Anti-Inflammatory Activity of Two Diterpenes of *Hyptis suaveolens* from El Salvador

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Separation and isolation of the two main compounds suaveolol and methyl suaveolate from leaves of chichinguaste (*Hyptis suaveolens* Poit., Lamiaceae) could be achieved by means of repeated column chromatography and repeated preparative thin layer chromatography. Their chemical structures were approved by MS, ^{1}H NMR, ^{13}C NMR and 2D-NMR experiments. The anti-inflammatory activity of the two compounds was tested for the first time as inhibition of croton oil-induced dermatitis of the mouse ear. Suaveolol and methyl suaveolate showed nearly the same dose-dependent topical anti-inflammatory activity, only two to three times lower than that of the reference drug indomethacin. The anti-inflammatory properties of these compounds could contribute to the antiphlogistic activity of extracts of *Hyptis* species and confirm the rational use of *Hyptis suaveolens* extracts in dermatological diseases.

Key words: Hyptis suaveolens, Diterpenes, Anti-Inflammatory Activity

Introduction

Hyptis (Lamiaceae) is a genus comprising almost 400 species mainly in the tropical Americas (Harley, 1988). Various species of this genus are used in traditional medicine in Latin America mainly as wound remedies and for the treatment of gastrointestinal disorders (Martinez, 1969; Rojas *et al.*, 1992; Garcia Barriga, 1975; Gonzalez Ayala, 1994; Zamora-Martinez and Nieto de Pascual Pola, 1992; Weimann and Heinrich, 1997). Some of them were also used against internal parasites, fever and rheumatic pains (Morton, 1981; Grenand *et al.*, 1987). Different species of *Hyptis* are not always correct distinguished in ethnomedicine and even in Salvadorian herbariums, which is impeded by the same popular name given to different species. In El Salvador *Hyptis suaveolens* and *Hyptis mutabilis* both are called "chichinguaste". *Hyptis suaveolens*, a weedy, strong aromatic annual species, is mainly used in the hot Pacific lowlands and in the coastal region of El Salvador. The leaves are used as a skin disinfectant and as a carminative and the seeds are prepared for the treatment of gastrointestinal disorders (Pereda-Miranda, 1995; Heinrich, 1992). In the ethnomedicine of Latin America the less aromatic *H. mutabilis*, growing in rather temperate regions, is primarily used as an antipyretic remedy in the cases of malarial infections (Garcia Barriga, 1975; Silva Morales, 1980). Surprisingly, the antimalarian diterpenes dehydroabietinol and 13α-epi-dioxiabiet-8(14)en-18-ol were isolated from *Hyptis suaveolens* and not from *Hyptis mutabilis* (Ziegler *et al.*, 2002; Chukwujekwu *et al.*, 2005). Dehydroabietinol revealed an *in vitro* antiplasmodial effect against *Plasmodium falciparum* 3D7 and Dd2 but this compound showed an undesired side effect on erythrocyte membranes (Ziegler *et al.*, 2002).

Furthermore, antimicrobial activities against various bacteria and fungi were described for the essential oil of *H. suaveolens* (Kishore *et al.*, 1996; Iwu *et al.*, 1990; Singh *et al.*, 1992; Jain *et al.*, 1974; Pandey and Dubey, 1994). An anti-phospholipase A_2 activity was observed for an aqueous extract of *Hyptis suaveolens* (Bernard *et al.*, 2001) and an antiphlogistic effect in the HET-CAM assay (hen's egg) was shown with a *Hyptis verticillata* crude extract (Kunth *et al.*, 1992).

The aim of this study was to isolate the main compounds of the methanol extract of *Hyptis suaveolens* leaves and to evaluate their anti-inflammatory activity against the contact dermatitis induced by croton oil in the mouse ear.

Material and Methods

Plant material and reagents

Plant material was collected from three locations of El Salvador, representing three essential oil chemotypes (Grassi *et al.*, 2005) of *Hyptis suaveolens* Poit. (Lamiaceae), as authenticated by Dr. Paolo Grassi, Institute for Applied Botany, University of Veterinary Medicine, Vienna, in cooperation with the Jardín Botanico La Laguna, San Salvador. Voucher specimens are deposited in the Museum of Natural History, Vienna, Austria.

The following reagents were used: Methanol *p.a.*, chloroform *p.a.*, petroleum ether, ethyl acetate, diethyl ether *p.a.*, *n*-hexane *p.a.*, dichloromethane *p.a.*, toluene *p.a.*, acetone *p.a.*, anisaldehyde, acetic acid 100%, sulfuric acid 100%.

Extraction and isolation

The air-dried leaves of *Hyptis suaveolens* (70 g) were extracted for 48 h by maceration with methanol at room temperature. After filtration and evaporation to dryness, the crude extract (14 g) was separated with chloroform/water in a separatory funnel. The chloroform fraction was separated by chromatography on silica gel 60 (110 g column; column dimensions: 80 × 3 cm), using a five step petroleum ether/ethyl acetate elution system (5%, 10%, 20%, 40%, 100% ethyl acetate in petroleum ether). All 20 ml fractions were monitored by TLC: CC-monitoring and preparative TLC; stationary phase: HPTLC, silica gel 60 F254, MERCK; mobile phases: diethyl ether/*n*-hexane/methanol (7 + 2 + 1) and dichloromethane/diethyl ether/toluol (6 + 3 + 1); detection: anisaldehyde reagent (Dequeker, 1964).

Structure elucidation

NMR: Bruker DRX400 WB (400 MHz; $CDCl_3$; ^1H NMR: $\delta = 7.26$ ppm; ^{13}C NMR: $\delta = 77.0$ ppm). MS: Finnigan MAT 900 S.

Suaveolol (13β-abiet-8-ene-14α,18-diol) (**1**) (comp. Manchand *et al.*, 1974; Matsumoto *et al.*, 1982): $[\alpha]_D^{25}$ + 80° (c = 0.6, $CHCl_3$). – ^1H and ^{13}C NMR: see Table I. – EI-MS (70 eV): m/z = 306 (16%, $[M^+]$), 288 (19), 273 (26), 257 (10), 153 (35), 123 (100), 91 (22), 55 (20).

Methyl suaveolate (methyl 14α-hydroxy-13β-abiet-8-en-18-oate) (**2**) (comp. Manchand *et al.*, 1974; Matsumoto *et al.*, 1982): $[\alpha]_D^{25}$ + 68° (c = 0.5, $CHCl_3$). – ^1H and ^{13}C NMR: see Table I. – EI-MS (70 eV): m/z = 334 (21%, $[M^+]$), 320 (28), 302 (67), 287 (53), 259 (48), 234 (33), 213 (29), 182 (31), 153 (86), 123 (91), 91 (64), 59 (100).

Anti-inflammatory activity

The croton oil ear test was performed as already described (Tubaro *et al.*, 1985), in accordance with the Italian Decreto di Legge n. 116 of January 27, 1992 and associated guidelines of the European Communities Council Directive of November 24, 1986 (86/609/ECC) for laboratory animal use and care. Groups of 10 male CD-1 mice (28–30 g; Harlan Italy, Udine, Italy) were anaesthetized with ketamine hydrochloride (145 mg/kg, intraperitoneally; Virbac Santé Animale, Virbac Group, Milan, Italy). An acetonic solution (15 μl) containing the irritant (80 μg of croton oil; Sigma-Aldrich, Milan, Italy) and the appropriate amount of the substances under testing were applied to the inner surface of the right ear of mice (surface: about 1 cm^2), the left ear remaining untreated. Control animals received only the irritant. The animals were sacrificed by cervical dislocation 6 h later and a plug (6 mm in diameter) was removed from both the treated and the untreated ear. Oedema was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percentage of the oedema reduction in mice treated with the tested substances compared to control mice. As reference, the non-steroidal anti-inflammatory drug indomethacin was used.

Statistical analysis

Pharmacological data were analyzed by one-way analysis of variance followed by the Dunnett's test for multiple comparisons of unpaired data, and a probability level lower than 0.05 was considered as significant. ID_{50} values (dose giving 50% oedema inhibition) were calculated by graphic interpolation of the dose-effect curves.

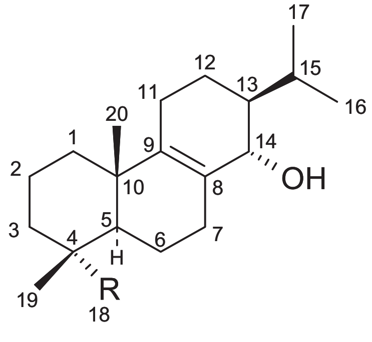
Results and Discussion

*Isolation and characterization of **1** and **2***

Screening of the three lipophilic leaf extracts of *Hyptis suaveolens*, originating from three different essential oil chemotypes, by means of TLC showed no significant differences in the pattern of diterpene compounds. One of the extracts (from the department La Unión, El Salvador) was further purified by means of CC on silica gel monitored by analytical TLC. **1** was eluted from fraction 120 and **2** from fraction 124 out of 140 fractions. The fractions were re-chromatographed by preparative TLC giving 14.3 mg suaveolol (**1**) and 11.1 mg methyl suaveolate (**2**), both chromatographically pure.

The structures of **1**, suaveolol, and **2**, methyl suaveolate (Fig. 1), were confirmed by MS, ^{1}H NMR, ^{13}C NMR and 2D-NMR studies. Both compounds were mentioned only twice in literature, no ^{13}C NMR data were given, and the ^{1}H NMR resonances listed have not been assigned (Manchand *et al.*, 1974; Matsumoto *et al.*, 1982). Using 2D-NMR we have now assigned all ^{1}H NMR resonances of **1** and the ^{13}C NMR resonances of **1** and **2** (Table I).

By means of the ^{1}H and *J*-modulated ^{13}C NMR spectra of **1**, in combination with C/H correlation (HMQC), H/H COSY, NOESY, and HMBC (long range couplings) all 4 CH_3, 8 CH_2, 4 CH groups, and 4 quaternary C atoms could be identified. The EI mass spectrum with a molecular mass of m/z = 306 indicated two additional oxygen atoms. The assignment of the resonances was started with the CH_3 groups, followed by CH, C, and finally the most critical CH_2 groups. In the argumentation either ^{1}H or ^{13}C NMR resonances were assigned, the corresponding ^{13}C or ^{1}H NMR resonances could be easily determined from the 2-D C/H correlation (HMQC).



1 R = CH_2OH: suaveolol
2 R = $COOCH_3$: methyl suaveolate

Fig. 1. Structure of suaveolol (**1**) and methyl suaveolate (**2**).

Discrimination of the two ^{1}H NMR methyl singlets 19 and 20 was possible due to the NOESY cross peak 18-H_2 to 19-H_3. The two methyl doublets 16 and 17 are magnetically not equivalent. The isopropyl side chain adopts a conformation with one methyl group (16-H_3) close to 14-H indicated by a strong NOESY contact between 14-H and 16-H_3. On the other hand, the NOESY cross peak 14-H to 17-H_3 is very small.

Concerning the four CH groups, the ^{1}H chemical shift of the doublet at 3.80 ppm was typical for the secondary alcohol at position 14 (14-H, compare also the NOESY cross peak mentioned above). 13-H shows a COSY cross peak with 14-H, and 15-H is coupling with the two methyl groups 16-H_3 and 17-H_3 showing the corresponding COSY contacts. The fourth one is necessarily **5**-H. This assignment agrees very well with the ^{13}C NMR data of the similar diterpene isopimara-8,15-dien-7ß,18-diol (Urones *et al.*, 1988) with an identical A and a similar B ring (C-5 44.4 ppm, reference 43.3 ppm).

Two of the four quaternary C atoms are olefinic (128.5 and 142.7 ppm). C-9 could be clearly identified by a strong long range coupling (HMBC) to 20-H_3 and further HMBC cross peaks to 12-H_2 and 1-H_2 (see below). C-8 shows the expected HMBC contact to 6-H_2. The remaining quaternary C-4 and C-10 are almost coinciding in the ^{13}C NMR spectrum (37.61 and 37.59 ppm) and cannot be discriminated. They show several long range HMBC cross peaks to different CH_2 groups (*e.g.* 19-H_2, 6-H_2).

Most of the eight CH_2 group resonances are overlapping with each other or other signals. However, careful observation of C/H correlation (HMQC), H/H COSY, and NOESY allowed a clear assignment (Table I). In the case of the low field diastereomeric 18-H_2 resonance pair at 3.08 and 3.44 ppm matters are clear. They belong to the primary alcohol of the A ring. For the assignments of C-1 and C-3 of the A ring ^{13}C NMR data were used. They agree very well with the corresponding C resonances of isopimara-8,15-dien-7β,18-diol (Urones *et al.*, 1988), the comparable values are

Table I. ^{1}H and ^{13}C NMR data of suaveolol (**1**) and methyl suaveolate (**2**) ($CDCl_3$, δ/ppm)[a].

	^{1}H NMR		^{13}C NMR	
	1	**2**	**1**	**2**
1	a: 1.71 ddd, b: 1.06 ddd	a: 1.66 m, b: 1.09 m	35.7 t	35.7 t
2	a: 1.63 m, b: 1.55 m	a: 1.56 m, b: 1.46 m	18.2 t	18.6 t
3	a: 1.43 m, b: 1.26 m	a: 1.62 m, b: 1.50 m	34.8 t	37.6 t
4			37.6 s	48.1 s
5	1.45 m	1.88 m	44.4 d	46.5 d
6	a: 1.68 m, b: 1.08 dddd	a: 1.64 m, b: 1.37 m	21.7 t	21.5 t
7	a: 2.02 m, b: 1.87 m	a: 1.99 m, b: 1.84 m	24.3 t	24.0 t
8			128.5 s	128.9 s
9			142.7 s	142.9 s
10			37.6 s	37.2 s
11	a: 2.42 m, b: 1.87 m	a: 2.36 m, b: 1.78 m	28.0 t	28.4 t
12	a: 1.65 m, b: 1.47 m	a: 1.61 m, b: 1.49 m	18.2 t	18.6 t
13	1.23 m	1.24 m	48.8 d	48.4 d
14	3.80 d	3.74 d	73.3 d	72.8 d
15	1.99 m	2.00 m	27.1 d	26.9 d
16	0.81 d	0.78 d	17.1 q	17.1 q
17	0.95 d	0.93 d	21.3 q	21.4 q
18	a: 3.44 d, b: 3.08 d		71.5 t	187.0 s
19	0.77 s	1.07 s	17.5 q	17.6 q
20	0.99 s	0.95 s	19.5 q	19.5 q
Ester Me		3.65 s		50.8 q

[a] Coupling constants: **1**: $J(13,14) = 8.3$ Hz, $J(18a,18b) = 11.1$ Hz, $J(1a,1b) = 14.1$ Hz, $J(1a,2a) = J(1a,2b) = 2.2$ Hz, $J(1b,2a) \sim 2$ Hz, $J(1b,2b) \sim 12$ Hz, $J(6a,6b) \sim 14$ Hz, $J(5,6a) \sim J(6a,7b) \sim 12$ Hz, $J(6a,7a) \sim 2$ Hz, $J(15,16) = J(15,17) = 6.8$ Hz; **2**: $J(13,14) = 8.2$ Hz, $J(15,16) = J(15,17) = 6.7$ Hz; due to line broadening and overlap of signals no further coupling constants could be determined.

35.7 and 34.8 ppm for **1** and 36.0 and 34.9 ppm for the reference compound with identical A ring. The ^{1}H NMR resonances for 7-H_2 and 11-H_2 in the region of about 2 ppm (1.87–2.42 ppm) are typical for methylene protons next to an olefinic double bond. The axial proton of 7-H_2 could be identified by a strong NOESY contact to the axial methyl group 20-H_3 across the B ring. Similarly, the axial H of 11-H_2 showed a strong NOESY cross peak with the axial H atom at position 13. The methylene group resonances of 12-H_2 (a and b) were identified by strong H/H COSY cross peaks to 11-H_2 (also a and b). The long range interaction of 12-H_2 with the quaternary C atom C-9 is also in agreement with the assignments. 6-CH_2 could be identified by H/H COSY crosspeaks to 7-H_2 and a long range coupling to C-8. The remaining protons of the methylene group 2-H_2 show COSY cross peaks to 3-H_2.

The ^{13}C NMR spectrum of methyl suaveolate (**2**) shows several characteristic differences to the one of suaveolol (**1**) (Table I). The triplet at 71.5 ppm for OCH_2 (18-H_2) has disappeared, a new quaternary CO group at 187.0 ppm is typical for an ester carbonyl. The methyl group of the ester at 50.8 ppm is also new. Further changes were observed for C-4 and to a less extent for C-3 and C-5. All other ^{13}C NMR resonances are very similar or almost identical in both compounds **1** and **2**. The most striking difference in the ^{1}H NMR spectra of the compounds is the lack of the diastereomeric OCH_2 resonances (18-H_2) and the dramatic shift of the methyl group resonance 19 (19-H_3) from 0.77 ppm to 1.07 ppm due to the anisotropy of the close carbonyl group. Additionally the ^{1}H NMR chemical shifts in the vicinity of the ester group (number 1 to 6) have changed in comparison with the ones of compound **1**. The results of the 2D NMR analysis are compiled in Table I.

Anti-inflammatory activity

The results on the anti-inflammatory activity of suaveolol (**1**) and methyl suaveolate (**2**) are presented in Table II, in comparison to those of the non-steroidal anti-inflammatory drug indomethacin. At doses ranging from 0.1 to 1 $\mu mol/cm^2$, the two diterpenes induced a dose-dependent oedema

Table II. Anti-inflammatory activity of suaveolol and methyl suaveolate isolated from *Hyptis suaveolens.*

Substance	Dose [μmol/cm^2]	Oedema [mg] Mean ± S.E.	Inhibition (%)	ID$_{50}$ [μmol/cm^2]
Controls	–	7.0 ± 0.3	–	–
Suaveolol	0.1	5.9 ± 0.2*	16	0.71
	0.3	4.7 ± 0.2*	33	
	1.0	3.0 ± 0.2*	57	
Methyl suaveolate	0.1	5.8 ± 0.2*	17	0.60
	0.3	4.5 ± 0.2*	36	
	1.0	2.8 ± 0.3*	60	
Indomethacin	0.1	5.8 ± 0.3*	17	0.26
	0.2	4.5 ± 0.4*	36	
	0.3	2.9 ± 0.3*	59	

* $p < 0.05$ at the analysis of variance, as compared with controls.

inhibition. At the lowest administered dose (0.1 μmol/cm^2), both suaveolol and methyl suaveolate reduced the oedematous response by about 16%, reaching about 60% reduction at 1 μmol/cm^2. As reference, doses of the reference anti-inflammatory drug indomethacin ranging from 0.1 to 0.3 μmol/cm^2 induced from 17% to 59% oedema inhibition. The obtained results allowed to calculate the ID$_{50}$ (dose giving 50% oedema inhibition) values of the tested compounds as an index of their anti-inflammatory potency. These values revealed that suaveolol (ID$_{50}$ = 0.71 μmol/cm^2) and methyl suaveolate (ID$_{50}$ = 0.60 μmol/cm^2) were only two to three times less active than indomethacin (ID$_{50}$ = 0.26 μmol/cm^2).

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