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Essential oils of *Dennettia tripetala* Bak. f. Stem Bark and Leaf – Constituents and Biological Activities

Adebayo A. Gbolade[a,*], Teresita Arcoraci[b], Manuela D'Arrigo[b], Francis O. Olorunmola[c], Daniela M. Biondi[d] and Giuseppe Ruberto[d,*]

[a]*Department of Pharmacognosy, Faculty of Pharmacy, Olabisi Onabanjo University, Sagamu campus, Ogun State, Nigeria*

[b]*Dipartimento Farmaco-Biologico, Università di Messina,Contrada Annunziata, I-98128 Messina, Italy*

[c]*The Drug Research and Production Unit, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria*

[d]*Istituto del C.N.R. di Chimica Biomolecolare, Via del Santuario, 110 I-95028 Valverde CT, Italy*

giuseppe.ruberto@icb.cnr.it (G. Ruberto); adegbolade@yahoo.com (A.A. Gbolade)

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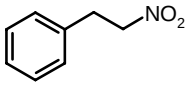
The essential oil from the stem bark and leaves of *Dennettia tripetala* Bak. f. (Annonaceae) growing wild in Ondo State, Nigeria, has been characterized by combined gas chromatography (GC) and gas chromatography-mass spectrometry (GC- MS) analyses. Overall, thirty-six components have been fully identified, thirty-two in the stem-bark oil, and only seven in the leaf oil. In both oils, 2-phenylnitroethane was the main component, ranging between 70 - 76% of the total oils. The profile of the stem bark oil was characterized by a large number of sesquiterpenes, whereas among the few components in the leaf oil, linalool reaches over 17%. When both oils were assayed for antimicrobial activity, only *Staphylococcus aureus* was susceptible to the stem-bark oil which was more active than leaf oil. For protective effects against UV radiation–induced peroxidation in phosphatidylcholine (PC) liposomes, stem-bark oil also showed greater effectiveness. Activity of the leaf oil against *Trichomonas gallinae*, was also remarkable.

Keywords: *Dennettia tripetala*, stem bark, leaf, essential oil, 2-phenyl nitroethane, antimicrobial activity, UV radiation, anti-trichomonal activity.

Dennettia tripetala Bak. f. (Annonaceae) [syn. *Uvariopsis tripetala* (Bak. f.) G.E. Schatz], is a rain forest tree and occasionally of the savannah, that can grow to 18 m with 60 cm girth. It has a limited distribution and is found from Cote d'Ivoire to western Cameroon [1]. Both the fruits and young leaves are chewed on account of their pungent spicy taste. Concerning the volatile components of this plant, most studies have focused on the essential oil composition of fruits [2-4] and only one report dealt with the essential oil of *D. tripetala* leaves growing in Benin [5]. In all cases, 2-phenylnitroethane (**1**) was the preponderant component. This compound is quite an unusual essential oil component. In fact, it has only been detected in the ornamental plant Madagascar jasmine, *Stephanotis floribunda* Brongn. (Asclepiadaceae), which is particularly appreciated for its delicate appearance and odor [6]. It has also been detected in relatively lower levels in the essential oil of *Cananga odorata* Hook Fil. et Thomson, *forma genuina* (Annonaceae), the ylang ylang oil, an important perfume raw material [7]. Furthermore, two derivatives, namely 2-(4-methoxyphenyl)-1-nitroethane (**2**) and 2-(4-hydroxyphenyl)-1-nitroethane (**3**), have been isolated from the skunk cabbage, *Lysichitum americanum* Hultén and St. John (Araceae) [8].



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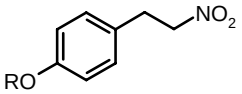

2 R = Me; 3 R = H

Nitro compounds are mostly associated with synthetic chemistry rather than natural products. However, several natural nitro compounds are

known, and most of them show interesting biological activities [9], as well as some level of toxicity [10].

Essential oil constituents of *D. tripetala* are scarcely reported. As part of our continued investigations into the aromatic flora of Nigeria, we here report the results of a study on the chemical composition of the oils from the stem-bark and leaves of *D. tripetala* and the evaluation of their antimicrobial and anti-trichomonal activities, as well as the protective effect against UVC-induced peroxidation (UV-IP test).

The oil is characteristically denser than water (1.18 g/mL) representing one of the few exceptions among the essential oils. The oil yield from leaves (0.44%) is double that of the stem bark, but both are high enough to encourage local cultivation. Nonetheless, these yields were less than that from the fruits (3.6-4.0%) reported by Agbakwuru *et al*. [3]. In addition, this oil was observed to possess appreciable repellent activity against some pests of stored products and mosquitoes [11-13], insecticidal effectiveness [14] and, also, antifungal qualities [15,16].

Table 1 lists the chemical composition of the *D. tripetala* essential oils accounting for 96% and 99% of the stem bark and leaf oils, respectively. The stem-bark oil showed the presence of thirty-three components, whereas the leaf oil was constituted by only seven components. 2-Phenylnitroethane was the main component of both oils and constituted over 70% of the total oils in both cases. This same compound was reported as the dominant volatile component in the fruit from Nigeria [2-4] and leaves from Benin [5].

Both oils of the Nigerian variety of *D. tripetala* showed qualitative similarities, and quantitative differences in composition. In fact, the stem-bark oil is characterized by a large number of sesquiterpenes comprising twenty-five components which amounted to 24% of the total oil. Major sesquiterpenes included β-caryophyllene, α-humulene, β- and α-eudesmol and *cis*-isocaryophyllene. Linalool (1%) was the only oxygenated monoterpene detected in the stem bark oil.

With the exception of the main component, i.e. 2-phenylnitroethane, the composition of the leaf oil is rather simple and quite different since only seven components, dominated by the only oxygenated monoterpene linalool (18%), were identified. In comparison with the previous oil, the almost total absence of sesquiterpenes should also be underlined.

Table 1: Chemical composition of *D. tripetala* essential oils.

No.[a]	Compound	RI[b]	RI[c]	Stem-bark[d]	Leaf[d]
1	*trans*-3-Hexenol	858	851	-	1.2
2	α-Pinene[d]	937	939	-	0.8
3	Benzaldehyde[d]	958	960	0.2	-
4	Limonene[d]	1027	1029	0.3	-
5	Phenyl acetaldehyde	1041	1042	0.2	-
6	*trans*-β-Ocimene[d]	1049	1050	0.2	1.3
7	**Linalool**[d]	1096	1097	1.3	**17.8**
8	Phenyl acetonitrile	1133	1142	0.3	-
9	**2-Phenylnitroethane**	1298	1295	**70.2**	**76.1**
10	Cyclosativene	1354	1371	t	-
11	α-Copaene	1366	1377	0.3	-
12	β-Elemene	1382	1391	t	-
13	*cis*-Isocaryophyllene	1394	1409	1.3	-
14	β-Caryophyllene[d]	1406	1419	5.8	-
15	β-Copaene	1416	1432	0.2	-
16	α-*trans*-Bergamotene	1426	1436	0.9	-
17	α-Humulene[d]	1441	1455	3.0	-
18	β-Farnesene[d]	1450	1457	0.2	
19	γ-Muurolene	1466	1480	0.4	-
20	α-Selinene	1482	1498	0.2	-
21	α-Muurolene	1488	1500	0.3	-
22	β-Bisabolene	1497	1506	t	-
23	α-Farnesene[d]	1499	1508	-	0.9
24	Cadinene	1523	1512	1.0	-
25	Elemol	1538	1559	0.4	-
26	*trans*-Nerolidol[d]	1555	1563	0.3	-
27	Dendrolasin	1571	1574	-	0.8
28	Globulol[d]	1572	1585	1.4	-
29	Guaiol	1585	1601	0.4	-
30	Humulene epoxide II	1593	1608	0.5	-
31	*epi*-α-Cubebol	1617	1629	0.7	-
32	10-*epi*-γ-Eudesmol	1621	1632	1.2	-
33	Cubenol	1634	1646	0.8	-
34	β-Eudesmol[d]	1643	1651	2.2	-
35	α-Eudesmol	1646	1654	1.9	-
36	Bulnesol	1664	1672	0.2	-
37	α-Bisabolol	1680	1686	0.1	-
	Total			**96.4**	**98.9**

[a] The numbering refers to elution order on DB-5 MS capillary column; [b] Retention index relative to standard mixture of C_8-C_{24} *n*-alkanes on DB-5 MS capillary column; [c] Literature data [24]; [d] Values (area %) represent averages of three determinations (t = trace, <0.05%); [d] Co-injection with authentic sample.

The latter, in fact, were represented at levels below 1%, by α-farnesene and dendrolasin, a well known furano-sesquiterpene [17]. The essential oils from *D. tripetala* examined here are, thus, strongly characterized by 2-phenylnitroethane, and this is in agreement with the report of Osisiogu and Agbakwuru for fruit oil [2]. This report represents the first documentation on the chemical composition of the stem bark oil. The leaf essential oil instead showed a unique precedent, coming from a sample collected in a forest of Benin [5]. This oil showed the presence of forty-eight components, four of which, namely 2-phenylnitroethane (54%), methyl eugenol (23%), linalool (16%) and eugenol (2%) represented about the 95% of the total oil, whereas the remaining components occurred at levels below 1% or as trace.

Both oils have been tested for their antimicrobial activity against three microbial strains, the Gram

Table 2: Inhibition zones and MIC of *D. tripetala* oils.

Dennettia oils/ References	*Staphylococcus aureus* ATCC 6538P		*Escherichia coli* ATCC 25922		*Candida albicans* ATCC 10231	
	Inhibition zone (mm)[a]	MIC[b] (μg/mL)	Inhibition zone (mm)	MIC (μg/mL)	Inhibition zone	MIC (μg/mL)
Leaf	8 ± 0.5	250	–––	–––	–––	–––
Stem-bark	11 ± 0.9	62.50	–––	–––	–––	–––
Amikacin	26 ± 2.0	1.56	25 ± 3.0	3.12	–––	–––
Amphotericin B	–––	–––	–––	–––	14.0 ± 1.1	0.78

[a] Including disc diameter (6 mm); oil dilution (1:50).[b] Minimum Inhibitory Concentration.

Table 3: Protective effects of *D. tripetala* oils in the UV radiation-induced peroxidation in liposomal membranes, UV-IP test.

Dennettia oils	UV-IP test (μg/mL)[a]
Leaf	83.68 (74.61 – 93.86)
Stem-bark	70.91 (64.95 – 77.41)

[a] IC_{50} (LC 95%)

(+) *Staphylococcus aureus*, the Gram (–) *Escherichia coli* and the yeast *Candida albicans*. The results of this screening as listed in Table 2, revealed *S. aureus* to be the only susceptible microorganism to both essential oils. However, the stem-bark oil showed better antimicrobial activity (MIC, 62.50 mg/mL) than the leaf oil (MIC, 250 mg/mL) probably owing to the presence of sesquiterpenes, whose antimicrobial effectiveness is largely reported [18,19].

Since some plant phenolic compounds are known to act as strong UV absorbers [20], with the UV-IP test, it is possible to study the protective effect against UV radiation-induced damage in biomembranes. The two oils have been subjected to an *in vitro* cell-free system (liposomal particles) to establish their protective action on lipid peroxidation. High energy UVC radiation was used as an inducer of lipid degradation, and malondialdehyde (MDA), a final product of lipid degradation, was colorimetrically monitored as an index of peroxidation. Both oils showed moderate protective activity, with the stem bark oil being more active (Table 3).

D. tripetala leaf oil, when tested against *Trychomonas gallinae* at 0.003908 to 2.0 v/v, exhibited appreciable effectiveness (LC_{50}: 0.132 v/v and 0.111v/v at 24h and 48h. respectively) (Table 4). However, complete mortality was evident at tested concentrations of 0.25 to 2.0 v/v.

This activity was comparable with that of the foremost commercial anti-trichomonal agent, metronidazole (Table 5). Therefore, *Dennettia* oil could serve as an alternative source of anti-trichomonal agents for future clinical studies. Previous studies have, however, documented anti-trichomonal activity of natural products [21-22]. This study becomes significant in view of the recent discovery of resistance [22] of trypanosomes to metronidazole and its unpleasant adverse effects, which has necessitated the search for alternative anti-trichomonal agents that are well tolerated and effective.

Table 4: Anti-trichomonal activityof *Dennettia* leaf oil.

Concentration (% v/v)	24 hrs (% mortality ± SEM)	48 hrs (% mortality ± SEM)
2	100 ± 0	100 ± 0
1	100 ± 0	100 ± 0
0.5	100 ± 0	100 ± 0
0.25	100 ± 0	100 ± 0
0.125	36.67 ± 8.41	48.18 ± 21.54
0.063	28.93 ± 5.74	39.99 ± 18.48
0.031	15.38 ± 2.25	28.18 ± 5.74
0.016	13.86 ± 9.23	15.35 ± 3.48
0.008	13.01 ± 8.68	6.17 ± 4.97
0.004	0.00 ± 0	8.19 ± 4.11
Control (50% DMSO)	0	0
LC_{50}	**0.13% v/v**	**0.11% v/v**

Table 5: Effect of metronidazole on parasites.

Concentration (μg/mL)	24 hrs (% mortality ± SEM)	48 hrs (% mortality ± SEM)
40	100	100
20	100	100
10	100	100
5	100	100
2.50	93.02 ± 0.09	80.87 ± 2.37
1.25	76.14 ± 1.79	62.92 ± 3.17
0.625	33.11 ± 2.16	47.93 ± 2.37
LC_{50}	**1.02 μg/mL**	**0.43 μg/mL**
Control (50% DMSO)	0	0

This study complements earlier publications on *D. tripetala* essential oil by reporting the constituents of the leaf and stem bark oils. Furthermore, the potential of the oil as an anti-trichomonal, an antibacterial and a radical scavenger agent is also documented.

Experimental

Plant material: Fresh samples of stem bark and leaves of *D. tripetala* were harvested from trees growing in Olomitoto village, Ondo/ Akure Road, Ondo State, Nigeria. Authentication was done at the Department of Botany (OAU) herbarium where voucher specimen no. OAU 14672 was deposited.

Essential oil extraction: The essential oils were obtained by hydrodistillation of fresh stem-bark (100 g) and leaves (100 g) by a Clevenger-type apparatus and stored in screw-capped vials under refrigeration until needed. Yellowish oils in yields of 0.23% v/w (fresh weight) and 0.44% v/w were recovered from the stem barks and leaves, respectively.

GC and GC-MS analyses: The oils were analyzed on a Shimadzu gas chromatograph, Model 17-A equipped with a flame ionization detector (FID). Analytical conditions: DB-5 MS capillary column (30 m x 0.25 mm x 0.25 μm), helium as carrier gas. Injection in split mode (1:50), injected volume 1 μL, injector and detector temperature 250 and 280°C, respectively. Linear velocity in column 19 cm/sec. The oven temperature was held at 60°C for six minutes, then programmed from 60 to 300°C at 2°C/min. Percentages of compounds were determined from their peak areas in the GC-FID profiles.

Gas-chromatography-mass spectrometry (GC-MS) was carried out in the fast mode on a Shimadzu GC-MS mod. GCMS-QP5050A, ionization voltage 70 eV, electron multiplier 900 V, transfer line temperature 280°C. Analytical conditions: SPB-5 capillary column (15 m x 0.10 mm x 0.15 μm), helium as carrier gas. Injection in split mode (1:100), injected volume 1 μL, injector and detector temperature 250°C. Constant linear velocity in column 50 cm/sec. The oven temperature was held at 60°C for one minute, then programmed from 60 to 280°C at 10°C/min.

Identification of components was based on GC retention indexes [23], computer matching with Wiley 275 and NIST (Versions 21 and 75) libraries, comparison of the fragmentation patterns with those reported in the literature and whenever possible, co-injection with authentic samples. Pure standards were purchased from Aldrich Chemical Co., Extrasynthese, France, and Fluka Chemie AG, Switzerland.

Antimicrobial assay

Test organisms: American Type Culture Collection (ATCC) standard strains were used: *Staphylococcus aureus* ATCC 6538P, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231.

Disk diffusion assay: Screening of essential oils for antibacterial and antifungal activity was done by the disk diffusion method, which is normally used as a preliminary check in order to select active essential oils. It was performed using an 18 h culture at 37°C in 10 mL of Mueller Hinton Broth for bacteria and at 35°C in 5 mL of RPMI 1640 (with L-glutamine and without sodium bicarbonate, supplemented with 0.165 M of MOPS) for yeast. The cultures of bacteria were adjusted to approximately 10^5 CFU/mL with sterile saline solution, whereas the culture of *C. albicans* was adjusted to approximately 10^3 CFU/mL with RPMI 1640.

Five hundred microliters of the suspensions were spread over the plates containing Mueller-Hinton agar and Sabouraud dextrose agar for bacteria and yeast, respectively, using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. The essential oils were dissolved in dimethylsulfoxide (DMSO) and sterilized by filtration through a 0.45 μm membrane filter. Under aseptic conditions, empty sterilized discs (Whatman no. 5, 6 mm dia) were impregnated with 20 μl of concentration 1:50 of the respective essential oils and placed on the agar surface. Paper disc moistened with DMSO was placed on the seeded Petri plate as a vehicle control. A standard disc containing amikacin (30 μg/disc) and amphotericin B (200 μg/disc) were used as reference controls. All Petri dishes were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 min at room temperature to allow the diffusion of oil, and then incubated at 37°C for 18 h for bacteria, and at 35°C for 24-48 h for yeast. After the incubation period, the zone of inhibition was measured with a calliper. Studies were performed in triplicate, and the mean value was calculated.

MIC assay: The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) according to the M100-S17 for bacteria and M27-A2 for yeast guidelines, established by the National Committee for Clinical Laboratory Standards [20,21]. The MIC values (minimum concentration that inhibits the inoculum growth) against the bacterial and yeast strains were determined on 96 well culture plates by a microdilution method. Eight two-fold dilutions of the samples were carried out starting from the concentration of 1 mg/mL as final concentration with a concentration of 1% DMSO The reference standards were used at 50 μg/mL as final concentration with a concentration of 1% of DMSO for amphotericin B whereas the amikacin was

solubilized in saline solution. All preparations were sterilized with a 0.45 µm filter. The wells were inoculated with a microorganism suspension at a density of 10^5 CFU/mL for bacteria and 10^3 CFU/mL for yeast. The plates containing bacteria were incubated at 37°C for 24 h and the plates containing the yeast were incubated at 35°C for 24-48 h . After incubation the plates were observed in order to determine the MICs. Proper blanks were prepared simultaneously. Samples were tested in triplicate.

UVC radiation-induced peroxidation in liposomal membranes (UV-IP test): The protective effect of oils against UVC-induced peroxidation was evaluated on phosphatidylcholine (PC) multilamellar vesicles (MLVs) by monitoring malondialdehyde (a final product of fatty acid degradation; MDA) production [15]. Briefly, 100 mg of PC, dissolved in chloroform, were transferred to a small stoppered tube. The lipid was thoroughly dried under nitrogen. It was then dissolved in warm ethanol (80 mg), and 25 mM Tris-HCl, pH 7.4 (200 mg) was added to yield a (100:80:200 w/w/w) lipid/ethanol/water mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature (20°C) yielding a proliposome mixture. The proliposome mixture was finally converted to a liposome suspension by the drop wise addition of 25 mM Tris-HCl, pH 7.4, to a final volume of 10 mL. The suspension was vortex-mixed throughout this last stage. Liposome dispersion (1 mL in a glass flask with a 3 cm^2 exposure surface area) was maintained at room temperature and exposed, for 1.5 h to UV-radiation from a 15 watt Philips germicidal lamp (254 nm) at a distance of 10 cm; the dose rate of UV-radiation was 105 erg/mm^2/sec. Different concentrations of oils were added to the system; an equal volume (50 µL) of the vehicle alone (ethanol) was added to control tubes. MDA concentration in the mixture was measured by using a colorimetric assay kit (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). All experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentage decrease with respect to control values; mean inhibitory concentrations (IC_{50}) and 95% confidence limits (95% C.L.) were calculated by using the Litchfield & Wilcoxon test.

Anti-trichomonal assay

Culturing of Trichomonas gallinae: *T. gallinae* was isolated from domestic pigeon (*Columba livia*) by gently inserting a sterile cotton swab into the crop of the pigeon and rolling it round the crop twice. The swab was then withdrawn and dipped into a test tube containing some normal saline. It was then rolled again to allow the parasite to go into solution and the resulting mixture was then checked for the presence of parasite by wet mount observation under the light microscope. The parasite was then cultured in the Ringer-egg-serum medium according to the method of Boeck and Drbohlav [22], as modified by Levine [23]. The medium was essentially a coagulated egg slant overlaid with a fluid nutrient solution. The egg slant was prepared by mixing 12.5 mL of Ringer's solution with albumin of one chicken egg, blended and dispensed in aliquots of 2 mL each into cotton stoppered test tubes. The test tubes were then placed in slanting position and autoclaved at 15 lb pressure for 20 minutes. The overlay fluid consisted of 500 mL. of sterile Ringer's solution, 10 mL sterile 10% w/v glucose solution and 10 mL of sterile bovine serum. Sufficient overlay fluid was then added to each egg slant to cover the whole slant. The inoculums of *T. gallinae* were then added to the prepared nutrient media and inoculated at 37°C for 24 to 48 hrs.

Anti-trichomonal assay: The assay was performed as described by Meingasser and Thurner [24]. One mL. sample of the volatile oil was dissolved in 1 mL. of 50% DMSO to serve as stock solution. This was serially diluted to give concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01563, 0.007813 and 0.003908 % used for the anti-trichomonal assay. Fifty microliter of each concentration and 150 µl of the inoculated nutrient solution were pipetted into micro wells and incubated at 37°C for 24 to 48 h. The number of organisms per mL. of each well after incubation for 24 and 48 h. respectively were enumerated under the microscope. Metronidazole (Flagyl®) at concentrations ranging from 40 to 0.625 % in DMSO: normal saline (1:1) served as the positive control, while the negative control was normal saline. The readings were done in triplicate and mean ±SEM recorded.

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