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Chemical Compositions and Biological Activities of Leaf Essential Oils of Six Species of Annonaceae from Monteverde, Costa Rica

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Abstract: The leaf essential oils of six members of the Annonaceae from Monteverde, Costa Rica (*Desmopsis bibracteata*, *Desmopsis microcarpa*, *Guatteria costaricensis*, *Guatteria diospyroides*, *Guatteria oliviformis*, and *Unonopsis costaricensis*) have been obtained by hydrodistillation and analyzed by GC-MS in order to compare and contrast the volatile chemical compositions of these species. The essential oils were screened for *in-vitro* cytotoxic activity against MDA-MB-231 and Hs 578T human breast tumor cells, and antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. The principal components of *D. bibracteata* were germacrene D (29.9%), (*E*)-caryophyllene (11.5%), and δ-cadinene (9.2%). *D. microcarpa* was dominated by bicyclogermacrene (45.5%) and germacrene D (28.3%). *G. costaricensis* was rich in α- and β-pinenes (36.3% and 48.2%, respectively). The leaf oil of *G. diospyroides* was composed largely of germacrene D (46.4%), (*Z*)-β-ocimene (17.4%), (*E*)-β-ocimene (12.0%), and (*E*)-caryophyllene (10.3%). Germacrene D dominated the leaf oil of *G. oliviformis* (73.3%) as well as *U. costaricensis* (62.9%). The leaf essential oils of *D. bibracteata*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis*, showed notable cytotoxicity on MDA-MB-231 cells (≥ 99% kill at 100 µg/mL) but only *D. bibracteata* leaf oil was cytotoxic to Hs 578T. *D. bibracteata*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis* leaf oils showed marginal antibacterial activity against *B. cereus* (MIC = 156 µg/mL). A cluster analysis of *Guatteria* species, based on the abundant essential oil components, has revealed a spathulenol-rich cluster (Brazilian species) and a germacrene D cluster (Costa Rican species).

Keywords: *Desmopsis bibracteata*; *Desmopsis microcarpa*; *Guatteria costaricensis*; *Guatteria diospyroides*; *Guatteria oliviformis*; *Unonopsis costaricensis*; Annonaceae; essential oil composition; cytotoxicity; antibacterial, germacrene D; cluster analysis

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1. Introduction

The Annonaceae is one of the largest and most diverse plant families, composed of around 112 genera and 2150 species, mostly from tropical regions [1]. The genus *Desmopsis* contains about 23 species, most of which are found in Central America [2], while there are around 69 species of *Unonopsis* distributed throughout the Neotropics. The genus *Guatteria*, with approximately 279 species, is one of the largest genera of the Neotropics [1,3]. Many members of the Annonaceae have been characterized in terms of volatile oil analysis [4,5] including *Guatteria* [6,7] and *Unonopsis* [8], but apparently not *Desmopsis*. Some members of the family are important in local traditional medicine including, for example, *Guatteria leiophylla* in Mexico to treat gonorrhea and leucorrhea [9], *G. gaumeri* in Mexico to treat hypercholesteremia [10], *G. pteropus* as a tonic in Peru [11], *Unonopsis stipitata* and *U. veneficiorum* in northwestern Amazonia to treat speaking disorders [12], and *U. floribunda* in Peru for arthritis and rheumatism [11]. In this report, we present the chemical compositions of the leaf essential oils of six species of Annonaceae collected from the Monteverde region of northwestern Costa Rica: *Desmopsis bibracteata* (B.L. Rob.) Saff., *Desmopsis microcarpa* R.E. Fr., *Guatteria costaricensis* R.E. Fr., *Guatteria diospyroides* Baill., *Guatteria oliviformis* Donn. Sm., and *Unonopsis costaricensis* R.E. Fr. To our knowledge, no previous phytochemical investigations have appeared on these species.

2. Materials and Methods

2.1. Plant Material

Leaves of *D. bibracteata*, *D. microcarpa*, *G. costaricensis*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis*, were collected from mature trees in the Monteverde region of the Cordillera de Tilarán in northwestern Costa Rica. The plants were identified by W. A. Haber. Voucher specimens have been deposited in the herbarium of the Missouri Botanical Garden. The fresh leaves of each plant were chopped and hydrodistilled using a Likens-Nickerson apparatus to give the essential oils (Table 1).

2.2 Gas Chromatography-Mass Spectrometry

The leaf oils of the Annonaceous species were subjected to gas chromatographic-mass spectral analysis on an Agilent system consisting of a model 6890 gas chromatograph, a model 5973 mass selective detector (EIMS, electron energy, 70 eV), and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a (5% phenyl)-methylpolysiloxane 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. Inlet temperature was 200°C and MSD detector temperature was 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, hold for 10 min; increased at 3°/min to 200°C; increased 2°/min to 220°C. Each sample was dissolved in $CHCl_3$ to give a 1% w/v solution; 1 μL injections using a splitless injection technique were used. Identification of oil components was achieved based on their retention indices (RI, determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns with those reported in the literature [13] and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.08)]. The chemical compositions of the essential oils are compiled in Table 2.

Table 1. Collection and hydrodistillation of leaves of Annonaceae from Monteverde, Costa Rica.

Plant	Voucher number	Collection Site (Date)	Mass of leaves	Mass of leaf oil
D. bibracteata	Haber 9765	San Luis Valley below Monteverde 10.2807 N, 84.8112 W, 960 m asl (May 9, 2008)	40.7 g	84.9 mg (0.209%)
D. microcarpa	Haber 4917	San Luis Valley below Monteverde 10.2818 N, 84.8008 W, 1120 m asl (May 9, 2008)	44.3 g	13.5 mg (0.030%)
G. costaricensis	Haber 8060	Peñas Blancas River Valley 10.3010 N, 84.7444 W, 900 m asl (May 14, 2008)	48.2 g	31.2 mg (0.065%)
G. diospyroides	Bello 558	Peñas Blancas River Valley 10.3091 N, 84.7162 W, 800 m asl (May 14, 2008)	21.8 g	32.8 mg (0.150%)
G. oliviformis	Bello 4153	Monteverde Cloud Forest Preserve 10.3483 N, 84.7633 W, 1530 m asl (May 10, 2007)	58.1 g	209.3 mg (0.360%)
U. costaricensis	Bello 2229	Peñas Blancas River Valley 10.2974 N, 84.7617 W, 1100 m asl (May 14, 2008)	30.4 g	60.9 mg (0.200%)

2.3. Numerical Cluster Analysis

The seven *Guatteria* samples were treated as operational taxonomic units (OTUs). The percentage composition of the main essential oil components was used to determine the chemical relationship between the different *Guatteria* leaf oil samples by cluster analysis using the NTSYSpc software, version 2.2 [14]. Correlation was selected as a measure of similarity, and the unweighted pair-group method with arithmetic average (UPGMA) was used for cluster definition.

2.4. Cytotoxicity screening

Human MDA-MB-231 breast adenocarcinoma cells (ATCC No. HTB-26) [15] were grown in an air environment at 37°C in Leibovitz's L-15 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 30 mM Hepes, pH 7.35. Human Hs 578T breast ductal carcinoma cells (ATCC No. HTB-129) [16] were grown in a 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 10.0 mg streptomycin per liter of medium, and buffered with 44 mM $NaHCO_3$, pH 7.35. Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22) [17] were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units penicillin and 10.0 mg streptomycin per liter of medium, 15mM of Hepes, and buffered with 26.7 mM $NaHCO_3$, pH 7.35.

Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 μL. After 48 h, supernatant fluid was removed by suction and replaced with 100 μL growth medium containing 1.0 μL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 μg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 μL DMSO/mL) were used. Tingenone [18] was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2; 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 MTT assay for cell viability was carried out [19]. After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%\text{kill}_{\text{cmpd}}/\%\text{kill}_{\text{DMSO}}$) were calculated. Cytotoxic activities of the essential oils are summarized in Table 3.

2.5. Antibacterial Screening

Essential oils and major components were screened for antibacterial activity against *Bacillus cereus* (ATCC No. 14579), *Staphylococcus aureus* (ATCC No. 29213), and *Escherichia coli* (ATCC No. 25922). Minimum inhibitory concentrations (MIC) were determined using the microbroth dilution technique [20]. Dilutions of the crude extracts were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 µL of 1% w/w solutions of crude extracts in DMSO plus 50 µL CAMHB. The extract 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 hr; the final minimum inhibitory concentration (MIC) was determined as the lowest concentration without turbidity. Gentamicin was used as a positive antibiotic control; DMSO was used as a negative control. Antibacterial results are listed in Table 3.

3. Results and Discussion

From the hydrodistillation, clear to light yellow essential oils were obtained. A total of sixty-two compounds were identified in the leaf oils, accounting for 99.5-100% of the total compositions of the essential oils. The chemical compositions of the leaf oils are compiled in Table 2.

The leaf essential oils of *D. bibracteata* and *D. microcarpa* were made up largely of sesquiterpene hydrocarbons (86.5% and 90.6%, respectively), with smaller amounts of oxygenated sesquiterpenoids (12.1% and 8.1%, respectively). The most abundant components of the essential oil of *D. bibracteata* were germacrene D (29.9%), (*E*)-caryophyllene (11.5%), and δ-cadinene (9.2%), while *D. microcarpa* was dominated by bicyclogermacrene (45.5%) and germacrene D (28.3%). *U. costaricensis* leaf oil was also characterized by abundant sesquiterpene hydrocarbons (85.8%), chiefly germacrene D (62.9%) and bicyclogermacrene (10.0%); oxygenated sesquiterpenoids (14.2%), principally viridiflorol (12.1%); but was completely devoid of monoterpenoids.

Two of the *Guatteria* species, *G. dispyroides* and *G. oliviformis*, were rich in sesquiterpene hydrocarbons (66.7% and 87.1%, respectively) with lesser amounts of monoterpene hydrocarbons (30.1% and 8.6%, respectively), with only small amounts of oxygenated sesquiterpenoids (3.2% and 4.3%, respectively. Conversely, the essential oil of *G. costaricensis* was dominated by monoterpene hydrocarbons (86.0%) with lesser amounts of sesquiterpene hydrocarbons (9.7%) and oxygenated sesquiterpenoids (4.3%). The leaf essential oil of *G. costaricensis* was rich in α-pinene (36.3%) and β-pinene (48.2%). Germacrene D (46.4%) dominated the leaf oil of *G. diospyroides*, but the monoterpenes (*Z*)-β-ocimene (17.4%) and (*E*)-β-ocimene (12.0%), in addition to (*E*)-caryophyllene (10.3%), were also abundant. *G. oliviformis* oil was also dominated by germacrene D (73.3%).

Table 2. Leaf oil chemical compositions of Annonaceae from Monteverde, Costa Rica.

RI[a]	**Compound**	**Percent Composition**					
		D bi	***D mi***	***G co***	***G di***	***G ol***	***U co***
939	α-Pinene	t[b]	---	36.3	---	3.4	---
956	Camphene	0.2	---	---	---	---	---
976	Sabinene	---	---	---	---	t	---
979	β-Pinene	---	---	48.2	---	4.4	---
990	Myrcene	---	---	1.4	---	t	---
1025	Limonene	---	---	t	---	---	---
1026	*p*-Cymene	---	---	---	0.4	---	---
1028	β-Phellandrene	---	---	---	---	0.8	---
1030	1,8-Cineole	---	---	---	---	t	---
1040	(*Z*)-β-Ocimene	0.6	1.1	t	17.4	t	---
1050	(*E*)-β-Ocimene	t	0.2	---	12.0	---	---
1059	γ-Terpinene	---	---	---	0.3	---	---
1283	Isobornyl Acetate	0.5	---	---	---	---	---
1350	α-Cubebene	0.6	0.3	---	---	---	0.4
1364	Cyclosativene	0.3	---	---	---	---	---
1376	α-Copaene	1.9	0.7	1.8	0.5	---	1.0
1384	β-Bourbonene	0.7	0.1	---	---	---	0.3
1390	β-Cubebene	---	0.1	t	0.5	t	0.2
1391	β-Elemene	3.5	0.9	t	1.2	1.5	0.4
1409	α-Gurjunene	---	---	---	---	---	0.2
1418	(*E*)-Caryophyllene	11.5	0.6	5.4	10.3	2.5	2.3
1429	β-Copaene	0.6	0.2	---	0.1	t	0.2
1439	Aromadendrene	1.0	3.0	---	---	---	---
1444	6,9-Guaiadiene	0.1	---	---	0.2	---	---
1450	*cis*-Muurola-3,5-diene	---	---	t	---	---	---
1453	α-Humulene	1.5	---	t	0.8	t	0.3
1460	Alloaromadendrene	0.3	0.2	---	---	---	1.1
1462	*cis*-Muurola-4(14),5-diene	---	0.1	---	---	---	---
1483	Germacrene D	29.9	28.3	1.9	46.4	73.3	62.9
1486	β-Selinene	4.7	0.8	---	---	---	0.2
1494	*trans*-Muurola-4(14),5-diene	0.4	0.4	---	0.2	0.7	0.1
1500	Bicyclogermacrene	3.8	45.5	---	2.2	4.5	10.0
1502	α-Muurolene	1.3	---	---	0.6	t	0.7
1503	Valencene	7.6	---	---	---	---	---
1503	*trans*-β-Guaiene	---	---	---	0.4	---	---
1507	Germacrene-A	---	0.4	---	---	1.8	---
1509	δ-Amorphene	---	---	---	---	t	---
1512	γ-Cadinene	2.0	2.0	---	0.3	0.9	1.4
1518	β-Cadinene	4.0	---	---	---	---	---
1523	δ-Cadinene	9.2	6.3	0.6	2.2	1.9	3.9
1532	*trans*-Cadina-1,4-diene	0.7	0.4	---	---	t	0.1
1538	α-Cadinene	0.7	0.3	---	---	t	0.2
1542	Selina-3,7(11)-diene	0.1	---	---	---	---	---
1548	Unidentified	0.5	---	---	---	---	---
1551	Elemol	---	---	3.4	---	4.3	---
1560	Germacrene B	---	---	---	0.9	t	---
1563	β-Calacorene	t	---	---	---	---	---
1574	Germacrene D-4-ol	---	---	---	---	---	0.1
1576	Spathulenol	1.5	1.1	---	0.1	t	0.8
1580	Caryophyllene oxide	---	---	---	1.5	---	---
1582	Globulol	1.4	2.5	---	---	t	---

Table 2 continued

RI[a]	Compound	Percent Composition					
		D bi	***D mi***	***G co***	***G di***	***G ol***	***U co***
1590	Viridiflorol	---	0.7	---	---	t	12.1
1598	Guaiol	1.5	---	t	---	---	0.6
1627	1-*epi*-Cubenol	0.8	0.3	---	---	---	---
1628	γ-Eudesmol	2.0	---	t	---	---	---
1640	τ-Cadinol	2.9	1.9	---	0.6	---	0.1
1641	Hinesol	0.1	---	---	---	---	---
1643	*epi*-α-Muurolol	0.1	---	---	---	---	---
1647	α-Muurolol (= Torreyol)	0.3	---	---	---	---	---
1649	Cubenol	0.4	---	---	---	---	---
1652	α-Eudesmol	---		0.9	---	---	---
1653	α-Cadinol	---	1.5	---	1.1	---	0.4
1679	Khusinol	0.5	---	---	---	---	---
	Total identified	99.5	100.0	100.0	100.0	100.0	100.0
	Monoterpene hydrocarbons	0.8	1.3	86.0	30.1	8.6	0.0
	Oxygenated monoterpenoids	0.5	0.0	0.0	0.0	0.0	0.0
	Sesquiterpene hydrocarbons	86.5	90.6	9.7	66.7	87.1	85.8
	Oxygenated sesquiterpenoids	12.1	8.1	4.3	3.2	4.3	14.2

[a] RI = "Retention Index" based on a homologous series of normal alkanes.

[b] t = trace (<0.1%)

Of the sixty-two compounds identified in this study, there were only four compounds that were common to all of the Annonaceae species: germacrene D (abundant in all species except for *G. costaricensis*), (*E*)-caryophyllene (abundant in *D. bibracteata* and *G. diospyroides*), β-elemene (relatively small amounts in any of the Annonaceae), and δ-cadinene (more abundant in *Desmposis*, less abundant in *Guatteria* or *Unonopsis*). Both germacrene D and (*E*)-caryophyllene seem to be omnipresent if not abundant in *Guatteria* species [6,7], and δ-cadinene and β-elemene are present in most. Flavonoids [21], isoquinoline alkaloids as well as the oxygenated sesquiterpenoids spathulenol and caryophyllene oxide [22-24] have been suggested to be chemotaxonomic markers for the Annonaceae. In this present work, we find little (*D. bibracteata*, *D. microcarpa*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis*) or no (*G. costaricensis*) spathulenol in the leaf oils of Monteverde Annonaceae, and caryophyllene oxide was detected in only one species (*G. diospyroides*). Erkens and co-workers [3], based on DNA phylogenetic analyses, have suggested that the diversification of the genus *Guatteria* can be attributed to Miocene migration from Central America into South America, diversification within South America, and then subsequent migrations back into Central America. A cluster analysis, based on 21 abundant volatile components, of *Guatteria* leaf oils (Fig. 1), does indeed show a spathulenol-rich cluster (*G. juruensis* and *G. poeppigiana*, from Brazil), as well as a germacrene-D-rich cluster (*G. diospyroides* and *G. oliviformis*, from Costa Rica). The clustering of *G. microcalyx* with *G. juruensis* and *G. peoppigiana* is due primarily to humulene epoxide II found in those three samples (2.0, 2.0, and 5.7%, respectively), but in no others.

Four of the six leaf oils showed notable *in-vitro* cytotoxic activity against MDA-MB-231 human breast tumor cells. Thus, *D. bibracteata*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis* oils killed ≥ 99% of the tumor cells at a concentration of 100 μg/mL. *G. diospyroides*, *G. oliviformis*, and *U. costaricensis*, are all characterized by high levels of germacrene D. *D. bibracteata* was abundant in both germacrene D and (*E*)-caryophyllene, both of which are cytotoxic to MDA-MB-231 cells (IC_{50} = 54 and 32 μg/mL, respectively). *G. diospyroides* was also rich in (*Z*)- and (*E*)-β-ocimene, which are also cytotoxic. Notably, *D. bibracteata* leaf oil was also cytotoxic on Hs 578T cells, whereas the other essential oils showed little or no activity. Both germacrene D and (*E*)-caryophyllene are cytotoxic to Hs 578T cells (IC_{50} = 55 and 78 μg/mL, respectively). None of the essential oils was particularly antibacterial, but *D. bibracteata*, *G. diospyroides*, *G. oliviformis*, and *U. costaricensis* leaf oils did show marginal activity (MIC = 156 μg/mL) against *B. cereus*.

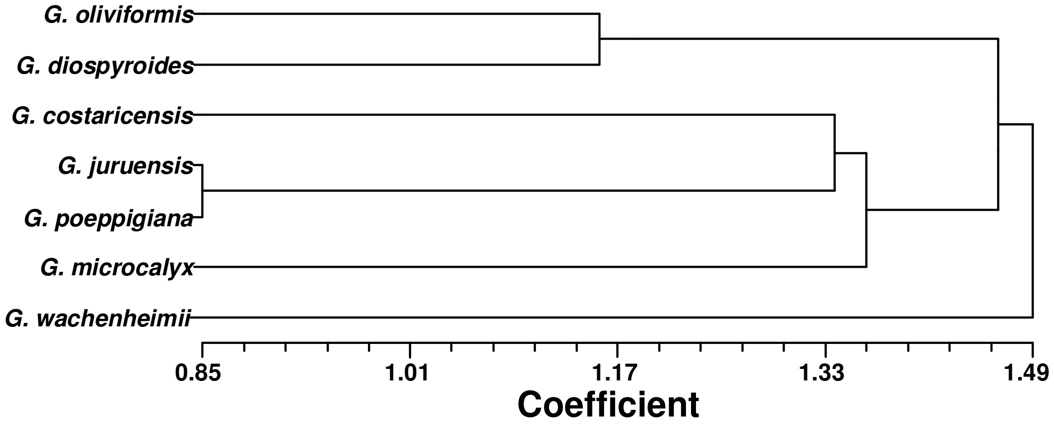

Figure 1. Dendrogram obtained by cluster analysis of the percentage composition of essential oils from *Guatteria* leaf essential oil samples, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA).

Table 3. Biological activities of leaf essential oils of Annonaceae from Monteverde, Costa Rica.

Material	**Cytotoxicity** (% kill at 100 μg/mL, standard deviations in parentheses)		**Antimicrobial activity** (MIC, μg/mL)		
	MDA-MB-231	**Hs 578T**	***B. cereus***	***S. aureus***	***E. coli***
D. bibracteata	99.3(0.7)	100	156	625	2500
D. microcarpa	53.0(9.6)	8.2(14.0)	312	1250	1250
G. costaricensis	54.6(5.7)	0	625	1250	1250
G. diospyroides	98.8(1.2)	21.1(8.2)	156	312	1250
G. oliviformis	100	35.6(1.9)[a]	156	1250	625
U. costaricensis	100	17.0(10.3)	156	625	1250
α-Pinene	0	26.7(7.7)	625	312	312
β-Pinene	0	30.4(9.3)	312	312	625
β-Ocimene	97.4(2.6)	98.5(0.3)	1250	1250	2500
Germacrene D	100	94.4(3.0)	625	156	625
(*E*)-Caryophyllene	100	78.3(8.3)	156	312	312

[a] MCF-7 cells rather than Hs 578T.

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