Differences in Essential Oil Production and Leaf Structure in Phenotypes of Damiana (*Turnera diffusa* Willd.)

Lilia Alcaraz-Meléndez[1]*, Sergio Real-Cosío[1], Václav Suchý[2], and Emil Švajdlenka[2]
[1]Centro de Investigaciones Biológicas del Noroeste, La Paz, Baja California Sur, México
[2]Department of Natural Drugs, Faculty of Pharmacy, Veterinary and Pharmaceutical University, Brno, Czech Republic

Plants of damiana (*Turnera diffusa* Willd.) are important to industry and traditional medicine in semi-arid climates. Although all populations are wild, no reports have been made previously of their different phenotypes. Here, we investigated various micromorphological characteristics and the levels of essential oils in two phenotypes. Oils were extracted from fresh leaves via hydrodistillation and analyzed by gas chromatography-mass spectrometry. Morphological analyses were conducted under a stereoscopic microscope and with a scanning electron microscope. In all, 56 compounds were identified, enabling us to distinguish separate phenotypes. DL1 plants mainly contained 1,8-cineole, 10-epi γ eudesmol, and guaiol; whereas those of DL2 primarily constituted β-pinene, β-caryophyllene oxide, cadinene, and α-cadinol. These two phenotypes also differed in their morphologies, with DL1 leaves showing elevated essential oil concentrations, but lacking trichomes. In contrast, the DL2 plants had lower contents of essential oils but did possess trichomes on their abaxial and adaxial leaf surfaces. This documentation of individual damiana phenotypes is the initial process toward validating the quality of essential oils from this species as well as inherent structural variations.

Keywords: essential oils, gas chromatography, mass spectrometry, phenotypes, structure and ultrastructure, *Turnera diffusa*

Damiana (*Turnera diffusa* Willd.) is a deciduous shrub found in arid and semi-arid regions of the West Indies, South America, Mexico, and the United States (Wiggins, 1980). Its leaves are added as flavoring in food and liquors, and infusions are used for a variety of medicinal purposes, including nervous system stimulants, aphrodisiacs, and diuretics (Johnson, 1999). Because damiana is a wild plant, its overexploitation, natural scarcity, and the near-impossibility of utilizing traditional forms of propagation to maintain a regular supply have led to large fluctuations in availability and market price. Our laboratory has been developing efficient propagation techniques to domesticate this species (Díaz-Rondero and Alcaraz-Meléndez, 1987; Alcaraz-Meléndez et al., 2002). These plants have revealed variability in their morphology and biochemical composition, and we have been working to identify its essential oils in the past few years (Alcaraz-Meléndez, et al., 2004). These oils, which are complex mixtures of various terpenoids, aromatic substances, aldehydes, ketones, alcohols, and esters (Schulz et al., 2004), are also important for the botanical classification of many other aromatic plants, e.g., *Thymus vulgaris* (Linhart and Thompson, 1999). Here, we analyzed differences in the content of essential oils and microstructures from cloned damiana plants as a starting point for distinguishing phenotypes for future cultivation and conservation.

MATERIALS AND METHODS

Plant Material

Samples were collected from plants originally propagated via tissue culture (Díaz-Rondero and Alcaraz-Meléndez, 1987; Alcaraz-Meléndez et al., 2002), then further cultivated under field conditions, at CIBNOR, 17 km west of La Paz, Baja California Sur, Mexico. Climatic conditions included an average annual temperature of 24.8°C (maximum annual average, 31.5°C; minimum annual average, 17.3°C), and annual precipitation of 170 mm. Plants were grown in sandy soil and irrigated every 15 d with 33 dm^3 water per m^2 volume. They were fertilized twice a year with triple 17 fertilizer (17- 17-17, N-P-K), but no insecticides were required. After five years, the leaves from three plants each of two phenotypes were collected and labelled as DL1 and DL2. The DL1 were shrubs of ascending form, 100 to 150 cm tall, with pilose to tometose leaves (light-green in color) and stems (light-green to light-brown). The DL2 plants were diffuse shrubs, 60 to 100 cm tall, with glabrous, dark-green leaves, and glabrous stems that were green to reddish in color. Both phenotypes had perfect, 8- to 12-mm-long yellowish flowers that were axillary and solitary, with green, thin-walled capsules.

Essential Oil Extraction

For each sample, 100 g of fresh leaves were processed. These materials were distilled for 1.5 h in a Clevenger-type hydrodistillation unit (VWR 14205-246; Wildmad-LabGlass, USA). Essential oils were measured and stored in 2-mL amber vials. They were then packed and shipped via air courier service to a laboratory at the Veterinary and Pharmaceutical University in Brno, Czech Republic.

GC-MS Analysis

These oil samples (0.2 or 1.0 μL) were diluted 100× with hexane if the peaks were overloaded during analysis by gas chromatography (Shimadzu GC-17A; Shimadzu Scientific Instruments, USA) coupled with a mass spectrophotometer (Shimadzu QP5050A). After an initial processing time of 4

*Corresponding author; fax +52-612-125-3625
e-mail lalcaraz04@cibnor.mx

min at 40°C, the injector temperature followed a gradient of 5°C min^{-1} until the final temperature of 250°C was reached for 4 min. The transfer line temperature was set at 280°C, with an He flow rate of 1.3 mL min^{-1} (i.e., 41.4 cm s^{-1}). The split ratio was 15, for a total flow of 21.5 mL min^{-1} at 4.0 kPa. We used a capillary column (CP-WAX 52 CB, 30 m long, internal diameter 0.32 mm, diffusion film 0.50 µm). The mass spectrophotometer scanned from 35 to 420 amu, 0.5 scans s^{-1}, 1000 amu s^{-1}, with a solvent cut-time of 2.5 min for diluted samples and 0 min. for pure essential oils. The detector voltage was 1.5 kV. Compounds identified from the Wiley Library database were compared semi-quantitatively.

Morphological Characteristics

Fresh leaves were collected and analyzed both under a

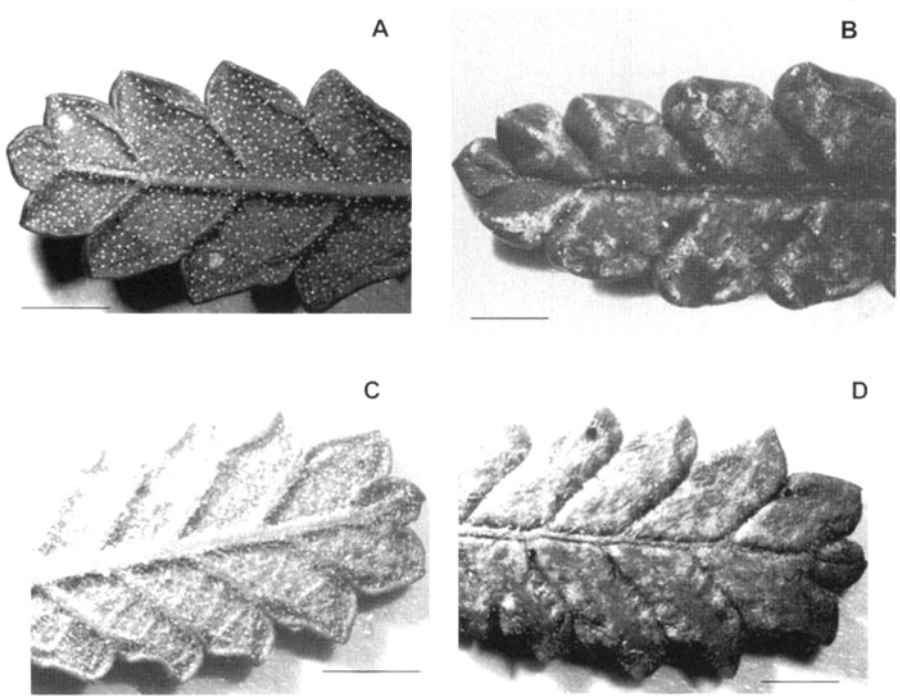

Figure 1. Leaf surfaces from two phenotypes of *I. diffusa* viewed under stereoscopic microscope (6.3 ×). (**A**) Damiana DL1 abaxial surface. (**B**) Damiana DL1 adaxial surface. (**C**) Damiana DL2 abaxial surface. (**D**) Damiana DL2 adaxial surface. Bar = 1 mm.

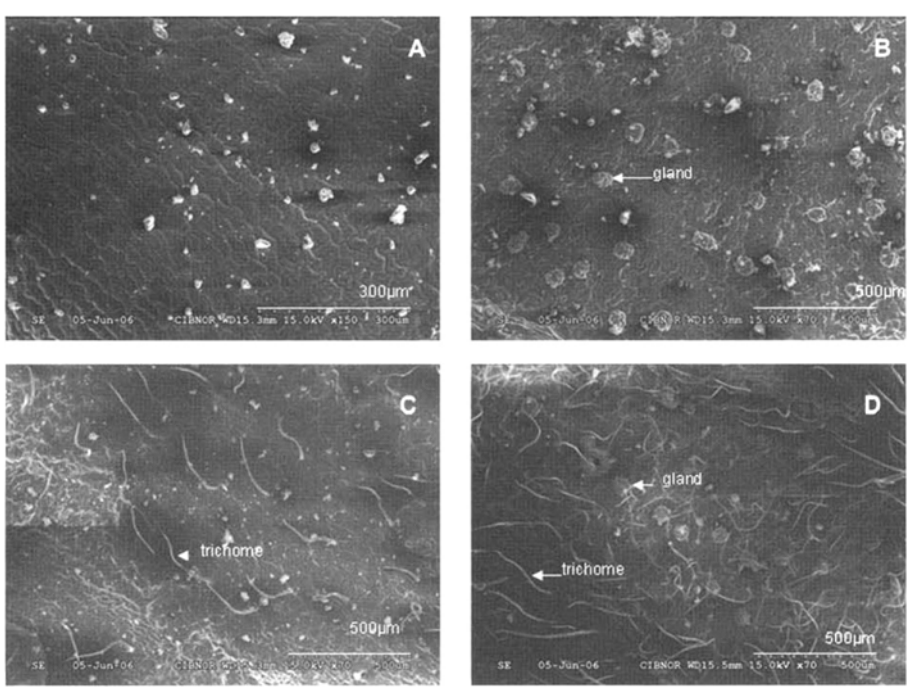

Figure 2. Leaf surfaces from two phenotypes of *I. diffusa* viewed under scanning electron microscope. (**A**) Damiana DL1 abaxial surface. (**B**) Damiana DL1 adaxial surface. (**C**) Damiana DL2 abaxial surface. (**D**) Damiana DL2 adaxial surface.

Table 1. Essential oils extracted from fresh leaves of two phenotypes of damiana (*T. diffusa*).

CAS No.	Library / ID	RT	DL 1	DL 2
		min	% Total	% Total
000080-56-8	α pinene	6.22	1.09 ± 0.60	2.23 ± 0.70
002867-05-2	α thujene	6.35	*	*
000079-92-5	camphene	7.41	*	*
000127-91-3	β pinene	8.76	0.65	3.87 ± 0.78
003387-41-5	Sabinene	9.26	*	*
001196-01-6	verbenone	9.38	*	*
000123-35-3	Myrcene	10.76	*	*
005989-27-5	Limonene	11.80		0.44
000470-82-6	1,8-cineole	12.31	17.20 ± 8.56	*
006728-26-3	trans-2-hexenal	12.54	0.26	0.48
000099-85-4	γ-terpinene	13.35	*	
000099-87-6	cymene	14.13	*	*
000586-62-9	α terpinolene	14.50	*	*
000111-27-3	1-hexanol	16.55	*	0.68 ± 0.23
000928-96-1	3-hexen-1-ol	17.50	0.54 ± 0.1	1.44 ± 0.16
020307-84-0	δ elemene	19.80	*	
001365-19-1	trans linalol oxide	20.00		0.79
003650-28-0	cycloisosativen	20.30		1.21
004501-58-0	α campholen aldehyde	20.60		*
005208-59-3	β bourbonene	21.33	*	
003650-28-0	sativene	21.57	*	*
016812-40-1	pinocarvone	22.71	*	2.23 ± 0.73
000078-70-6	linalool	21.99	*	1.49
015358-88-0	isopinocamphone	22.10		*
000498-81-7	dihydro terpineol	22.42	*	
000515-13-9	β elemene/germacrene A	23.30	1.16 ± 0.75	0.53
000087-44-5	β caryophyllene	23.43	0.56	1.01 ± 0.06
000547-61-5	pinen-3-ol	23.54	0.33	
006831-16-9	aristolene	23.66	3.47 ± 1.17	1.67 ± 0.14
095910-36-4	isoledene	23.91	4.24	1.21
007493-71-2	allyl tiglate	24.08	0.65 ± 0.09	0.68
000564-94-3	myrtenal	24.30		1.29 ± 0.76
025246-27-9	alloaromadendrene	24.24	0.36	1.18 ± 1.05
000099-83-2	α phellandrene	25.10		*
067883-79-8	cis 3 hexenyl tiglate	25.23	0.59 ± 0.06	
000483-75-0	α amorphene	25.78	1.68 ± 0.50	
000098-55-5	α terpineol	25.93	0.96 ± 0.59	1.07 ± 0.41
006485-40-1	carvone	26.91		*
000515-00-4	bicyclogermacrene	27.05	0.32	
000483-76-1	γ δ cadinene	27.61	2.56 ± 0.95	2.21 ± 0.30
000515-00-4	myrtenol	28.21	0.42	2.01 ± 0.54
032433-11-7	trans-carveole	29.30		*
001197-01-9	p-cymen-8-ol	29.50	*	0.53 ± 0.09
001139-30-6	β caryophyllene oxide	32.77	0.86 ± 0.43	9.62 ± 1.28
000489-41-8	globulol	33.64	0.88 ± 0.30	1.64 ± 0.64
	oplopenone	34.66	3.63 ± 0.37	5.97 ± 1.18
037526-88-8	benzyl tiglate	35.40		0.43
001209-71-8	10-epi γ eudesmol	35.41	4.54 ± 0.49	
006750-60-3	spathulenol	35.60	1.55 ± 0.59	2.31 ± 0.83
024405-05-1	cadinene	36.40		7.30

Table 1. Essential oils extracted from fresh leaves of two phenotypes of damiana (*T. diffusa*).

CAS No.	Library / ID	RT	DL 1	DL 2
		min	% Total	% Total
000481-34-5	α cadinol	36.84	2.23 ± 0.77	6.45 ± 1.98
036564-42-8	δ cadinol	36.90		2.59
000489-86-1	guaiol	37.67	8.92 ± 4.52	2.42
000473-04-1	juniper camphor	39.01	1.29 ± 0.19	2.25
003856-25-5	α copaene	39.32	1.61 ± 0.08	
000150-86-7	phytol	44.31	0.48 ± 0.14	1.85 ± 0.71
000057-10-3	palmitic acid	49.70		0.85 ± 0.18

RT, retention time; *, trace amounts only.

stereoscopic microscope (Leica MZ 7_5; Leica Microsystems, Switzerland) mounted with a digital camera system (Cool SNP-Procf; Media Cybernetics, USA), and by using a scanning electron microscope (model S-300N; Hitachi High Technologies America, USA) at a 5 to 15 Kv accelerating voltage. We examined abaxial and adaxial leaf surfaces.

RESULTS AND DISCUSSION

Although both phenotypes showed glands on their abaxial leaf surfaces, morphological and micromorphological differences were apparent (Fig. 1, 2). For example, the DL1 plants had leaves without trichomes while those of DL2 bore leaves with simple trichomes on their adaxial and abaxial surfaces. Wiggins (1980) has described leaf blade pubescence as sparse to subtomentose, with hairs <1 mm long. However, previous reports have made no mention of damiana without trichomes. The functions of trichomes are important, particularly for plants grown under arid conditions, because those structures regulate temperature, increase light reflection, and decrease subsequent water loss (Ehleringer, 1984; Wagner et al., 2004).

The essential oils of damiana (Table 1) were extracted by steam distillation. In DL1 plants, those contents varied from 301.0 to 399.5 µL per 100 g fresh leaves, whereas, for DL2 plants, the range was 41.0 to 53.9 µL per 100 g tissue.

The distinguishing criteria for chemotype identification are the major plant parts that supply essential oils (particularly the seeds and leaves). In DL1 plants, the greatest concentrations were of eucalyptol (1,8-cineole) (RT 12.01 min), 10-epi γ eudesmol (RT 35.41), and guaiol (RT 37.67). That phenotype contained only low concentrations of β-pinene and caryophyllene oxide. In contrast, the DL2 plants showed only traces of eucalyptol, but relatively high concentrations of β-pinene (RT 8.76 min), β-caryophyllene oxide (RT 32.77), cadinene (RT 36.4), and β-cadinol (RT 36.84). We noted that these differences did not depend on environment or season because our samples had been taken from plants grown in the same field under identical conditions. Only 28 compounds were common to both phenotypes, albeit with some present just as trace amounts.

Damiana plants with trichomes contained less essential oil than did those lacking trichomes – this difference in composition was so striking that it enabled easy distinction between the two forms. Because of their greater production of these oils, plants of phenotype DL2 are important in the liquor industry and in the manufacture of tea bags. Nevertheless, each phenotype is also the source of different compounds crucial for medicinal use, so that research on the effect of these various compounds will now be improved because of our experimental tests. This kind of morphological difference related to essential oil composition has also been reported with *Lippia alba* in Guatemala (Fischer et al., 2004). Jo and Kim (2005) analyzed intraspecific variability of 11 *Juniperus* L. species based on monoterpenoid composition and reported results by Phylogenetic Analysis Using Parsimony they identified four different groups with this method. As plant sources are exploited for new drugs based on traditional medicines and herbal remedies (Verpoorte, 2000), the identification of unique plant varieties and their range of essential oils will be a critical part of the process in validating effective ingredients and applications. Therefore, as we continue to determine the chemical composition of specific *T. diffusa* variants, more and clearer choices will become available when selecting native plants for economic uses or as medicinal treatments for which their essential oils have demonstrated efficacy.

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