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HPLC analysis of *neo*-clerodane diterpenoids from *Teucrium chamaedrys*

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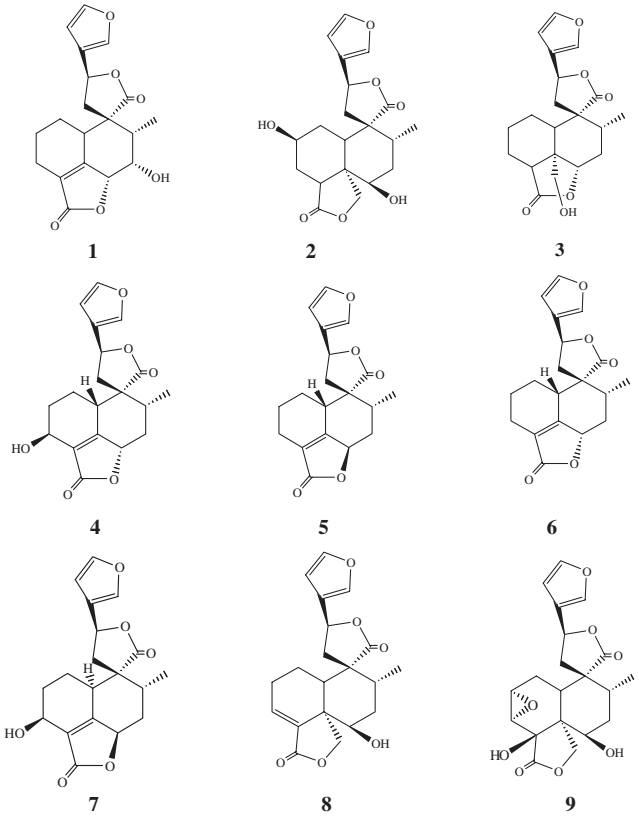
A simple, rapid analytical method for the quantitative determination of nine *neo*-clerodane diterpenoids was developed. The *neo*-clerodane diterpenoids present in the plant material and extracts were separated with an acetonitrile-water gradient at a flow rate of 1 mL per minute. The HPLC separation was performed on a Phenomenex Luna C18(2) (150 × 4.6 mm I.D., particle size 5 μm) reversed phase column with detection at 220 nm. The limit of detection was 0.24–0.90 μg/mL. The relative standard deviation (RSD) values for the determination of *neo*-clerodane diterpenoids in plant extracts were less than 3.20%. This is the first analytical method developed for qualitative and quantitative analysis of nine *neo*-clerodane diterpenoids by HPLC with PDA detection.

1. Introduction

Teucrium chamaedrys (Lamiacea), common name Germander is a perennial herb and grows to an average height of 30 to 60 cm. It prefers full sun and the soil should be moist. Native to Europe and southwest Asia [1], it has an ancient reputation as a cure for dyspepsia, anorexia, nasal catarrh, chronic bronchitis, gout, rheumatoid arthritis, intermittant fever and uterine infections [1]. *Neo*-clerodane diterpenoids are accepted to be chemotaxonomic markers of the *Teucrium* species which are known to be the most abundant natural source for these compounds [2].

The studies conducted in mice implicated that the cytochrome P450 metabolism of the *neo*-clerodane diterpenoids present in aerial parts of *T. chamaedrys*, resulted in hepatotoxicity [3, 4] which was further extended on Germander to show that the furan ring of one of the major *neo*-clerodane diterpenoids of Germander, teucrin A, is required to cause the hepatotoxicity in mice [3, 5]. Capsules containing either Germander alone or in combinations were marketed in France as a weight control supplement. While on the market 30 cases of heptatoxicity were reported as a result of the products [6] containing *Teucrium* species, which included a fatality due to fulminant hepatic necrosis [7]. As a consequence, sale of preparations containing Germander were prohibited in Europe and the USA. Toxicity was found to be reversible when use of the herb stops, but there has been one reported fatal case of hepatitis [6, 8].

Due to the hepatotoxic nature of Germander, an analytical method to quantitatively determine the *neo*-clerodane diterpenoids was established. Published chromatographic methods for the determination of *neo*-clerodane diterpenoids in plant extracts were based on TLC [9] and GC [10–13]. The techniques developed either involved the isolation of diterpenoids or the analysis of the essential oils in the aerial parts of the *Teucrium* species. The simple, precise method reported herein is the first HPLC method for the detection and quantitation of nine *neo*-clerodane diterpenoids [teucrin A (**1**), dihydroteugin (**2**), teucanadensin (**3**), isoteuflidin (**4**), teuflin (**5**), teucvidin (**6**), teucrin H1 (**7**), 6-β-hydroxy teuscordin (**8**) and teucrin G (**9**)] in *Teucrium* extracts.



Structures of *neo*-clerodane diterpenoids

2. Investigations, results and discussion

2.1. Chromatographic conditions

Optimal chromatographic conditions were obtained after running different mobile phases with a reversed phase C18 column. The different columns tried were Synergi Max-RP 80 A, Aqua, Luna, Lichrospere, Selectosil, Hypersil, Waters Resolve and XTerra. Solvents other than acetonitrile or the addition of modifiers such as THF, methanol, reagent alcohol or methyl *t*-butyl ether (MBE) did not improve the separation. The best separations were observed with Luna RP column using acetonitrile and water as mobile phase. Variation of the column temperature between 25–40 °C did not cause significant change in the resolution, however changes in retention time were observed. Thus, the column was used at room temperature at a flow rate of 1 mL/min. The method allowed for the separation of *neo*-clerodane diterpenoids in 13 mins.

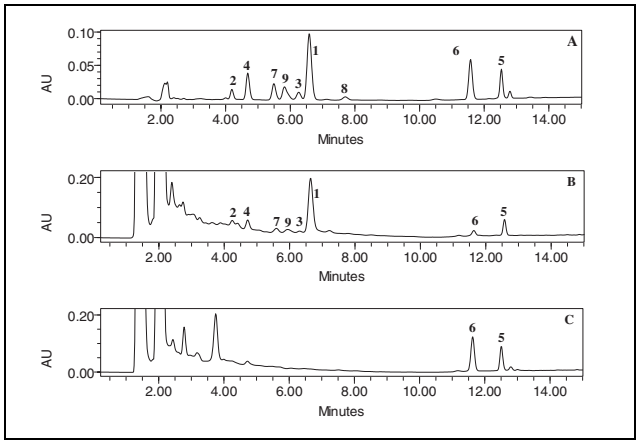


Fig.: A typical HPLC chromatogram of pure *neo*-clerodane diterpenoidal standards (1–9) (A), and plant extracts (B, C) at 220 nm

2.2. Accuracy, precision and linearity

Linear gradient elutions resulted in separation with injection volume of 10 μL at 220 nm. HPLC calibration curve showed a linear correlation between sample concentration and peak area. Intra and inter-day variation were determined with standards. It was performed thrice on three different days and each concentration point was injected in triplicate. The analytical parameters like accuracy, precision, peak purity, linearity and limit of detection are important for the assessment of the quality level of pharmaceutical products. Purity was determined by the PDA data of all peaks of interest. In order to determine the accuracy of the method one sample (sample **A**) was spiked with a known amount of the standard compounds and recovery rates were between 98.0% and 103.7%. An indicator for precision is the standard deviation (δ). All samples were injected in triplicate and the standard deviation of standard compounds was below 1.0% and for plant extracts was below 2.0% except for sample **C** (3.17%). Calibration data (Table 1) indicated the linearity of the detector response for all standard compounds from 0.800 to 300.0 μg/mL. The limit of detection was found to be between 0.24 and 0.90 μg/mL for the standard compounds.

Table 1: Calibration data [regression equation and correlation coefficient (R^2)] and limit of detection (LOD) for compounds 1–9

Compd.	Regression Equation	R^2	LOD (μg/ml)
1	$y = 2.64 \times 10^4 x$	0.9998	0.240
2	$y = 5.00 \times 10^3 x$	0.9999	0.800
3	$y = 4.70 \times 10^3 x$	0.9999	0.900
4	$y = 1.66 \times 10^4 x$	0.9998	0.240
5	$y = 1.99 \times 10^4 x$	0.9999	0.270
6	$y = 2.22 \times 10^4 x$	0.9999	0.500
7	$y = 1.55 \times 10^4 x$	0.9998	0.320
9	$y = 8.08 \times 10^4 x$	0.9997	0.800

2.3. Analysis of plant extracts

Table 2 shows the quantities of *neo*-clerodane diterpenoids in different species of plant extracts. The highest content of teucrin A was found in plant extract **F**. However, no peak of teucrin A was found in plant extracts **B**, **D** and **E**, shown to be hepatotoxic. The two species, **A** and **B**, were obtained from the same company listed as *T. canadense,* but the chemical profile of the two samples were not similar. All compounds were detected in sample A, but only compounds **5** and **6** were detected in sample **B**. This is a good indication that samples A and B were different species. There is also the possibility of environmental and seasonal differences, but the collection information is unknown. Further population studies must be carried out to determine possible chemical profile differences. The species **C**, **D** and **E** are authenticated samples. The chemical profile of sample **A** and **F** closely matches that of sample **C**, which was authenticated as *T. chamaedrys*.

The identification of the compounds in plant extracts was based on the retention times and the comparison of UV spectra with those of authentic standards. The method can be used to quantify major compounds in plant extracts and possibly products.

The method described in this paper can be applied as an analytical tool for determining the possible adulteration of

Table 2: Content (%) of neo-clerodane diterpenoids in six different plant materials

Compd.	A	B	C	D	E	F
1	0.140 (0.61)*	ND	0.046 (0.34)	ND	ND	0.158 (0.06)
2	0.030 (1.33)	ND	0.030 (0.59)	ND	ND	0.046 (0.59)
3	0.031 (0.44)	ND	0.027 (1.92)	ND	ND	0.046 (0.62)
4	0.028 (0.19)	ND	0.019 (0.93)	ND	ND	0.011 (0.70)
5	0.033 (0.61)	0.061 (0.34)	0.157 (0.26)	0.001 (1.07)	0.029 (0.39)	0.072 (0.29)
6	0.012 (0.91)	0.084 (0.25)	0.009 (0.85)	DUL	0.005 (0.06)	DUL
7	0.014 (0.89)	ND	0.109 (0.25)	ND	0.024 (0.68)	0.016 (0.93)
8	D	ND	D	ND	ND	D
9	0.015 (1.22)	ND	0.029 (3.17)	ND	ND	0.022 (0.19)

* percent RSD (n = 3) given in parenthesis
ND = not detected; D = detected; DUL = detected under limit of quantitation

commercial products (as in skullcap preparations) in order to establish quality and safety. Further studies will involve the identification and quantification of all species of *Teucrium* and a number of populations.

3. Experimental

3.1. Instrumentation and chromatographic conditions

The HPLC system consisted of Waters (Waters Corp., Milford, MA) model 6000A pumps, a Waters model U6K injector, a Waters model 680 automated gradient controller, a Waters model 996 photodiode array detector, and a computerized data station equipped with Waters Millennium software. Separation was achieved on a Luna C18(2) column (Phenomenex, 150 × 4.6 mm I.D.; 5 μm particle size) and operated at ambient temperature. The column was equipped with a 2 cm LC-18 guard column (Supelco, Bellefonte, PA, USA). The mobile phase delivered at a flow rate of 1 mL/min, comprised of the gradient system: 0 min, 62% water/38% acetonitrile; 8 min, 62% water/38% acetonitrile; 15 min, 10% water/90% acetonitrile. Each run was followed by a 5 min wash with 100% acetonitrile and an equilibration period of 15 min.

3.2. Chemicals

The standard compounds **1**–**9** were isolated at NCNPR, their identity and purity were confirmed by chromatographic (TLC, HPLC) methods and comparison with published spectral data (IR, 1D- and 2D-NMR, HRESIMS). HPLC-grade acetonitrile was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water for the HPLC mobile phase was purified in a Milli-Q system (Millipore, Bedford, MA). Samples A and B were obtained from American Merchantile Cooperation (Memphis, TN, USA). Other samples *T. chamaedrys* (C), *T. polium* (D) and *T. flavum* (E) were collected in Italy and sample F was purchased from Botanical Liaisons (Boulder, CO, USA).

3.3. Standard solution

Individual stock solutions of diterpenoids were prepared at a concentration of 0.1 mg/mL in methanol. The quantification was performed using seven levels of external standards. The ranges obtained were 0.8–2.5 μg/mL to 300–100 μg/mL depending on the concentration of each diterpenoid stock solution. Table 1 shows the calibration data and the calculated limit of detection. The calibration data for analyte **8** could not be established due to insufficient quantity but was identified in various plant extracts.

3.4. Sample preparation

Ground aerial parts (500 mg) of the plant material were sonicated in 2.5 mL of methanol for 20 min followed by centrifugation for 10 min at 1500 rpm. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated thrice and respective supernatants combined. The final volume was adjusted to 10 mL with methanol.
Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.45 μm nylon membrane filter. The first 1 mL was discarded and the remaining volume was collected in an HPLC sample vial. Each sample solution was injected in triplicate.

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References

1 Grieves, M.; A Modern Herbal, Vol. **1**, 351–352, Hafner Publishing Co., New York 1959
2 Piozzi, F.; Rodriguez, B.; Savona, G.: Heterocycles **25**, 807 (1987)
3 Kouzi, Samir, A.; McMurtry, Randolph J.; Nelson, Sidney D.: Chem. Res. Toxicol. **7**, 850 (1994)
4 Loeper, J.; Descatoire, V.; Letterton, P.; Moulis, C.; Degott, C.; Dansette, P.; Fau, D.; Pessayre, D.: Gastroenterology **106**, 464 (1994)
5 Popa, D. P.; Reinbol'd, A. M.; Rezvukhin, A. I.: Khim. Prir. Soedin. **9**, 169 (1973)
6 Larrey, D.; Vial, T.; Pauwels, A.; Castot, A.; Biour, M., David, M.; Michel, H.: Ann. Intern. Med. **117**, 129 (1992)
7 Dao, T.; Peytier, A.; Galateau, F.; Valla, A.: Gastroenterol. Clin. Biol. **17**, 609 (1993)
8 Laliberte, L.; Villeneuve, J. P.: Can. Med. Ass. J. **154**, 1689 (1996)
9 Wang, Lei.; Luo, Shurong.; Li, Guangyi.: Yaoxue Xuebao **35**, 294 (2000)
10 Chialva, F.; Gabri, G.; Liddle, P. A. P.; Ulian, F.: Aromat. Plants **7**, 183 (1982)
11 Tateo, F.; Di Cesare, L. F.; Bononi, M.; Cunial, S.; Trambaiolo, F.: Flavour Science **197**, 158 (1996)
12 Tzakou, O.; Roussis, V.; Loukis, A.; Harvala, C.; Galati, E. M.; Germano, M. P.: Flav. Fragr. J. **12**, 113 (1997)
13 Kamel, A.; Sandra, P.: Biochem. System. Ecol. **22**, 529 (1994)