and FHI 107411, respectively) were deposited. The plant materials were air-dried under a laboratory shade prior to extraction.

2.2 Extraction of the volatile oils

Essential oils were obtained by hydrodistillation of the pulverized air-dried plant samples (500 g) in an all glass Clevenger-type apparatus in accordance with the British Pharmacopoeia specifications [26]. The distillation time was 4 h in all experiments. The oils obtained were stored under refrigeration until moment of analyses.

2.3 Gas chromatography-mass spectrometry analyses (GC/MS)

The volatile oils were subjected to GC-MS analyses on an Agilent system consisting of a model 6890 Gas chromatograph, a model 5973 Mass Selective Detector (MSD) and an Agilent ChemStation Data system. The GC Column was an HP-5ms fused silica capillary coated with (5% phenyl)-methylpolysiloxane (equivalent to USP phase G27) stationary phase, film thickness of 0.25 μm , a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 8.28 psi and flow rate of 1.0 mL/min. The inlet and MSD detector temperatures were maintained at 200 °C and 230 °C, respectively, while the MS transfer-line temperature was 280 °C. The GC oven temperature was programmed as follows: 40 °C initial temperature held for 10 min; increased at 3°/min to 200 °C; increased 2°/min to 220 °C. The samples were dissolved in CH_2Cl_2 to give a 1% w/v solution and a split injection technique was used. The split ratio was 1:30. Mass spectra were recorded at 70 eV. Identification of each individual constituent of the essential oils was achieved based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns (NIST database/ ChemStation data system) [27].

2.4 Cell culture media

Human Hs578T breast ductal carcinoma cells (ATCC. No. HTB-129) [28] were grown in 3% CO_2 environment at 37 °C in DMEM with 4500 mg glucose per liter of medium, supplemented with 10% fetal bovine serum, 10 μg bovine insulin, 100,000 units penicillin and 100 mg streptomycin per liter of medium, and buffered with 44 mM NaHCO_3 , pH 7.35. Human PC-3 prostatic carcinoma cells (ATCC No. CRL-1435) [29] were grown in 3% CO_2 environment at 37 °C in RPMI-1640 medium with l-glutamine, supplemented with 10% fetal

bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, and buffered with 15 mM Hepes and 23.6 mM NaHCO_3 .

2.5 Cytotoxicity screening

Hs578T cells were plated into 96-well cell culture plates at 1.0×10^5 cells per well and PC-3 cells at 1.9×10^4 cells per well. The volume in each well was 100 μL for both cell types. After 48 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 2.5 or 1.0 μL of DMSO solution of oil (1% w/w in DMSO), giving a final concentration of 250 or 100 $\mu\text{g}/\text{mL}$ respectively for each oil. Hs 578T cells were tested at final concentration at 250 $\mu\text{g}/\text{mL}$ and PC-3 cells at final concentration of 100 $\mu\text{g}/\text{mL}$. Solutions were added to wells in four replicates. Medium controls and DMSO controls (25 and 10 μL DMSO/mL) were used. Tingenone (250 and 100 $\mu\text{g}/\text{mL}$) was used as a positive control [30]. After the addition of compounds, plates were incubated for 48 h at 37 °C. The medium was then removed by suction and 100 μL of fresh medium was added to each well. In order to establish percent kill rates, the cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay was performed [31]. After colorimetric readings were recorded in triplicates (using a molecular Devices SpectraMAX Plus microplate reader, 490 nm), average absorbances, standard deviations, and percent kill ratios (% kill_{oil}/% kill_{DMSO}) were calculated.

2.6 Antimicrobial screening

Essential oils were screened for antibacterial activity against the Gram-positive bacteria, *Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No 29213) and the Gram negative bacteria, *Pseudomonas aeruginosa* (ATCC No 27853) and *Escherichia coli* (ATCC No 254922). Minimum inhibitory concentration (MIC) was determined using microbroth dilution technique [32]. Dilutions of the oils were prepared in cation-adjusted Mueller Hinton Broth (CAMHB) beginning with 50 μL of 1% w/w solutions of essential oils in DMSO plus 50 μL CAMHB. The oil solutions were serially diluted (1:1) in CAMHB in 96-well plates. Organisms at a concentration of approximately 1.5×10^8 colony forming units (CFU)/mL were added to each well. Plates were incubated at 37 °C for 24 h; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Gentamicin was used as a positive control while DMSO was used as a negative control. Antifungal activity was determined as described above using *Candida albicans* (ATCC No. 10231) in yeast nitrogen base growth medium with approximately 7.5×10^7 CFU/mL. Antifungal activity against *Aspergillus niger* (ATCC No 16401) was determined as above using potato dextrose broth inoculated with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control. Both *C. albicans* and *A. niger* were maintained at 32 °C and 25 °C respectively for 24 h.

3. Results & Discussion

The identified leaf volatiles of *F. mucoso* are listed in Table 1. A total of thirty five constituents were identified which represent 100% of the leaf essential oil. It was found that the terpenoid content of the leaf is high (89.6%), primarily four monoterpene hydrocarbons accounted for 29.7% while fourteen sesquiterpene hydrocarbons made up 39.9% of the volatile oil. Seven oxygenated monoterpenoids constituted 16.1% and five oxygenated sesquiterpenoids accounted for

3.9% of the leaf oil. The aromatic compounds represented by methyl salicylate, dill apiole and (3*Z*)-hexenyl benzoate constituted 4.1% of the oil; two aliphatic oxygenated compounds were identified in 6.4% concentration. The main constituents in this oil were α -phellandrene (13.0%), *p*-cymene (11.3%), germacrene D (10.5%), β -caryophyllene (9.7%), 1,8-cineole (9.5%), α -copaene (8.7%) and terpinolene (4.9%). (*E*)-3-methyl-non-2-en-4-one and two oxygenated sesquiterpenoids, spathulenol and carotol, were detected as components of *F. mucoso* leaf essential oil. Previous studies on Nigerian grown *Ficus* species have revealed the abundance of 1,8-cineole (13.8%), (*E*)-phytol (13.7%) and *p*-cymene (11.4%) in *Ficus exasperata* [11]; acorenone (20.7%) and phytol (16.2%) in *Ficus lutea*, with *Ficus polita* consisting mainly of phytol (23.3%) and 6,10,14-trimethyl-2-pentadecanone (15.0%), and *Ficus thonningii* rich in 6,10,14-trimethyl-2-pentadecanone (18.8%) and phytol (14.7%) [12]; β -caryophyllene (37.0%), ethyl octanoate (14.9%) and methyl octanoate (8.3%) in *Ficus mucosa* [13]; 6,10,14-trimethyl-2-pentadecanone (25.9%), geranyl acetone (9.9%), heneicosane (8.4%) and 1,8-cineole (8.2%) in *F. elastica* [14]; α -cadinol (25.1%), germacrene-D-4-ol (14.9%), γ -cadinene (11.8%) and α -muurolene (9.6%) in *F. benghalensis* [15]. Both 6,10,14-trimethyl-2-pentadecanone and phytol have been described as marker components of the oils of Nigerian grown *Ficus* species [12].

Some major constituents of previous studies on the essential oil of *Ficus* plants from Nigeria such as acorenone B, (*E*)-phytol, 6,10,14-trimethyl-2-pentadecanone, hexadecanoic acid, ethyl octanoate, tricosane, hexacosane and cyclotetradecane were not identified in the *F. mucoso* leaf oil. Adebayo *et al.* [15] also reported similar variation of *F. benghalensis* oil composition from previous published data. This observed variation in compositional profile may be attributed to chemotype as well as climatic and ecological conditions in the place of collection.

Chemical analyses of *C. equisetifolia* leaf essential oil revealed the occurrence of twenty five components accounting for 100% of the oil. Identified compounds characterized in the oil and their percentage concentrations are shown in Table 2. The oil was comprised of monoterpenoids, C₁₃-norterpenoids, sesquiterpenoids, aromatic and simple aliphatic compounds. Moderate amounts of alcohols (17.9%), ketones (3.4%), aldehydes (1.3%), aromatic esters and ether (4.4%) were identified in the oil. The monocyclic compounds of the *p*-cymene group and bicyclic compounds of the camphene group were present in the monoterpenoid class. In the *p*-cymene group, α -phellandrene, *p*-cymene, terpinolene and the oxides, 1,8-cineole and α -terpineol were representative compounds while in the camphene group, α -pinene and β -pinene were detected. The C₁₃-norterpenoids identified were (*E*)- β -damascenone, geranyl acetone and (*E*)- β -ionone. It was found that the terpenoid content of the leaf oil was high (94.7%). Five monoterpene hydrocarbons (69.2%) made up the bulk of the oil with their oxygenated derivatives (18.4%). Six sesquiterpene hydrocarbons (3.8%), three oxygenated sesquiterpenes (3.3%), four aromatic compounds (4.4%) and one oxygenated aliphatic compound (0.9%) were also classified in the oil. The aromatic ether and esters detected

were methyl salicylate, (3*Z*)-hexenyl benzoate, hexyl benzoate and dill apiole, which occurred as minor components of the leaf oil. The quantitatively significant compounds were α -phellandrene (40.6%), *p*-cymene (15.7%), 1,8-cineole (14.1%) and terpinolene (8.4%). Ogunwande *et al.* [14] reported that the major compounds in *C. equisetifolia* leaf essential oil were pentadecanal (32.0%) and 1,8-cineole (13.1%); significant quantities of α -phellandrene (7.0%), apiole (7.2%) and α -terpinene (6.9%) were present. The main constituents of the fruit were caryophyllene-oxide (11.7%), *trans*-linalool oxide (11.5%), 1,8-cineole (9.7%), α -terpineol (8.8%) and α -pinene (8.5%).

In vitro cytotoxic assays carried out in order to evaluate the biological potentials of the investigated oils revealed potent inhibitory effects of *F. mucoso* leaf essential oil against Hs578T cells (98.18% kill at 250 μ g/mL), however no significant effect on PC-3 cells (Table 3). *Casuarina equisetifolia* oil showed no cytotoxic activity on either of the cell lines. It is well known that the biological activities of an essential oil may depend on the main constituents or a synergy between the major and some minor compounds [33]. The chemical profiles of both leaf oils are qualitatively and quantitatively distinct (Table 1 and 2). Major sesquiterpenes constituents in *F. mucoso* oil, such as α -copaene, β -caryophyllene and germacrene D, were either not detected or found in trace amount in *C. equisetifolia* oil, have shown cytotoxic activity in previous studies [33-35]; however, α -phellandrene, *p*-cymene, 1,8-cineole and terpinolene were common to both oils. Hence, the compositional pattern of *F. mucoso* leaf oil may have influenced such notable anti-proliferative effect against the Hs578T cell line. α -Copaene, a tricyclic sesquiterpene, exhibited cytotoxic effects on MCF-7 breast carcinoma [33] and N2a-NB neuroblastoma [36] cell lines; β -caryophyllene has been described as a potent cytotoxic compound over a wide range of cell lines [33-37]; Salvador *et al.* [38] attributed the potent cytotoxic activity of *Casuarina lasiophylla* leaf essential oil against several cell lines to a high content of germacrene D and β -caryophyllene; and germacrene D has shown cytotoxic activity to both Hs578T and PC-3 cell lines [34]. Potent cytotoxic activities were considered at $\geq 90\%$ lethality at tested concentrations of 250 and 100 ppm for both breast and prostate carcinoma cells, respectively, in this report. The antibacterial and antifungal activities of the leaf volatile oils of *F. mucoso* and *C. equisetifolia* demonstrated broad spectrum antimicrobial activity (Table 4). *Casuarina equisetifolia* oil exhibited stronger antibacterial effect on *B. cereus*, *S. aureus* (39 μ g/mL) and *E. coli* (312 μ g/mL) compared with *F. mucoso* oil. *Candida albicans* was more sensitive to *C. equisetifolia* oil while *A. niger* showed more susceptibility to *F. mucoso* volatile oil. It has been documented that Gram-positive bacteria are more sensitive to chemical compounds than Gram-negative bacteria due to the relative thickness of their cell walls [39]. This is obvious by the sensitivity of the Gram-positive bacteria to the tested essential oils in the assay. It should also be noted, however, that prominent constituents common to both oils such as α -phellandrene, *p*-cymene, 1,8-cineole and terpinolene, along with other constituents have exhibited antimicrobial activity [40-43].

3.1 Tables

Table 1: Chemical constituents of *Ficus mucoso* leaf essential oil

RI ^a	RI ^b	Compound ^{c,d}	Area (%)	QI ^e (%)
870	875	(3Z)-Hexenol	3.4	91
940	939	α -Pinene	0.5	91
1004	1005	α -Phellandrene	13.0	94
1024	1026	<i>p</i> -Cymene	11.3	97
1031	1033	1,8-Cineole	9.5	98
1087	1088	Terpinolene	4.9	96
1102	1098	Linalool	2.1	93
1190	1189	α -Terpineol	0.6	92
1194	1190	Methyl salicylate	1.2	95
1199	1198	Safranal	0.7	95
1217	----	(<i>E</i>)-3-Methyl-non-2-en-4-one	3.0	98
1218	1218	β -Cyclocitral	1.0	96
1349	1368	α -Cubebene	0.6	98
1376	1376	α -Copaene	8.7	99
1390	1390	β -Cubebene	0.4	91
1392	1391	β -Elemene	0.3	98
1408	1409	α -Gurjunene	0.2	93
1419	1418	β -Caryophyllene	9.7	99
1428	1426	α -Ionone	0.6	98
1452	1454	α -Humulene	3.5	99
1459	1461	Alloaromadendrene	2.0	99
1477	1477	γ -Muurolole	0.2	91
1482	1480	Germacrene D	10.5	96
1486	1485	β -Ionone	1.6	96
1495	1494	Bicyclogermacrene	0.5	93
1500	1499	α -Muurolole	1.3	98
1510	1508	(<i>E,E</i>)- α -Farnesene	0.2	87
1523	1524	δ -Cadinene	1.8	99
1564	1564	(<i>E</i>)-Nerolidol	0.5	94
1572	1570	(3Z)-Hexenyl benzoate	1.3	98
1580	1576	Spathulenol	1.7	95
1593	1594	Carotol	0.9	96
1625	1622	Dill Apiole	1.6	96
1640	1640	τ -Cadinol	0.3	90
1653	1653	α -Cadinol	0.5	95

^aRI, calculated retention indices; ^bRI, retention index from literature; ^cOrder of elution on HP-5ms capillary column; ^dIdentification by comparison of the mass spectral and retention index data; ^eQI, 'quality index', reflects the fit comparison of experimental mass spectrum and NIST library mass spectrum.

Table 2: Chemical constituents of *Casuarina equisetifolia* leaf essential oil

RI ^a	RI ^b	Compound ^{c,d}	Area (%)	QI ^e (%)
855	854	(2E)-Hexenal	0.9	95
940	939	α -Pinene	3.7	95
977	980	β -Pinene	0.8	91
1003	1005	α -Phellandrene	40.6	93
1023	1026	<i>p</i> -Cymene	15.7	97
1031	1033	1,8-Cineole	14.1	99
1087	1088	Terpinolene	8.4	98
1190	1189	α -Terpineol	0.5	94
1194	1190	Methyl salicylate	0.5	96
1218	1218	β -Cyclocitral	0.4	94
1385	1380	(<i>E</i>)- β -Damascenone	0.4	78
1392	1391	β -Elemene	1.0	96
1418	1418	β -Caryophyllene	0.8	99
1455	1453	Geranyl acetone	0.5	91
1460	1459	Aromadendrene	0.8	90
1480	1480	Germacrene D	0.6	93
1486	1485	(<i>E</i>)- β -Ionone	2.5	96
1510	1508	(<i>E,E</i>)- α -Farnesene	0.3	96
1541	1542	α -Calacorene	0.3	93
1564	1564	(<i>E</i>)-Nerolidol	1.4	99
1570	1570	(3Z)-Hexenyl benzoate	1.1	92
1577	1576	Hexyl benzoate	0.8	78
1593	1594	Carotol	1.5	89
1626	1622	Dill apiole	2.0	96
1723	1722	(<i>E,E</i>)-Farnesol	0.4	94

^aRI, calculated retention indices; ^bRI, retention index from literature; ^cOrder of elution on HP-5ms capillary column; ^dIdentification by comparison of the mass spectral and retention index data; ^eQI, 'quality index', reflects the fit comparison of experimental mass spectrum and NIST library mass spectrum

Table 3: Cytotoxic activity of *F. mucoso* and *C. equisetifolia* leaf essential oils^a

Sample	Hs578T ^b	PC-3 ^c
<i>F. mucoso</i>	98.18 ± 1.17	47.17 ± 1.63
<i>C. equisetifolia</i>	0	1.81 ± 2.37
β-Caryophyllene	100.0 (IC ₅₀ = 78.2 ± 8.3 μg/mL) ^[34]	100 (IC ₅₀ = 31.3 ± 1.1 μg/mL) ^[34]
Germacrene D	100.0 (IC ₅₀ = 55.2 ± 5.3 μg/mL) ^[34]	100 (IC ₅₀ = 26.0 ± 2.0 μg/mL) ^[34]
Tingenone	100.0	100.0

^a% kill at tested concentrations in triplicate (standard deviations in parentheses); negative controls, medium and DMSO had no effects in the assay; ^bHuman breast (ductal) tumor cells (% kill at 250 μg/mL); ^cHuman prostate tumor cells (% kill at 100 μg/mL). Reference compound; tingenone at concentration of 250 or 100 ppm.

Table 4: Antimicrobial activity of *F. mucoso* and *C. equisetifolia* volatile oils (MIC, μg/mL)

Sample	B.c	S.a	E.c	P.a	C.a	A.n
<i>F. mucoso</i>	625	312	625	625	625	78
<i>C. equisetifolia</i>	39	39	312	625	312	156
Positive control	1.22 ^a	0.61 ^a	2.44 ^a	1.22 ^a	0.61 ^b	0.61 ^b

B.c., *Bacillus cereus* (ATCC No. 14579); S.a, *Staphylococcus aureus* (ATCC No 29213); E.c, *Escherichia coli* (ATCC No. 25922); P.a, *Pseudomonas aeruginosa* (ATCC No. 27853); C.a, *Candida albicans* (ATCC No. 10231); A.n, *Aspergillus niger* (ATCC No. 16401). ^aGentamicin sulphate; ^bAmphotericin B; Negative control, DMSO had zero effect in the assay.

4. Conclusions

The biological activities of *F. mucoso* and *C. equisetifolia* essential oils are a function of their distinct chemical profiles. This study indicates that *F. mucoso* leaf oil possesses strong cytotoxic effects against Hs578T carcinoma cells. Furthermore, *F. mucoso* and *C. equisetifolia* oils demonstrated significant broad spectrum *in-vitro* antimicrobial activity against medicinally important pathogens in the assay. The present findings would provide further insight on the plants therapeutic applications and enhance the exploitation of these oils for other biological purposes.

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