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Use of high-performance liquid chromatographic peak deconvolution and peak labelling to identify antiparasitic components in plant extracts

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ABSTRACT

Artemisia absynthium L. is a commonly used medicinal plant for parasitic diseases all over the world. By means of high-performance liquid chromatography with diode-array detection and the PU6100 solvent optimization system, two sesquiterpene lactones, α-santonin and ketopelenolid-A, were tentatively identified in methanolic extracts of this plant. α-Santonin is a well known antiparasitic compound and could be one of the active principles of this plant species. Reconstructed spectra are potentially useful in scanning a complex chromatogram for pharmacologically active compounds.

INTRODUCTION

For many years, the isolation and identification of natural products from plants widely used in folk medicine, or with a clearly demonstrated pharmacological activity, have been intensively studied. A major role in this kind of research is played by chromatographic methods, in particular liquid chromatography.

Many species of the large genus *Artemisia* (family Asteraceae) have been used in folk medicine all over the world, and the antiparasitic activity of several species is well known [1–3]. The Asteraceae family is characterized by structurally diverse sesquiterpene lactones (a large class of terpenes). Reports dealing with the isolation and structure elucidation of sesquiterpene lactones have increased during the last decade for two main reasons: first, these components have been used as chemical markers in chemotaxonomy, and second, a number of compounds received considerable attention owing to their various biological activities [4,5]. A detailed review of the sesquiterpene lactones of the *Artemisia* genus has been published [6] and may more compounds have since been isolated.

The species *Artemisia absynthium* L., growing in different regions of the world, has been intensively studied because of several reports on its antiparasitic medicinal properties and because of its use in the preparation of wines and beverages. Many terpenes have been identified in the essential oil, in addition to sesquiterpene lactones, coumarins and other compounds. Toxic effects have also been reported [4,5,7–10]. In recent years, this species, and many others of the genus *Artemisia*, have been investigated to find the sesquiterpene lactone artemisinin (Qinghaosu), which so far has been isolated only from *Artemisia annua* L. from China, and which has unique clinical properties against malaria [11–14].

Aqueous, aqueous alcoholic and methanolic ex-

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tracts of *Artemisia absynthium* growing in Havana, Cuba, showed consistent and reproducible antiparasitic activity against three different parasites in tests carried out at the Institute of Tropical Medicine (IPK) in Havana. We considered it of interest to develop some phytochemical work-up of this plant material, to establish some of the active principles elaborated by this Cuban plant. One important approach was the chromatographic comparison of some standards with extracts, or fractions, of this plant by means of high-performance liquid chromatography (HPLC) with diode-array detection (DAD), and the use of software for data handling, solvent optimization and peak comparison.

EXPERIMENTAL

Plant extraction and fractionation

Aerail parts of *Artemisia absynthium* growing in Guira de Melena, Havana, were collected in July 1990, air dried and finely ground. The plant species was determined by comparison with authentic herbarium specimens and voucher samples are deposited in the Herbarium of the Botany Institute of the Cuban Academy of Sciences. A 5-g amount of fine powdered material was extracted first with water (50 ml) at 50°C for 8 h, filtered, dried and extracted with methanol (50 ml) at 50°C for 8 h. Both water and methanolic extracts were used for chromatographic studies.

Standards

α-Santonin, ketopelenolid-A, absynthin and artemisin, four sesquiterpene lactones isolated from different species of the genus *Artemisia* growing in Europe, were kindly provided by Dr. J. Harmatha (Terpenes Department, Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences). Chemical structures of these lactones were determined by spectroscopic methods (infrared, nulear magnetic resonance, mass, circular dichroism, optical rotatory dispersion, ultra violet, etc.) and chemical correlation [5]. The standards were dissolved in methanol at concentrations of 1 and 0.1 mg/ml.

HPLC equipment and conditions

The liquid chromatograph consisted of an HPLC pumping system with quaternary solvent capability (Unicam Analytical, Cambridge, UK), an injection valve fitted with a 20-μl sample loop (Rheodyne) and a 250 × 4.0 mm I.D. column (Superspher Spherisorb 100 RP-18, 4 μm; Merck). Diode-array data were collected on a PU6003 diode-array system, which consists of a PU4120 detector and a P3202 IBM PC-compatible computer (Unicam Analytical). This computer was also used for the data handling and processing with a PU6100 solvent optimizer (Unicam Analytical). Helium degassing was used and all injected samples were filtered through Anotop 10 plus 0.2-μm disposable filters.

RESULTS AND DISCUSSION

Previous work on the extraction and fractionation of fresh and dried aerial parts of *A. absynthium* with solvents such as water, water–methanol and methanol, partitioning with immiscible solvents such as chloroform, ethyl acetate, *n*-butanol, etc., and even open-column chromatography with several stationary phases was not very successful in isolating and identifying active compounds in this plant. This was due mainly to the presence of high-polarity compounds and a lack of resolution. On the other hand, the biological testing of fractions against three types of parasites indicated that the methanolic fraction was the most active, but activity was also present in other polar fractions. The availability of some sesquiterpene lactone standards isolated from the same genus species growing in Europe, and the possibility of working with HPLC coupled to a DAD system and PU6100 software for solvent optimization and peak comparison, provided a good opportunity to investigate the chemical composition of this medicinal plant. Dried plant powder was first extracted with water to eliminate very polar compounds with short retention times on reversed-phase columns, and then extracted with pure methanol. This methanolic extract was used for HPLC without further preparation.

The standards were prepared at a concentration of 1 mg/ml for artemisin, santonin and ketopelenolid-A and 2 mg/ml for absynthin because of its lower absorption at 210 nm. The mixture of standards at this concentration, or diluted tenfold, was used for the solvent optimization software and peak comparison as described [15,16]. The first experiment for optimization was a gradient from meth-

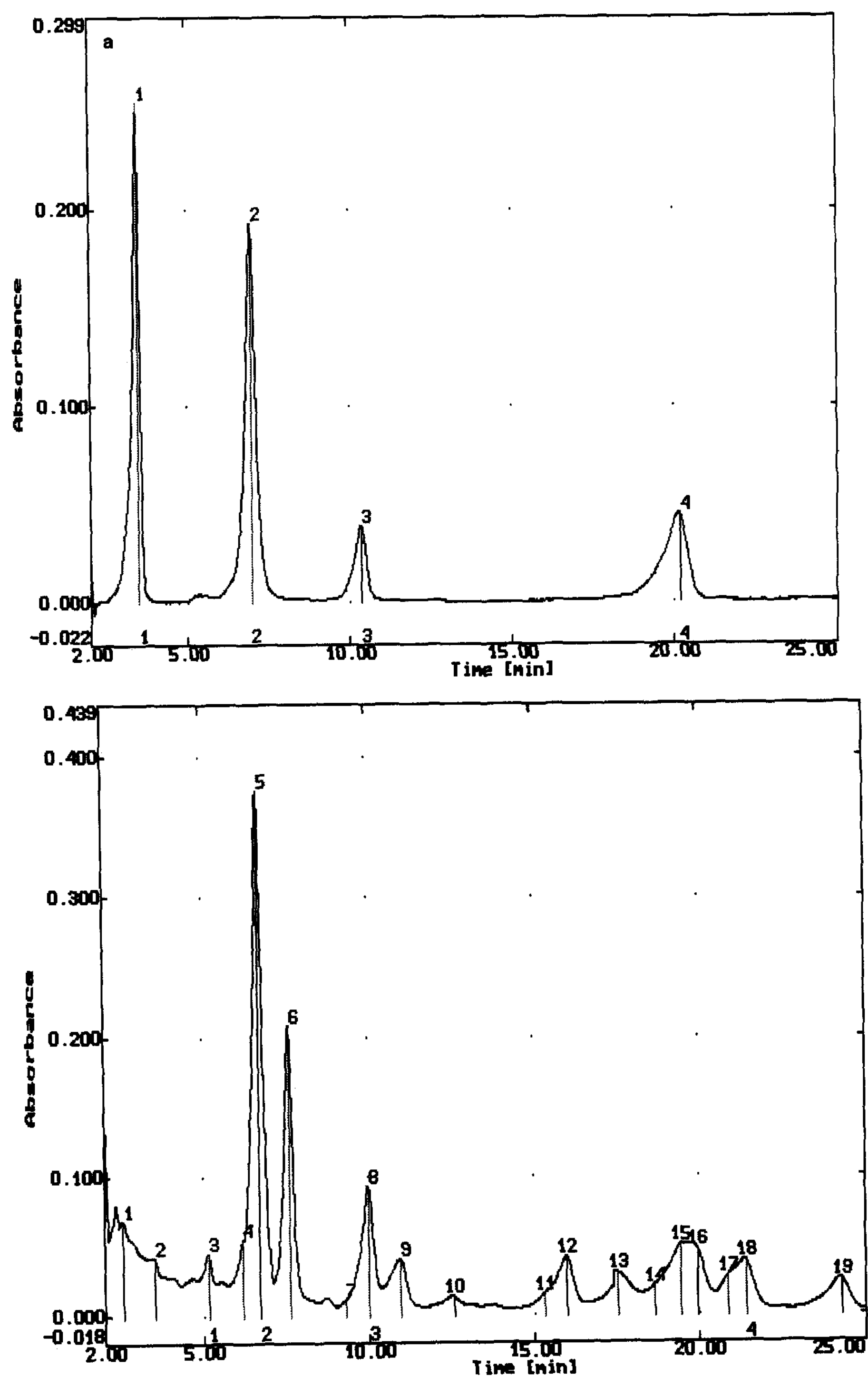

Fig. 1. (a) Chromatogram of the four standards, (1) artemisin, (2) santonin, (3) ketopelenolid-A and (4) absynthin in methanol–water (53:47). (b) Chromatogram of the methanolic extract from *Artemisia absynthium*, methanol–water (53:47). Wavelength: maximum absorbance above 210 nm.

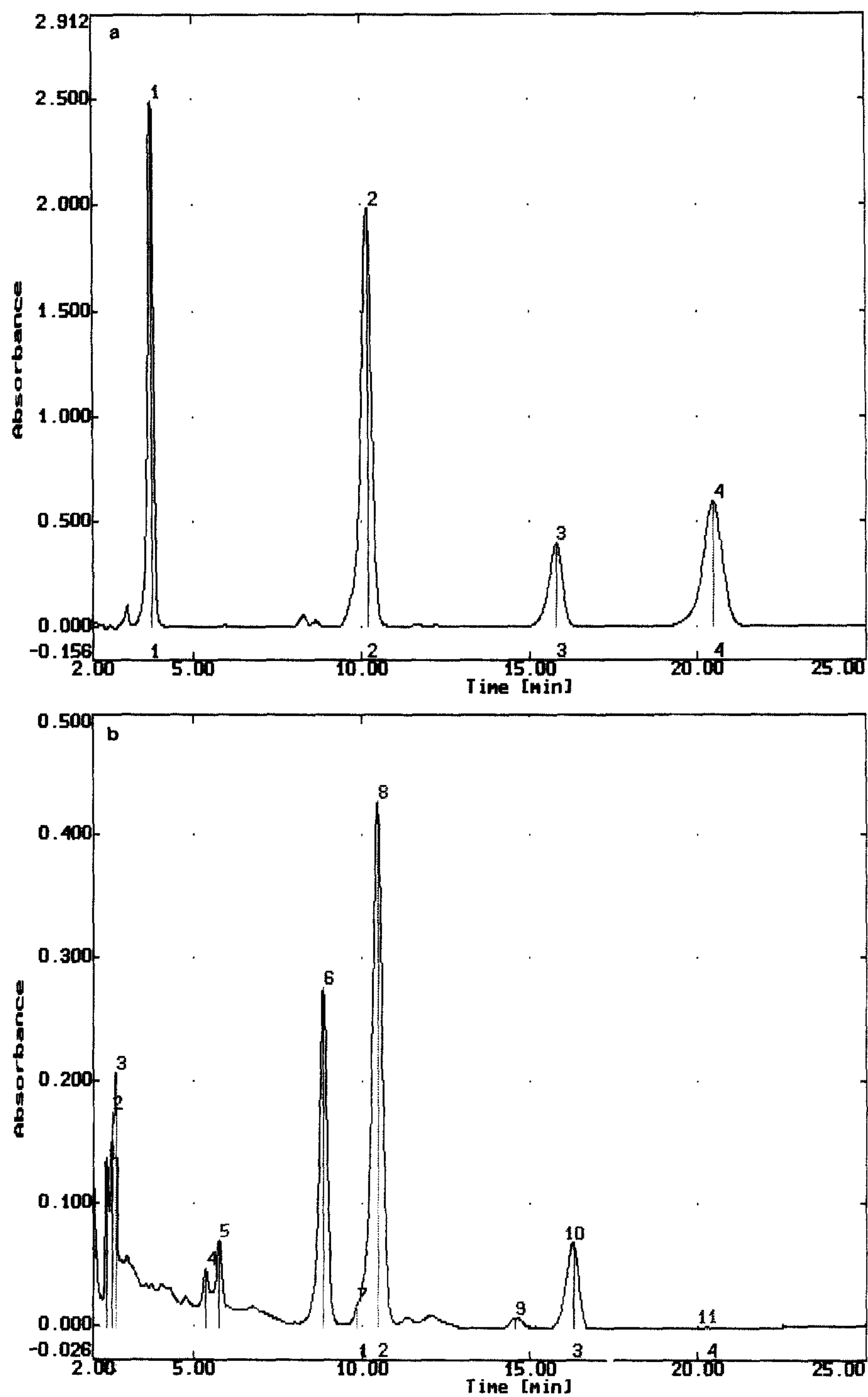

Fig. 2. As Fig. 1, but with the solvent acetonitrile–water (40:60).

anol–water (40:60) to pure methanol in 20 min at a flow-rate of 1 ml/min. Retention times from this run were used by the software to predict the methanol–water composition; after some updating we obtained a good separation with methanol–water (53:47). The order of elution was artemisin (3 min 19 s), santonin (6 min 37 s), ketopelenolid-A (9 min 55 s) and absynthin (18 min 50 s) (Fig. 1a).

After program prediction and some updating, a solvent composition of acetonitrile–water (40:60) proved to be convenient for the standards: artemisin (3 min 37 s), santonin (10 min 13 s), ketopelenolid-A (15 min 37 s) and absynthin (20 min 25 s) (Fig. 2a). With this acetonitrile–water composition we obtained almost the same retention times for the first- and last-eluting compounds, and a convenient spacing for the second and third. The prediction for the tetrahydrofuran (THF)–water composition according to the PU6100 was THF–water (25:75). In this experiment we obtained the following retention times: artemisin 5 min 43 s, santonin 9 min 37 s, ketopelenolid-A 14 min 25 s and absynthin 17 min 43 s. This was also a good separation but the presence of THF in the solvent is less appropriate for the detection of ketopelenolid-A and absynthin, lactones with only a low UV end absorption. Because of this we tried a ternary mixture of water–methanol–acetonitrile and after some updates of the program data we obtained a satisfactory composition of water–methanol–acetonitrile (50:30:20) with the following retention times: artemisin 3 min 37 s, santonin 8 min 43 s, ketopelenolid-A 13 min 58 s and absynthin 26 min 16 s.

The next step was to separate the plant methanolic extract using these solvents and to use the PU6100 to match the labelled standard peaks and spectra with those present in the plant extract.

The solvent optimization software contains peak identification, or labelling functions, which enable the correct assignments to be made as the solvents change. In this instance we are using the peak-labelling functions as a peak identification tool. The procedure is first to deconvolute the three-dimensional chromatogram and to obtain the "pure" spectra of the peaks by iterative target transformation factor analysis (ITTFA) [15]. These spectra and

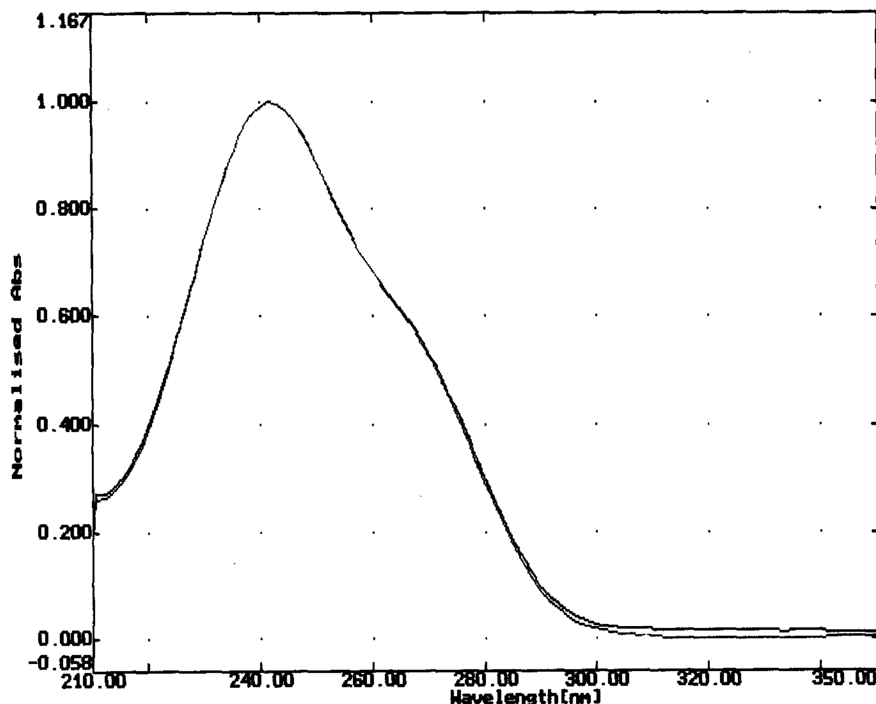

Fig. 3. Comparison of the spectrum of santonin (bottom trace) with that of peak 5 of the extract in methanol–water (53:47).

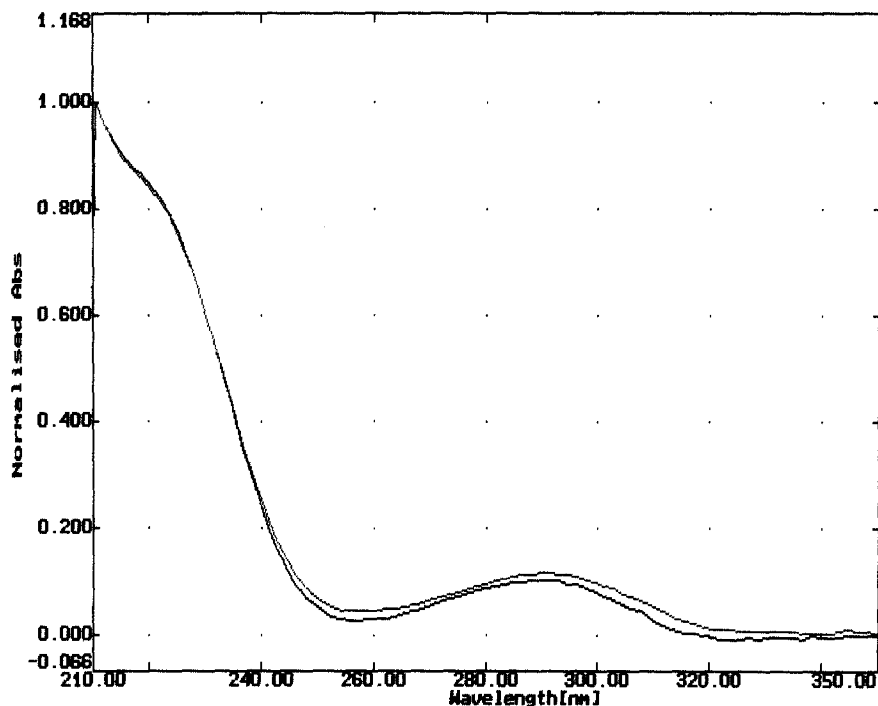

Fig. 4. Comparison of the spectrum of ketopelenolid-A (top trace) with that of peak 8 of the extract in methanol–water (53:47).

Peak Labelling

Data name : NESTOR_NMET53-3.CSN
Solvent : 53.0% MeOH 47.0% Water

REF	POSITION	SIZE	WIDTH	SPEC	CONC	TIME	MATCH	NAME
1	0.000	0.000	0.000	0.00	0.00	0.00	0.00	ARTEMIS
2	6.670	247.980	0.187	1.00	1.00	0.96	0.95	SANTONI
3	9.956	38.837	0.239	0.98	1.00	0.96	0.94	KETOPEL
4	21.362	14.482	0.441	0.88	1.00	0.93	0.82	ABSYNTH

Data name : NESTOR_NMETAC40.CSN
Solvent : 40.0% ACN 60.0% Water

REF	POSITION	SIZE	WIDTH	SPEC	CONC	TIME	MATCH	NAME
1	0.000	0.000	0.000	0.00	0.00	0.00	0.00	ARTEMIS
2	10.439	285.820	0.263	1.00	1.00	0.90	0.90	SANTONI
3	16.242	34.915	0.346	0.96	1.00	0.88	0.85	KETOPEL
4	20.261	1.595	0.641	0.71	1.00	1.00	0.71	ABSYNTH

Size : peak volume (Abs * nm * Time)

Fig. 5. The peak labelling information from the PU6100 solvent optimizer, showing the spectral matching factors and retention matching factors for each peak of interest. Reference 1 was not assigned.

peak areas are then matched. More details of this process are given in ref. 15. In Fig. 1a and b chromatograms of the standard mixture (top) and methanolic extract from the plant (bottom) in the same solvent, methanol–water (53:47), are compared. Fig. 2a and b show a similar comparison using acetonitrile–water (40:60). Standard 2 (α-santonin) seems to be present in the methanol extract as peak 5 and standard 3 (ketopelenolid-A) as peak 8 in the same extract. UV spectra of the compounds in peaks 2 and 5, and 3 and 8, are almost completely superimposable (Figs. 3 and 4) and the retention times agree within 3.9% and 3.5%, respectively. The same situation was found when the mixture of standards was compared with methanolic extracts in the other two solvent systems, acetonitrile–water (40:60) and water–methanol–acetonitrile (50:30:20); the spectrum matching factors for standards 2 and 3 were close to unity with corresponding peaks in the plant extract. The retention times agreed within 2.8% (santonin) and 3.0% (ketopelenolid-A).

Fig. 5 shows the peak labelling output of the PU6100 with the solvents methanol–water (53:47) and acetonitrile–water (40:60). There are high matching factors for both santonin and ketopelenolid-A in both solvents. However, the matching factor for peak 4 is lower and its identity is inconclusive. Absynthin has a spectrum which is very different from those of peaks 15–17 in Fig. 1b. The spectrum of peak 18 is similar, and has an intermediate matching factor of 0.88 in Table I. However, the retention times are substantially different and its presence cannot be confirmed.

CONCLUSIONS

Using the described equipment and software we developed chromatographic methods for the analysis and preparation of four sesquiterpene lactones and for the analysis of the methanolic extract of *Artemesia absynthium*. The results suggest that α-santonin and ketopelenolid-A are present in these extracts. The presence of santonin in Cuban *Artemisia absynthium* aerial parts is a possible explanation for the antiparasitic properties of this medicinal plant, because α-santonin has been used for many years as a medicine for parasitic diseases.

The spectral reconstruction algorithms are potentially useful in highlighting compounds with known or similar chromophores in a complex chromatogram. The use of the algorithms in this way avoids lengthy and unnecessary method development in the search for pharmacologically active compounds.

REFERENCES

1 E. Rodriguez, G. H. N. Towers and J. C. Mitchell, *Phytochemistry*, 15 (1976) 1573.
2 Qinghaosu Antimalarial Co-ordinating Research Group, *Chin. Med. J.*, 92 (1979) 811.
3 N. Katz, presented at the *Simposio Brasil–China de Quimica e Farmacologia de Produtos Naturais, Rio de Janeiro, December 14, 1989*, paper L-44.
4 F. C. Seaman, *Bot. Rev.*, 48, No. 2 (1982) 121.
5 N. H. Fischer, E. J. Olivier and H. D. Fischer, *Prog. Chem. Org. Nat. Prod.*, 38 (1979) 49.
6 R. G. Kelsey and F. Shafizadeh, *Phytochemistry*, 18 (1979) 1591.
7 T. Sacco and F. Chialva, *Planta Med.*, 54 (1988) 93.
8 M. C. Delahaye, in *Artemisie, Ricerca ed Applicatione, Supplemento al Quaderno Agricolo*, Federagrario, Turin, 1985, p. 233.
9 R. Truhaut, in *Artemisie, Ricerca ed Applicatione, Supplemento al Quaderno Agricolo*, Federagrario, Turin, 1985, p. 181.
10 J. Volak and J. Stodola, *Plantes Medicinales*, Artia, Prague, and Grund, Paris, 1983.
11 J. M. Liu *et al.*, *Acta Chim. Sinica*, 37 (1979) 129.
12 *4th Meeting of the Scientific Working Group on the Chemotherapy of Malaria, Beijing, 1981, WHO Report, TDR Chemal SWG (4) QHS/813*, p. 5.
13 D. L. Klayman, J. L. Ai and N. Acton, *J. Nat. Prod.*, 47 (1984) 715.
14 J. E. F. Reynolds (Editor), *Martindale. The Extra Pharmacopeia*, Pharmaceutical Press, London, 29th ed., 1989.
15 R. Lynch and G. Measures, *Lab. Pract.*, 39, No. 1 (1990) 61.
16 R. Lynch, S. D. Patterson and R. E. A. Escott, *LC · GC Int.*, 3, No. 9 (1990) 54.