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NMR spectroscopy: a useful tool for characterisation of plant extracts, the case of supercritical CO_2 arnica extract

Anna Rita Bilia *, Maria Camilla Bergonzi, Giovanni Mazzi, Franco Francesco Vincieri

Department of Pharmaceutical Sciences, University of Florence, Via G. Capponi 9, 50121 Florence, Italy

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Abstract

The efficiency of two-dimensional homonuclear $^1H-^1H$ correlated spectroscopy (COSY) and two-dimensional reverse heteronuclear shift correlation spectroscopy (i.e. heteronuclear multiple quantum correlation, HMQC) in characterising the content of the constituents of innovative extracts is demonstrated. These experiments were performed directly on a supercritical carbon dioxide (CO_2) commercial extract of arnica and were able to fully characterise the active constituents, sesquiterpenes, and other metabolites extracted with the supercritical CO_2, namely polyketides. Identification of constituents was performed by combining literature data and information obtained by 2D-NMR experiments.

Keywords: ^{1}H-NMR; ^{13}C-NMR; 2D-NMR experiments; Arnica; Supercritical carbon dioxide commercial extract; Constituent characterisation

1. Introduction

In continuing our studies on direct NMR analysis of complex plant mixtures, without purification or fractionation steps [1,2], we now report the analysis of an innovative supercritical carbon dioxide (CO_2) commercial extract of arnica. As previously reported [1,2], the analysis of herbal drugs, herbal drug preparations and herbal medicinal products are in general achieved through high-resolution chromatography techniques such as high performance liquid chromatography (HPLC), high performance thin layer chromatography (HPTLC), capillary gas chromatography (GC), and electrophoresis using specific detectors. However, these methods give a fingerprint of the markers or active constituents (and their percentage) but no information with regards to the other metabolites of the extract which can represent up to 95%. This is true especially in the unconventional extracts such as the innovative supercritical CO_2 extracts.

The Working Party of Herbal Medicinal Products (HMPWP) of the European Agency for Evaluation of Medicinal Products (EMEA) fixed for guidance on quality of herbal remedies [3]. The

* Corresponding author. Tel.: +39-055-275-7288; fax: +39-055-240-776.
E-mail address: ar.bilia@unifi.it (A.R. Bilia).

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control tests on the herbal drug preparations which also include extracts, must be such as to allow the qualitative and quantitative determination of the composition of the active substances and eventual other components such as diluents and preservatives. This aspect is truly important for the innovative extracts such as supercritical CO_2 extracts that are considered as new entities with respect to the traditional ones.

Arnica is well known in popular medicine for its anti-inflammatory action and many herbal drug preparations (HDPs) and herbal medicinal products (HMPs) are marketed in Europe [4].

The herbal drug 'Arnica flower', contains several classes of constituents such as flavonoids, quinic acid derivatives, sesquiterpenes, acetylenes and essential oil (0.2–0.35%) [5]. Sesquiterpenes represent the active constituents and the herbal drug should contain not less than 0.40% w/w of total sesquiterpene lactones calculated as helenalintiglinate with reference to the dried drug. They are quantified by a chromatographic procedure based on the separation of the sesquiterpene derivatives and using santonin as internal standard [5].

The marketed extracts are mainly represented by glicolic extract, fluid extract, tincture and the dried extracts and contain sesquiterpenes, flavonoids and quinic acid derivatives [6]. Among them, the tincture is mainly employed as such or as component of ointments, creams, gels or compresses containing 5–25% v/v tincture [7].

Recently, an innovative supercritical carbon dioxide (CO_2) extract with a high sesquiterpene content has also been marketed [6]. This sample was first evaluated by the authors using conventional HPLC analysis and in addition, it was directly analysed by NMR simply after its solubilisation in hexadeuterated dimethyl sulfoxide to fully characterise it.

2. Experimental

2.1. Chemicals

Dimethylsulfoxide-d_6 (99.8%) was purchased from Euriso-top (Gif-Sur-Yvette, France). Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA).

2.2. Sample

A commercial sample of the supercritical CO_2 extract of *Arnica montana* L. (lot n. CIH 06891-2) was kindly provided by Arkopharma (Carros Cedex, France).

2.3. NMR spectroscopy

The ^1H, ^1H–^1H COSY, and HMQC spectra were recorded at 300 K on a Bruker Avance-600 spectrometer operating at 600.13 MHz (14.1 T) using a 5-mm inverse probe equipped with a z-shielded gradient. Data processing was achieved with a SGI/02 computer using XWin-NMR software version 2.6. Samples (50 mg/0.8 ml) were dissolved in deuterated dimethylsulfoxide, and the solvent signal was used for spectral calibration (^1H: 2.49 ppm). Proton spectra were run using the standard pulse sequence program 'zg' for recording 1D experiments. The time domain size was 32K, number of scans four, spectral width 7440 Hz, Fid resolution 0.23 Hz, acquisition time 2.2 s, relaxation delay 1 s. Processing parameters were: number of points 64K, line broadening 3 Hz, FFT.

The ^1H–^1H COSY experiments were acquired using gradient pulses for selection and type COSY magnitude 90 degree. The acquisition parameters used were: time domain size of 2K, number of experiments 512, number of scans two, dummy scans 16, spectral width 7440 Hz in both dimensions. Fid resolution was 14.5 Hz in F2 and 29.1 Hz in F1, acquisition time 138 ms, relaxation delay 1 s: number of points was 1024 in F2 and 512 in F1, filter function applied was squared sine in both dimensions, with magnitude calculation of phase along columns. No phase correction was applied along rows.

The phase sensitive HMQC experiments were acquired via heteronuclear zero and double quantum coherence; using TPPI with decoupling during acquisition, peak type selection and gradient

Table 1
Time table of the HPLC programme

Time (min)	% H_2O	% CH_3OH	% CH_3CN	Flow (ml/min)
0.0	100.0	0.0	0.0	0.8
3.0	85.0	0.0	15.0	0.8
7.0	68.0	10.0	22.0	0.8
27.0	5.0	15.0	80.0	0.8
35.0	100.0	0.0	0.0	0.8
40.0	100.0	0.0	0.0	0.8

pulses with coherence selection step after t1. The acquisition parameters used were: acquisition time 138 ms, time domain size of 2K, number of experiments 256, number of scans 16, dummy scans 16, spectral width 7440 Hz (^{1}H) and 37730 Hz (^{13}C). Fid resolution was 3.633 Hz in F2 and 147.400 Hz in F1, F1 (^{13}C) at 110 ppm, relaxation delay 1 s. Processing parameters used were: number of points 1024 and 512, squared sine filter function of 60 degree. The total experimental time was 1 h 20 min.

2.4. HPLC analysis

The extract (8 mg/ml) was dissolved in a mixture of HPLC-grade solvents (methanol:acetonitrile:water, acidified to pH 3.2 with formic acid; 3:1:1). That was sonicated and filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane ($d = 13$ mm, porosity 0.45 μm, Lida manufacturing Corp., WI, USA) before HPLC analysis.

The HPLC system consisted of a HP 1100L instrument with a diode array detector controlled by a HP 9000 workstation (Hewlett and Packard, Palo Alto, CA, USA). The column was a 201 TP 54 RP-18 (5 μm, 254 × 4.6 mm, 300 Å Vydac Separation Group Hesperia, CA, USA) maintained at 26 °C. The experimental details are reported in Table 1, the injected volume was 20 μl solution, and chromatograms were monitored at 225, 254, 280, 350 nm.

The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett and Packard, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those of HPLC-DAD analysis. The same column, mobile phase, time period and flow rate were used. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: gas temperature used was 350 °C at a flow rate of 10 l/min. The nebulizer pressure was 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Spectra were fully scanned from m/z 100 to 800 in the negative and positive ion mode were obtained (scan time 1 s).

3. Results and discussion

HPLC analysis of an innovative extract of arnica (8 mg/ml), obtained by supercritical carbon dioxide extraction, showed the presence of several sesquiterpene lactones based on helenanin and

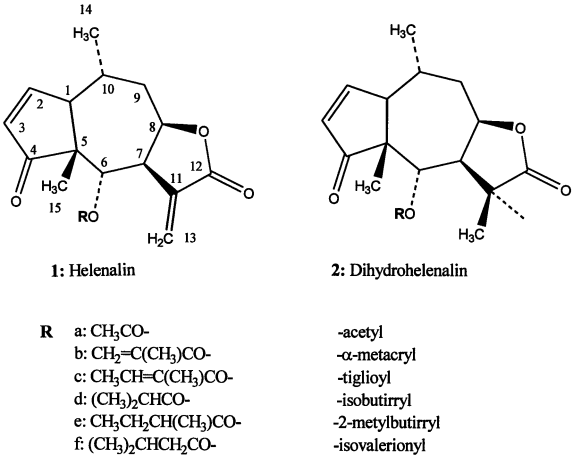

Fig. 1. Chemical structures of lactones sesquiterpenes **1a**–**1f** and **2a**–**2f**.

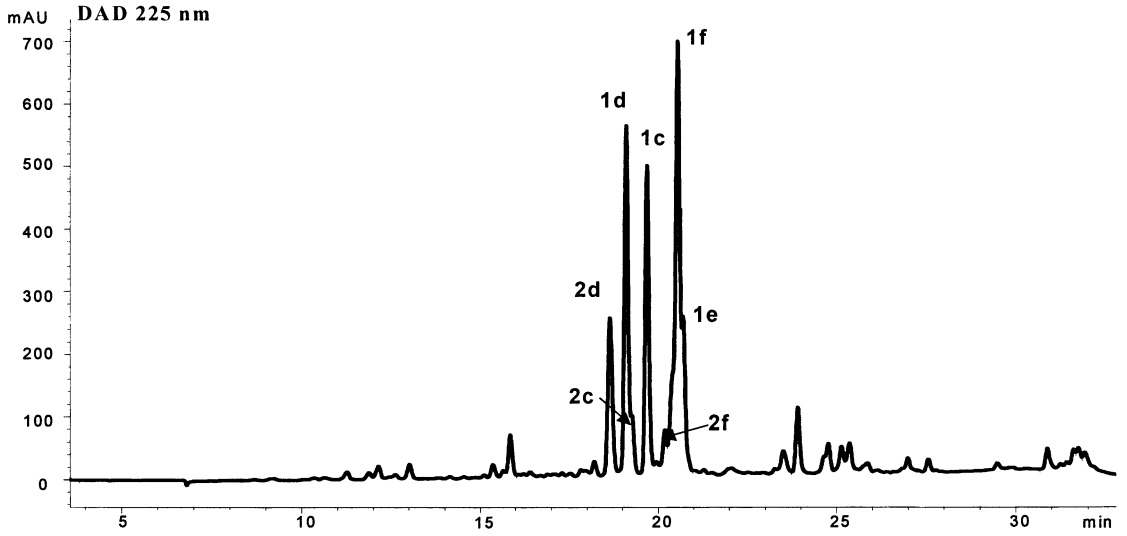

Fig. 2. HPLC chromatogram of arnica supercritical CO_2 extract.

dihydrohelenalin esters (Figs. 1 and 2). Neither flavonoids, nor caffeoyl quinic acid derivatives, characteristic constituents of the herbal drug, were detected by HPLC analysis.

The extract (50 mg) was also dissolved in hexadeuterated dimethylsulfoxide (0.8 ml) and directly analysed by NMR spectroscopy. Proton spectrum and 2D-NMR experiments were performed according to the methods reported in the experimental part. Spectral assignments of the constituents were carried out according to the data (chemical shifts and coupling constants) found in the literature [8–11] and by means of 1D-NMR and 2D-NMR spectra, and are presented in Table 2.

The ^{1}H-NMR spectrum (Fig. 3) was quite complex and it was characterised by four main regions: a low-field region between 5.6 and 8.2 ppm with signals principally due to olefinic protons of exocyclic acetylene of sesquiterpene lactone derivatives, and other conjugated olefinic protons; a mid-low-field region between 5.6 and 4.5 ppm with signals mainly due to olefinic protons of sesquiterpenoids and polyketides and carbinolic protons; a mid-field region between 4.5 and 3.6 ppm with signals due principally to the carbinolic protons of the polyacetilenes; and a high-field region between 3.6 and 0.7 ppm with signals due to aliphatic protons.

*3.1. Sesquiterpene lactones (**1**- and **2**- derivatives)*

Typical resonances of helenanolides were easily attributed due to their chemical shifts by comparison data reported in the literature [8,9], together with their splitting and couplings shown by cross peaks in the COSY experiments and reported in Table 2. On the basis of all the data, the presence of helenalin and 11α,13-dihydrohelenalin derivatives was stated.

The signals at 6.10 and 7.93 ppm were assigned to the olefin protons of the typical ketofurane ring of helenalin derivatives, namely H-3 and H-2, respectively (Fig. 3). Also their splittings and couplings obtained by COSY experiments (Fig. 4) confirmed their attribution: thus, both signals had a coupling with the signal at 2.98 ppm corresponding to H-1 [8,9]. The proposed assignments were unequivocally confirmed by HMQC experiments by the connectivities of the signals of protons H-2 and H-3 with the carbon resonances at 166.0 and 129.4, respectively and that of H-1 with carbon resonance at 53.6 ppm (Fig. 5), and were in agreement with the data reported in the literature [10,11]. In addition, proton resonance at 2.03 ppm was assigned to that of H-10 by the cross peak in the COSY experiments with the signal at 2.98 ppm (corresponding to that of H-1). As a

consequence, the cross peaks of the signal corresponding to H-10 with the signal at 1.24 ppm which was unequivocally allocated to protons of methyl-14. All these data were confirmed by the carbon resonances obtained by HMQC experiments.

In the region between 5.5 and 6.5 ppm of the proton spectrum, two other signals were easily distinguished from the others, the resonances at 5.98 and 6.26 ppm (Fig. 3). They were attributed to protons of α-methylene group exocyclic to the γ-lactone function of helenanin and its derivatives, namely H-13a and H-13b [8,9]. These data were unequivocally confirmed through HMQC experiments (Fig. 5) by the connectivities of these two proton resonances with the carbon resonance at 124.2 ppm [10,11]. In the COSY experiments (Fig. 4) were evidenced the cross peaks between the signals corresponding to H-13a and H-13b and the resonance at 3.52 ppm which was attributed to H-7. In addition the resonance of H-6 at 5.23 ppm was evidenced by the coupling of this resonance with the signal at3.52 of the H-7 (Fig. 4). The corresponding carbon resonances at 47.9 and 76.98 (Table 2) were found by the cross peaks in the HMQC experiments. The above data were confirmed by the literature [8].

By the analysis of proton and the COSY experiments (Figs. 3 and 4) was also evidenced a signal of H-2 of the dihydrohelenalin derivatives at 7.82

Table 2
Resonance assignments with chemical shift of constituents identified in 600 MHz ^{1}H and ^{1}H–^{13}C NMR spectra of arnica supercritical CO_2 extract

Compound	^{1}H shift (δ, ppm)	Assignment	^{13}C shift (δ, ppm)
Acetyl	2.06	CO–CH_3	21.3
Aromatic derivatives	2.26	C=C–CH	21.0
	7.31, 7.09, 6.94	C=CH–C=	129.7, 127.1, 124.0
11α,13-Dihydrohelenalin	7.82 and 6.10	H-2 and H-3	129.4 and 171.0
	5.28 and 2.95	H-6 and H-7	77.2 and 53.2
	3.29	H-11	40.0
	4.81	H-8	79.6
	3.03, 2.63, 1.69, 2.12	H-1, H-9a, H-9b and H-10	54.0, 40.0, 39.7, 27.2
	1.24, 1.21, 0.82	Me–13, Me–14 and Me-15	22.8, 19.3 and 12.2
Helenalin	7.93 and 6.10	H-2 and H-3	129.4 and 166.0
	5.23 and 3.52	H-6 and H-7	76.8 and 47.9
	5.98 and 6.26	H_2-13	124.2
	2.98, 2.03	H-1, H-10	53.6, 27.2
	5.04, 2.23, 1.69	H-8 and H-9a, H-9b	78.7, 39.7
	1.20 and 0.98	Me-14 and Me-15	20.6 and 19.1
Glycerol	4.09, 4.50	CH_2-OCOR	65.5
Isobutirryl	1.91, 0.98 and 0.99	CH_3–CH–CH_3	40.0, 22.2 and 16.5
Isovalerianyl	2.17 and 2.25	CH_2–	34.2
	2.04, 0.86	CH_3–CH–CH_3	32.9 and 18.8
Polyketides	1.69	CH_2–$(CH_2)_2$–CO	27.4
	2.23	CH=CH–CH_2–CO	34.6
	2.72	C=C–CH_2–C=C	26.2
	3.94	C=CH–CH–O	58.0
	4.68	C=CH_2	107.1
	5.08, 5.27	CH=CH–CH_2–CH=CH	124.8, 127.3
	5.32, 5.33	CH=CH–CH_2–CH_2	128.0, 130.2
α-Metacryl	5.64, 5.90	CH_2=	127.2
	1.54	CH_2=C–CH_3	16.0
2-Methyl-butirryl	2.05, 0.80	CO–CH–CH_3	38.9 and 22.9
	1.49 and 0.83	CH–CH_2–CH_3	25.0 and 14.5
Tiglioyl	6.66, 1.62, 1.67	CH_3–CH=C–CH_3	141, 26.1 and 23.8

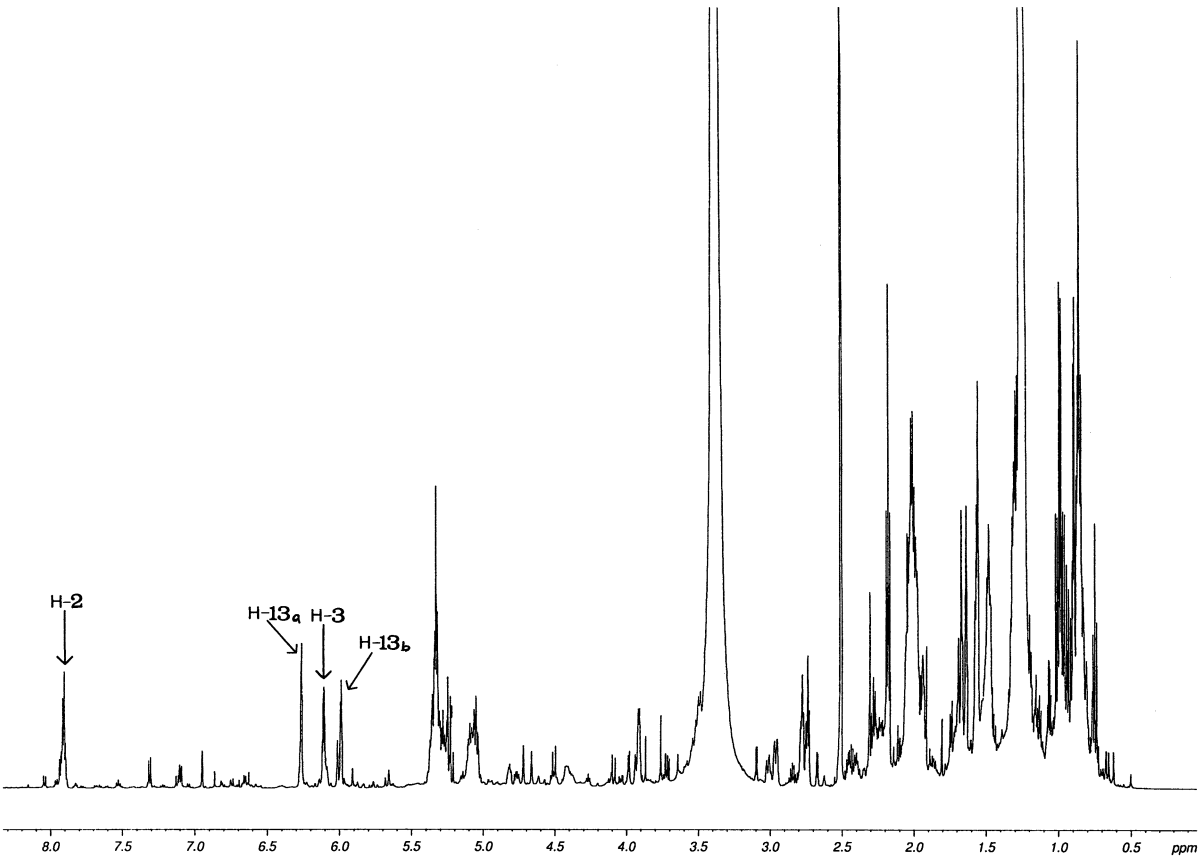

Fig. 3. Full resolution-enhanced 600 MHz ^{1}H NMR spectrum of arnica supercritical CO_2 extract.

ppm which was slightly shifted from that of helenalin derivatives at 7.93 ppm due to the lack of the α-methylene group exocyclic to the γ-lactone [8,9]. The resonance of H-3 was attributed to the same resonance of H-3 of helenalin derivatives at 6.10 ppm due to the cross peak in the COSY experiments between the signals at 7.82 and 6.10 ppm. Thus, the resonance of H-3 was not affected by the saturation of the of the α-methylene group, as also reported in the literature [8,9].

Another characteristic signal of dihydrohelenalin derivatives was found in the HMQC experiments (Fig. 6): a proton at 3.29 ppm which correlated with the carbon resonance at 40.0 ppm. These two resonances were characteristic and typical of those of proton and carbon 11, respectively, of the 11α,13-dihydrohelenalin moiety [8].

Signals due to H-7 and H-6 of 11α,13-dihydrohelenalin were attributed by the cross peaks in the COSY and HMQC experiments, confirmed by the analysis of the literature data [8] and reported in Table 2.

The signals corresponding to H-8 of helenalin and 11α,13-dihydrohelenalin derivatives, typical of the region from 4.8 to 5.1 ppm [8,9], were obtained through the cross peaks of H-7 of both moieties in the COSY experiments. These protons were identified by the signal at 5.04 ppm, attributable to the H-8 of helenalin, and the resonance at 4.81 ppm corresponded to the H-8 of 11α,13-dihydrohelenalin.

These assignments were also confirmed by the connectivities of each proton signal with the corresponding carbon signals in the HMQC experiments at 78.7 and 79.6, respectively, consistently with the reported data [10,11].

Based on data from literature [8,9,11], H-1 resonances of helenalin and 11α,13-dihydrohelenalin and H_2-9 of each helenalolide moiety were attributed by the COSY connectivities with H-2 and H-8, respectively. In addition, H-10 resonances of helenalin and 11α,13-dihydrohelenalin were obtained by the cross-peaks in the COSY spectra with H-1 and H-9a and H-9b. The corresponding resonances of C-1, C-9 and C-10 were subsequently obtained from HMQC connectivities (see Table 2) and were in accordance with the data reported in the literature [8,11].

Finally, both ^{1}H and ^{13}C NMR of Me-14 of helenalin and 11α,13-dihydrohelenalin were similar because this methyl is weakly affected by the reduction of the exocyclic methylene in 11α,13-dihydrohelenalin derivatives. This reduction, however, caused an upfield shift of carbon resonance of Me-15 in 11α,13-dihydrohelenalin, as reported in Table 2 [10].

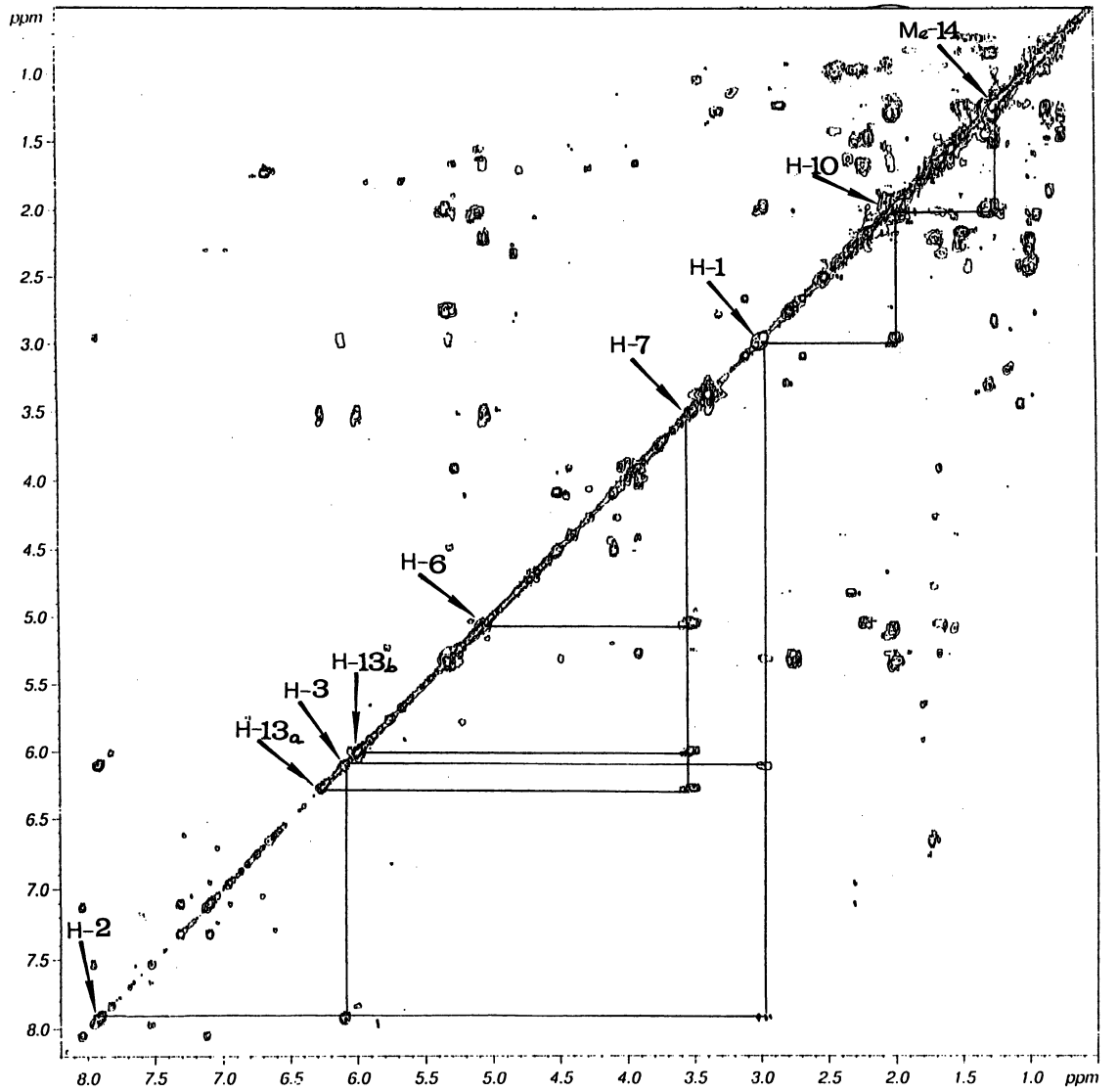

Fig. 4. $^{1}H-^{1}H$ COSY spectrum of arnical supercritical CO_2 extract. Cross peaks of some proton resonances of helenalin derivatives.

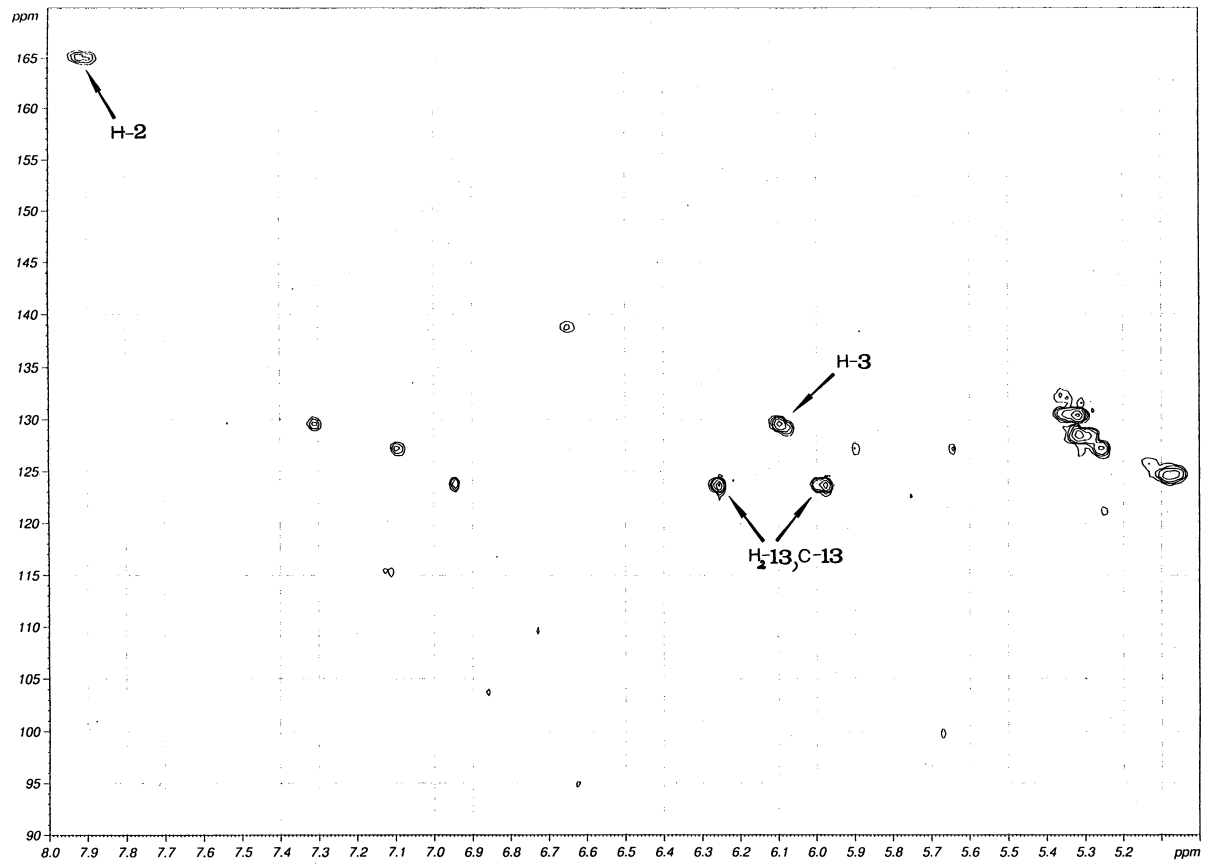

Fig. 5. Expanded region of the $^{1}H-^{13}C$ HMQC experiment: 5.0–8.0 ppm. Cross peaks of some resonances of helenalin derivatives are reported.

3.2. Ester moieties of helenanolides (*a–e*)

Signals of acetyl, metacrylic, tiglioyl, isobutirryl, 2-methyl-butirryl and isovalerionyl moieties were identified by their chemical shift, splitting and cross peaks in the COSY (Fig. 3) and HMQC (Fig. 6) experiments and confirmed by comparison with data reported in the literature [8–11].

The presence of acetyl moiety (**a**) was evidenced by the characteristic proton signal at 2.06 which correlated in the HMQC experiments with the carbon resonance at 21.3 ppm (Fig. 5). Olefinic protons of metacrylic moiety (**b**) were represented by two resonances at 5.64 and 5.90 ppm (carbon resonance at 127.2 ppm, obtained by HMQC experiments, Fig. 4) and correlated in the COSY experiments with the methyl at 1.54 ppm (carbon resonance at 16.0 ppm). A signal of an olefinic proton at 6.66 ppm which correlated in the COSY spectra with the resonances at 1.62 and 1.67 ppm was attributed to a tigloyl moiety. This matter was confirmed by the ^{13}C resonances obtained by the cross-peaks in the HMQC spectra (Table 2).

Thus, first proton resonance in the HMQC experiments showed a correlation with the carbon resonance at 141.0 ppm, while the two others showed a cross-peak with the signals at 26.1 and 23.8 ppm, respectively, and confirmed the presence of the tiglioyl (**c**) moiety. The presence of isobutirryl (**d**) and 2-methyl-butirryl (**e**) moieties were shown by the cross-peaks in the COSY spectra. The signal at 1.91 ppm that showed connectivities with the methyl protons at 0.98 and 0.99 ppm were attributed to the isobutirryl moiety

(**d**). The resonance at 2.05 ppm that showed connectivities with the methylene protons at 1.49 ppm were attributed to 2-methyl-butirryl moiety (**e**). All these assignments were confirmed by HMQC experiment (see Table 2) [8–11]. Finally, the methylene and methyne protons of isovalerionyl moiety (**f**) were evidenced by their cross-peaks in the COSY experiments at 2.17 and 2.25 ppm and 2.04 ppm, respectively and confirmed by the ^{13}C resonances obtained by the connectivities in the HMQC experiments (see Table 2) [8–11].

3.3. Polyketides

The signals of polyketides were evidenced by a set of correlated peaks in the COSY (Fig. 3) and HMQC (Fig. 6) spectra. These constituents are characterised by the presence of olefinic methine and aliphatic methylene protons resonances (CH=CH–CH_2–CH=CH) at 5.0–5.3 ppm and 2.72 ppm, respectively [12–14]. Thus these signals showed cross-peaks in the COSY experiments and correlated with the resonances at 124.0–127.3 ppm and 26.2 ppm in the HMQC spectra, respectively. The presence of CH=CH–CH_2–CO moieties were also evidenced by the cross-peak signals in the COSY spectra of the resonances at 5.0–5.3 ppm and the signal at 2.23 ppm. The proposed moieties were confirmed by HMQC experiments (Table 2).

The signals in the zone between 5.0 and 5.3 ppm also showed cross-peaks in the COSY spectra with a resonance of the carbinolic region at 3.94 ppm and evidenced the presence of the fol-

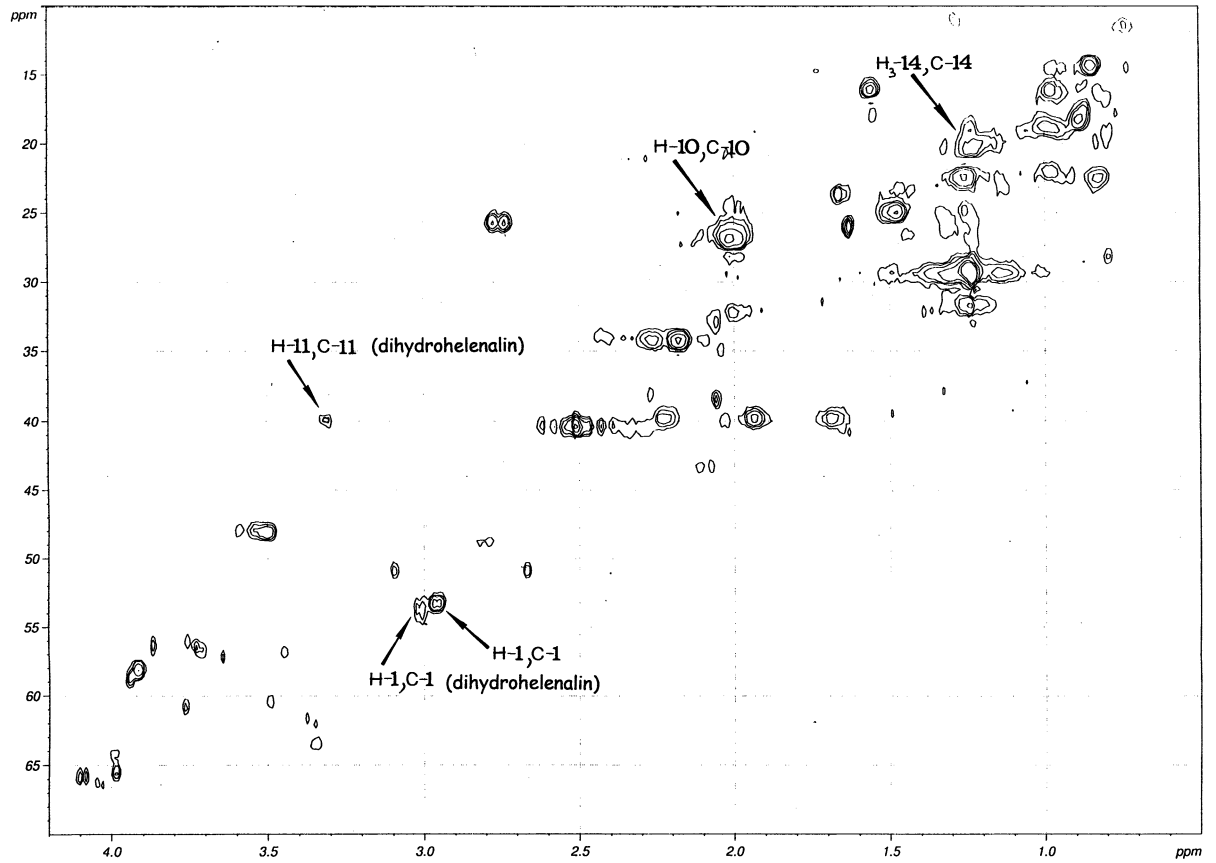

Fig. 6. Expanded region of the $^{1}H-^{13}C$ HMQC experiment: 0.5–4.0 ppm. Cross peaks of some resonances of helenalin and dihydrohelenalin derivatives are reported.

lowing moiety: =HC–CH–O–. The resonance at 3.94 ppm correlated in the HMQC spectra with the carbon resonance at 58.0 ppm and confirmed the above suggestion. Finally, a terminal vinyl moiety (=CH_2) was evidenced in HMQC spectra by the cross-peak between the proton signal at 4.68 ppm and the carbon signal at 107.1 ppm [12–14].

3.4. Other constituents

NMR signals due to aromatic derivatives were easily seen by the characteristic ^1H–^1H COSY connectivities of signals at 7.31, 7.09 and 6.94 ppm. These signals also showed long range connectivities with protons at 2.26 ppm due to aliphatic protons. The presence of glycerol derivatives was confirmed by the characteristic HMQC connectivities of carbinolic protons at 4.09 and 4.50 with the ^{13}C resonance at 65.5 (see Table 2) [12].

In order to verify the validity of all these findings, high performance liquid chromatography (not reported) analysis was also carried out. Peaks of the chromatogram were similar, with a very high content of sesquiterpenes (about 9% w/w) in the innovative CO_2 extract.

In this investigation high resolution NMR spectroscopy is used for the analysis of a new innovative commercial extract, the supercritical carbon dioxide arnica extract.

Many functional groups can be easily and conclusively identified by their characteristic ^1H and/or ^{13}C chemical shifts, determined by detailed analysis of 2D experiments and compared with the literature data. As expected, due to the type of process of extraction, no signals of solvents were evidenced. From these NMR results, especially two-dimensional experiments, the technique can be considered as a valid alternative method to obtain a fingerprint for the assurance of content and, therefore, safety and efficacy of innovative extracts of herbal drugs.

As previously evidenced [1,2], NMR experiments can provide a real and complete fingerprint of the extract, as required especially for the innovative ones due to the lack of pre-treatment, the tremendous versatility, not depending on the nature of the extract.

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