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Characterization of Commercial Kava-Kava Herbal Drug and Herbal Drug Preparations by Means of Nuclear Magnetic Resonance Spectroscopy

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The efficiency of one- and two-dimensional NMR experiments in characterizing the content of the constituents of both herbal drugs and herbal drug preparations is demonstrated for kava-kava. These experiments directly detect active constituents represented by kavalactones in both a finely powdered herbal drug and a commercial extract. In addition, NMR spectroscopy can detect all other compounds present in the extract. As previously evidenced, NMR experiments can represent a generally applicable technique for rapid screening and are a complement to the classical analytical techniques such as high-performance thin-layer chromatography, high-performance liquid chromatography, capillary gas chromatography, and electrophoresis. These experiments can be considered a very simple and fast analytical method to obtain a fingerprint of the herbal drugs and their preparations, and to quantify the content of the active principles of the extract.

KEYWORDS: Kava-kava; *Piper methysticum* G. Forst.; commercial herbal drugs and extracts; 1D-NMR and 2D-NMR experiments; constituent content characterization and quantification

INTRODUCTION

In recent years, an extensive use of herbal drugs (HDs), herbal drug preparations (HDPs), and herbal medicinal products (HMPs) in all countries of Europe has pointed out the need for overall appropriate methods of assay for a rapid screening and, in a few cases, for specific measurements of these commercial phytochemicals. In the European Pharmacopoeia the analysis of herbal drug constituents is generally achieved using UV spectroscopy and chromatographic techniques (TLC, HPLC, and GC). However, several problems arise from these techniques concerning the specificity and sensitivity of the method of revelation, time- and solvent-consuming separation, the accuracy, reproducibility, selectivity, and specificity of the method. In addition, generally crude extracts need a prepurification and thus face the possibility of losing some constituents, whereas herbal drugs need elaborate and tedious sample preparation with many steps to purify the constituents to be analyzed. Moreover, all these techniques cannot reveal eventual unknown plant metabolites that can also be present and may contribute to the biological activity of the phytochemicals, or other unexpected compounds.

In continuing our studies on NMR analysis of plant extracts (*1*), we now report the analysis of both a commercial herbal drug and an extract of kava-kava (*Piper methysticum* G. Forst.). Phytochemicals based on kava-kava are sold worldwide for the treatment of nervous anxiety, tension, and restlessness (*2*−*4*). The Deutscher Arzneimittel Codex (*5*) reports a 3.5% kavalactones content expressed as kavain. Commercial high-quality kava rhizome contains from 5.5 to 8.3% kavalactones, and kava-kava extracts contain from 30 to 70% kavalactones. They are represented by a mixture of more than 18 different α-pyrones, and the major constituents (**Figure 1**) are kavain, methysticin, demethoxyyangonin, yangonin, dihydrokavain, dihydromethysticin, and 5,6-dehydromethysticin (*6*−*11*). These kavalactones are characterized by different double-bond linkage patterns in positions 5,6 and 7,8, and so their pyrone moieties can be summarized by structures A−C as reported in **Figure 1**. In the literature (*12*, *13*) it is reported that the activity of kavalactones is related to the 5,6 and 7,8 unsaturations, so it is very important to quantify the content of each kavalactone group. On the other hand, it seems that the substitution patterns of aromatic rings do not influence their activity.

The aim of this work is to demonstrate that NMR spectroscopy is a suitable quali-quantitative analytical tool for HDs, HDPs, and HMPs composition without requiring fractionation or isolation steps.

MATERIALS AND METHODS

Solvents and Reagents. DMSO-d_6 (99.8%) was purchased from Euriso-top (Groupe CEA C. E Saclay Bât 547 91191 Gif-Sur-Yvette, France). Methanol was HPLC grade from Merck (Darmstadt, Germany), and 85% formic acid was provided by Carlo Erba (Milan, Italy). Water

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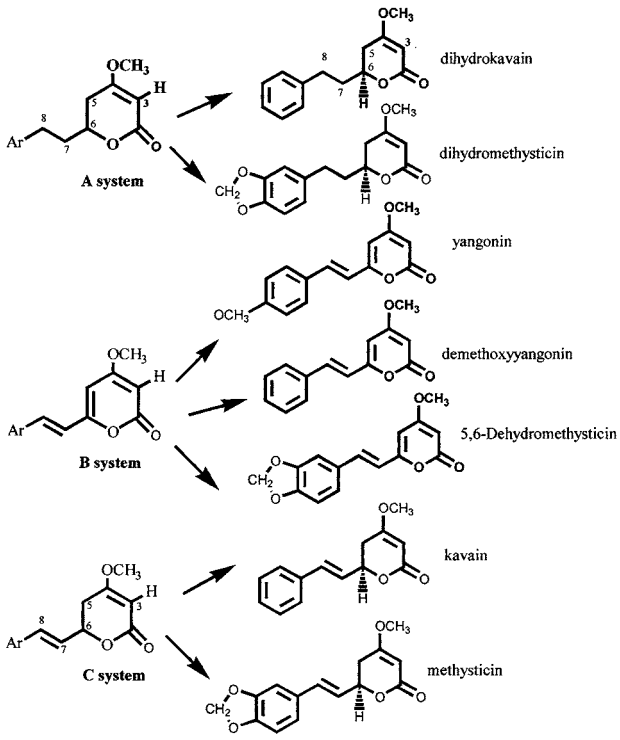


Figure 1. Structures of kavalactones and structures of A–C moieties.

was purified by a Milli-Q_{plus} system from Millipore (Milford, MA). CH_2Cl_2, hexane, and ethyl acetate (96%) were from Riedel-de Haen Rdh Soherchemikalien Gmbh & Co. KG Seelze, Germany. Maleic acid (98.6%) was purchased from Sigma-Aldrich (Fluka Chemicals, Sigma-Aldrich Division, Milano, Italy).

Plant Material. A commercial extract of kava-kava (lyophilized extract, lot 9A3847), and kava-kava finely powdered HD (lot L. 98/13353/1) were kindly offered by Aboca S.p.A. (Sansepolcro, Arezzo, Italy).

Isolation of Five Kavalactones from the Kava Extract. The lyophilized extract (3 g) was suspended in 4 mL of a solution of CH_2Cl_2/CH_3OH 1:1 and purified by chromatographic column (Silica 9385, Kieselgel 60 Merck) at room temperature. The elution mixture was hexane/ethyl acetate from 95:5 to 70:30. The fractions were collected, evaporated, and crystallized from hexane to obtain the yellow or white solids that were identified by HPLC/DAD/MS as methysticin, 7,8-dihydromethysticin, kavain, yangonin, and demethoxyyangonin. Kavain was recrystallized from hexanes−ethyl acetate to obtain a pure compound (pf 110 °C, >99%) which was used as external standard for the HPLC analysis.

NMR Apparatus and Parameters. The ^{1}H, COSY, HMQC, and TOCSY 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 were prepared by dissolving crude extracts in deuterated dimethyl sulfoxide without other treatment (50 mg/0.8 mL) and the solvent signal was used for spectral calibration (^{1}H: 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 was 4, spectral width was 7440 Hz, FID resolution was 0.23 Hz, achieved with 1 level of zero filling, acquisition time was 2.2 s, relaxation delay was 1 s. Processing parameters were as follows: number of points, 64K; line broadening, 3 Hz; FFT.

The COSY experiments were acquired with an internal pulse sequence for 2D homonuclear shift correlation, using gradient pulses for selection, 90 degree, absolute-value COSY experiment. Acquisition parameters were as follows: time domain size 2K; number of

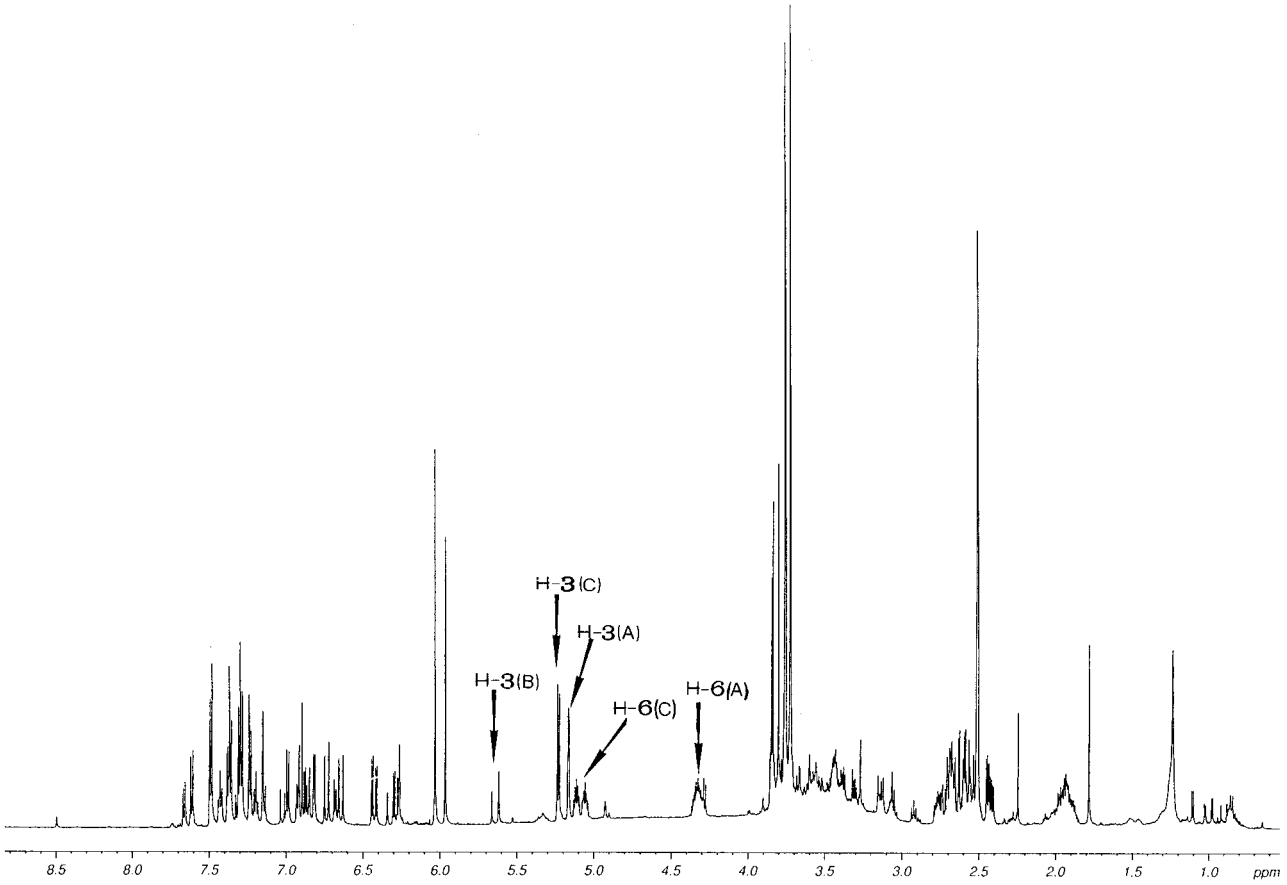
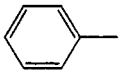
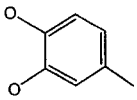


Figure 2. 600 MHz ^{1}H NMR spectrum of kava-kava commercial extract.

Table 1. Resonance Assignments with Chemical Shift of Constituents Identified in 600 MHz ^{1}H and ^{1}H–^{13}C NMR Spectra of *Piper methysticum* L. Dried Extract

Compound	^{1}H shift (δ,ppm)	Assignment	Observed	^{13}C shift (δ,ppm)
Kavalactones				
Pyrone A	5.16	H-3	1D, COSY, TOCSY, 1D-TOCSY, HMQC	91.8
Pyrone A	2.43 and 2.54	H-5b, H-5a	1D, COSY, TOCSY, 1D-TOCSY, HMQC	33.8
Pyrone A	4.35	H-6	1D, COSY, TOCSY, 1D-TOCSY, HMQC	75.8
Pyrone A	3.73	OCH_3	1D-TOCSY, HMQC	57.5
Pyrone B	5.61, 5.65	H-3	1D, COSY, TOCSY, 1D-TOCSY, HMQC	88.3, 89.6
Pyrone B ,	6.26, 6.33	H-5	1D, COSY, TOCSY, 1D-TOCSY, HMQC	102.0, 102.5
Pyrone C	5.22, 5.23	H-3	1D, COSY, TOCSY, 1D-TOCSY, HMQC	92.0, 92.2
Pyrone C	2.59-2.70	H-5	1D, COSY, TOCSY, 1D-TOCSY, HMQC	32.0,33.6
Pyrone C	5.07, 5.11	H-6	1D, COSY, TOCSY, 1D-TOCSY, HMQC	76.3, 76.4
Pyrone C	3.76	OCH_3	HMQC	57.5
Styryl of B structure	6.84, 6.90	H-7	1D, COSY, TOCSY, 1D-TOCSY, HMQC	118.6, 122.0
Styryl of B structure	7.27, 7.32	H-8	HMQC	135.0
Styryl of C structure	6.28, 6.42	H-7	HMQC	126.4, 128.3
Styryl of C structure	6.64, 6.74	H-8	HMQC	134.0
Styryl of A structure	1.95	H-7a, H-7b	COSY, HMQC	36.4
Styryl of A structure	2.71, 2.77	H-8a, H-8b	COSY, HMQC	32.3
Aromatic rings	7.50, 7.67		1D-TOCSY, HMQC	128.8, 128.3
	6.99, 7.61		1D-TOCSY, HMQC	117.2, 130.0
	6.68, 6.80		1D-TOCSY, HMQC	121.6, 116.0
	5.96, 6.03	O-CH_2-O	1D-TOCSY, HMQC	102.0, 102.2
Other constituents				
Lipids	0.85	CH_3-$(CH_2)_n$-	1D, COSY	
Lipids	1.22, 1.24, 1.28	-$(CH_2)_n$-	1D, HMQC	29.4, 29.1, 29.2
Methanol	3.09		1D, HMQC	54.0
Sucrose	5.18, 4.26	H-1 fructose and H-1 glc, respectively	1D, HMQC	91.8, 95.6

experiments 512; number of scans 2; dummy scans 16; and spectral width 7440 Hz in both dimensions. FID resolution was 7.3 Hz in F2 and 14.5 Hz in F1, acquisition time was 138 ms, and relaxation delay was1 s.The number of points was 1024 and 512, and the filter function applied was squared sine bell in both dimensions, with magnitude calculation of phase along columns. No phase correction was applied along rows.

The HMQC experiments were acquired with an internal pulse sequence for 2D H-1/X correlation via heteronuclear zero and double quantum coherence; phase sensitive using TPPI with decoupling during acquisition; and peak type selection using gradient pulses with coherence selection step after t 1. Acquisition parameters were the following: 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 7.3 Hz in F2 and 147.400 Hz in F1, acquisition time was 138 ms, F1 (^{13}C) at 110 ppm, relaxation delay 1 s. Processing parameters were number of points 1024 and 512; filter function applied 60 degree, squared sine.

Selective TOCSY experiments were performed using the eburp 1/25 selective pulse shape program (PW = 200, TRIMPWR = 54) and acquired as 16K datapoints (SW = 4400 Hz, AQ = 2.00 s). Offline data processing was performed using the manufacturer's NMR data processing software (Bruker WinNMR packages).

Internal Standard Solution. A sample (30 mg) of pure maleic acid (98.6%) was dissolved in 50 mL of DMSO-d_6. A 0.5-mL aliquot of this solution (corresponding to 0.30 mg of maleic acid) was added to 29 mg of kava extract prior to recording of the NMR spectrum.

Quantitation. After recording of the NMR spectrum and its integration, the concentrations of systems A, B, and C were expressed as kavain and calculated using the following equation:

$$\text{Concentration \%} = \frac{\text{area}(K) \times \text{MG}(K) \times \text{W(IS)}}{\text{area(IS)} \times 58.02 \times M} \times 100$$

where area(K) = the peak area of the kava system of interest; area(IS) = the peak area of the internal standard (maleic acid); MG(K) = kavain molecular weight; W(IS) = the net weight of the internal standard added to the sample; and M = weight of the dried extract (mg). The constant (58.02) is derived from the molecular weight of maleic acid divided by two.

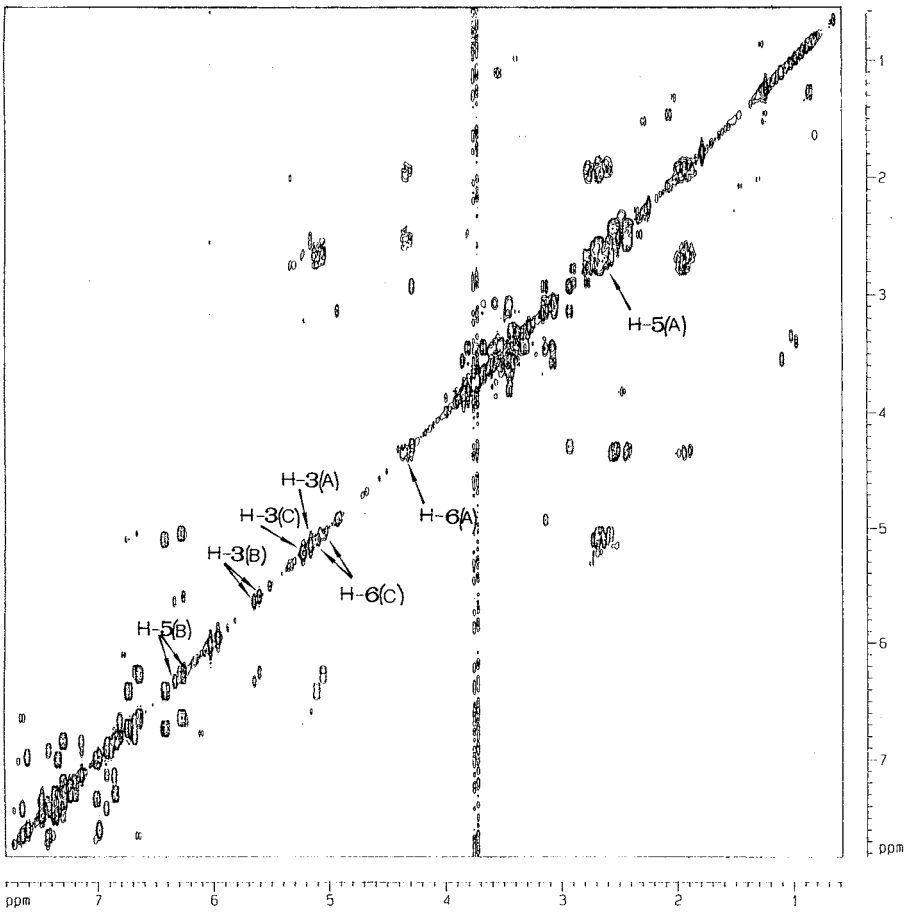


Figure 3. ^{1}H–^{1}H COSY spectrum of kava-kava commercial extract.

Reproducibility of Integration Process. An FID of a kava kava crude extract (with added internal standard) was Fourier transformed and integrated five times.

Reproducibility of the Recording and Integration Process. A sample of kava kava crude extract (with added internal standard) was analyzed, and the spectrum was Fourier transformed and integrated seven times, all on the same day.

HPLC−DAD and HPLC−MS Apparatus and Methods. The identification of constituents was performed using combined HPLC−diode array detection (DAD) analysis and HPLC−thermospray mass spectrometry.

The HPLC system consisted of a HP 1100L instrument with a diode array detector and managed by a HP 9000 workstation (Hewlett-Packard, Palo Alto, CA). The reverse-phase column was a 201 TP 54 RP-18 (250 mm × 4.6 mm, 5 μm, 300 Å, Vydac Separation Group, Hesperia, CA) maintained at 30 °C. The mobile phase was a seven-step linear solvent gradient CH_3OH/H_2O (pH 3.2, HCOOH) during a 40-min period at a flow rate of 0.8 mL/min. The mobile phase was as follows: 0 min, H_2O/CH_3OH 70:30; 5.0 min, H_2O/CH_3OH 60:40; 10.0 min, H_2O/CH_3OH 50:50; 15 mi, H_2O/CH_3OH 45:55; 35 min, H_2O/CH_3OH 40:60; 40 min, H_2O/CH_3OH 30:70. Before the HPLC analysis, each sample was filtered through a cartridge-type sample filtration unit with a poly(tetrafluoroethylene) (PTFE) membrane (d = 13 mm, porosity 0.45 μm, Lida manufacturing Corp.) and immediately injected. The injected volume of sample (3 mg/mL with respect to the dried extract) was 10 μL of solution. UV−vis spectra were recorded in the range 200−590 nm, and chromatograms were acquired at 240, 254, 270, 350, and 590 nm. Quantification was performed using kavain as external standard.

The HPLC system was interfaced with a HP 1100 MSD API-electrospray (Hewlett-Packard, Palo Alto, CA). 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 optimized in order to achieve maximum sensitivity values: gas temperature of 350 °C at a flow rate of 10 L/min, nebulizer pressure 30 psi, quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from m/z 100 to 800 in the negative and positive ion mode were obtained (scan time 1 s).

RESULTS AND DISCUSSION

In this work NMR spectroscopy is proposed as an alternative and integrative, nonchromatographic method for the quality assessment of a commercial extract and an herbal drug of kava-kava (*Piper methysticum* G. Forst.). Generally, kava-kava preparations are analyzed by HPLC and capillary GC, using mainly retention time (R_t) among the spectroscopic characteristics of the constituents (*14*−*18*). In general, the crucial point in these analyses is to find appropriate standards for the quantification of active constituents, and also, adequate conditions of elution and suitable columns. In addition, eventual unexpected or unknown constituents also may be present but are not detected because of the selectivity of the analytical method developed.

As a first step in our investigation, a qualitative NMR analysis of the kava kava extract was performed by dissolving it in hexadeuterated dimethyl sulfoxide. Proton spectrum and 2D-NMR experiments were carried out according to the methods reported in the experimental part of the present work.

Many functional groups were easily and conclusively identified by their characteristic ^{1}H and/or ^{13}C chemical shifts, determined by detailed analysis of 2D-NMR experiments of isolated constituents (not reported) and compared with the reported literature data (*19*−*22*). In addition, a quantitative

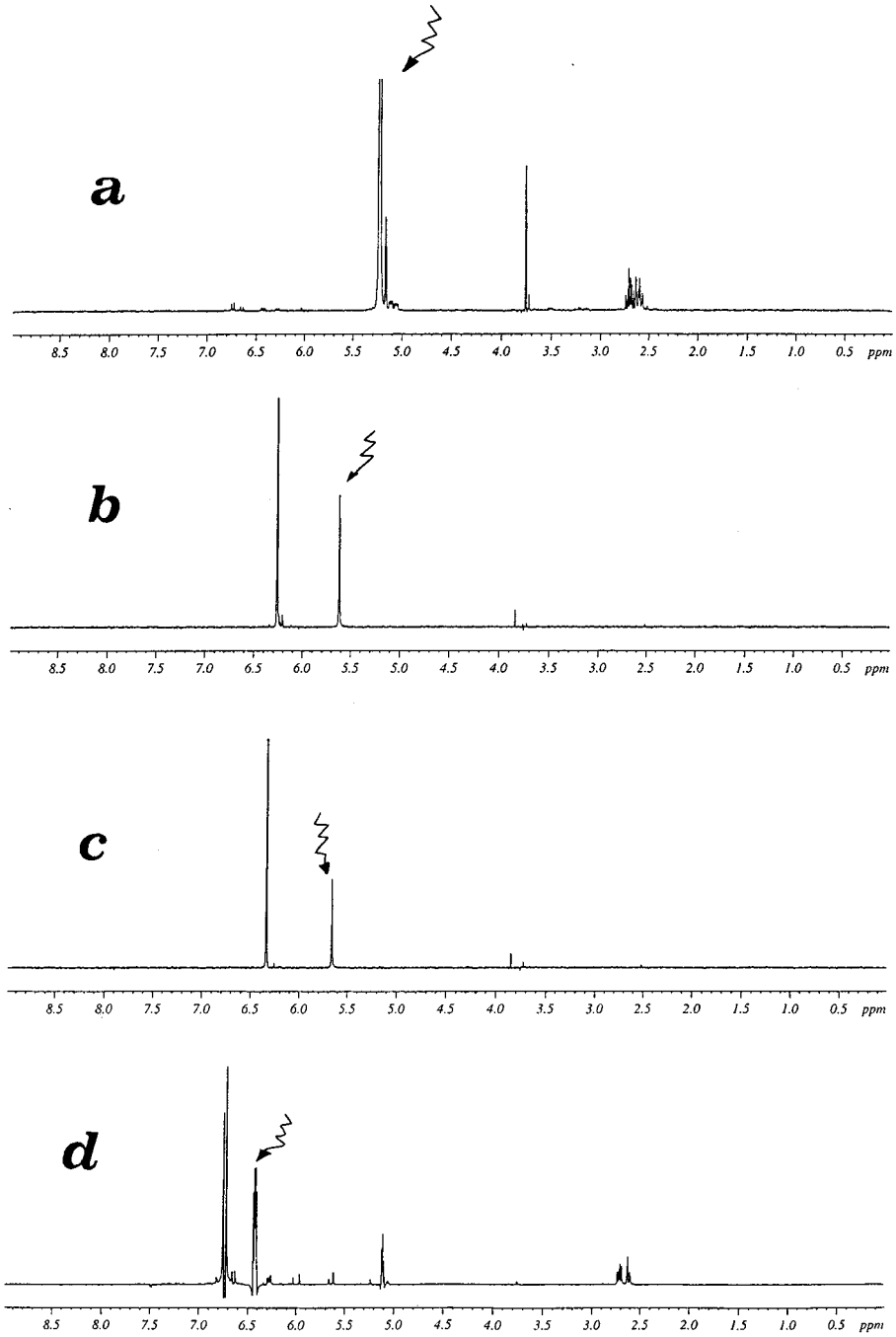


Figure 4. 1D-TOCSY obtained from selective irradiation of signal at (a) 5.22 ppm (H-3 of C moiety); (b) 5.61 ppm (H-3 of B moiety); (c) 5.65 ppm (H-3 of a second B system); and (d) 6.42 ppm (H-7 of C moiety).

analysis of kavalactones was also carried out using maleic acid as internal standard, and data obtained were also compared with those obtained by HPLC analysis.

In our experiments a number of typical kavalactones having different double bond patterns were observed and are reported in **Figure 1** as systems A−C. The A system is characterized by the absence of double-bond linkages in both the positions 5,6 and 7,8; B is a completely unsaturated system; and C has an unsaturation in the 7,8 position.

The ^{1}H NMR spectrum (**Figure 2**) was characterized by four main regions: a low-field region between 7.8 and 5.9 ppm with signals principally due to aromatic protons, olefinic protons of styryl moiety, conjugated olefinic protons, and protons of oxymethylene moieties; a mid-low-field region between 5.7 and 4.1 ppm with signals mainly due to olefinic and mainly carbinolic protons; a mid-high field region between 4.0 and 1.7 ppm with signals principally due to aliphatic protons of kavalactones and other carbinolic protons (mainly OMe of kavalactones); and the region lower than 1.7 ppm with signals probably due to aliphatic protons of lipids.

Signal Assignment in the Pyrone Moiety of the A−C Structures. In the analysis of the proton spectrum (**Figure 2**) it is quite easy to assign signals corresponding to protons 3 of A−C systems. Thus, the chemical shift of H-3 is in the region from 5.5 to 5.7 ppm for 5,6 unsaturated constituents (B system) and it is in the region from 5.1 to 5.3 ppm in 5,6 saturated ones (A and C systems). The signals corresponding to proton 6 were observed from 4.2 to 4.4 ppm in 7,8 saturated constituents (A system) and from 5.0 to 5.3 ppm in 7,8 unsaturated ones (C system) (*20*−*22*, **Table 1**).

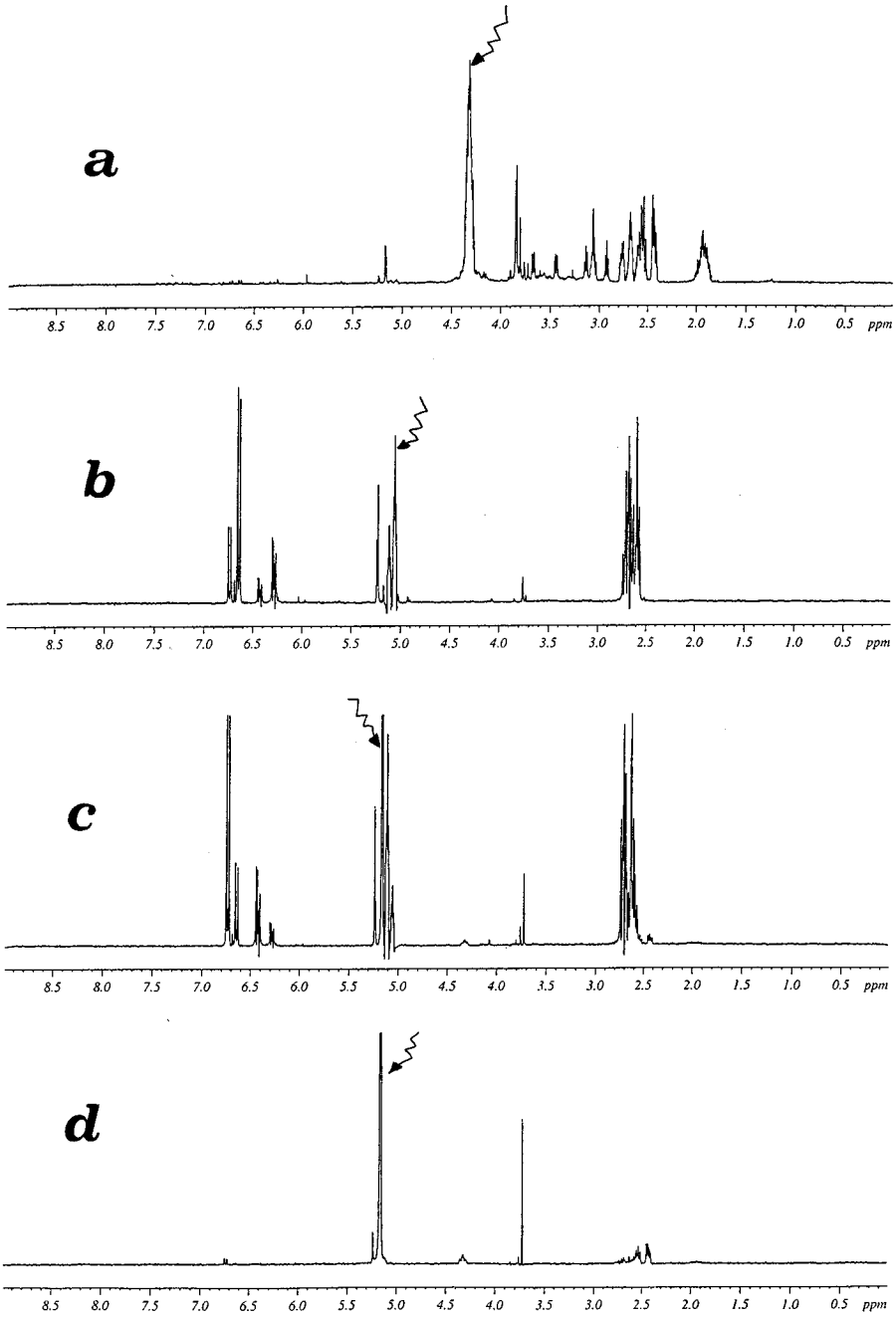


Figure 5. 1D-TOCSY obtained from selective irradiation of signal at (a) 4.35 ppm (H-6 of A moiety); (b) 5.07 ppm (H-6 of C moiety); (c) 5.11 ppm (H-6 of a second C moiety); and (d) 5.16 ppm (H-3 of A moiety).

In the fully unsaturated compounds (B derivatives) signals of H-3 of two B-derivatives were identified at 5.61 ppm ($J_{3,5}$ = 2.2 Hz) and 5.65 ppm ($J_{3,5}$ = 2.2 Hz). These signals are coupled with H-5 (see COSY, **Figure 3**) at 6.26 and 6.33 ppm, respectively (**Table 1**). Similar results were obtained by irradiation of H-3 signals at 5.61 ppm (**Figure 4b**) and at 5.65 ppm (**Figure 4c**).

H-3 of A and C systems were suggested by the signals at 5.16 (d, $J_{3,5a}$ = 14 Hz), 5.22 (br s), and 5.23 (d, $J_{3,5a}$ = 14 Hz). In the 2D-COSY experiments (**Figure 3**) the first signal was coupled with the signals at 2.43 (H-5b) and 2.54 (H-5a) ppm. These signals were also coupled with the multiplet at 4.35 ppm, assigned to H-6 of the A moiety, as also suggested by 1D-TOCSY (**Figures 4** and **5**). In addition, from the 1D-TOCSY experiments the resonances of the 4-OMe was evidenced at 3.73 ppm (**Figure 5d**). So the presence of the A moiety arose from the coupled signals at 5.16, 2.43, 2.54, and 4.35 ppm (**Table 1**).

The other two signals of H-3, i.e., the broad singlet at 5.22 and the doublet at 5.23 ppm, were assigned to the C moiety on the basis of the chemical shifts of the corresponding H-5 and H-6. Thus, the H-3 signals were coupled with protons in the region between 2.59 and 2.70 ppm (H-5) and they were also coupled with protons at 5.07 and 5.11 ppm (H-6) (**Table 1**). The 1D-TOCSY obtained from selective irradation of signal at 5.22 ppm (**Figures 4a**, **5b**, and **5c**) confirmed these results.

All these data were confirmed by the cross-peaks between carbon and proton resonances obtained by HMQC experiments. The degree of unsaturation and the double bond pattern in the kavalactones affect also the chemical shifts of C-2, C-3, C-4, and C-5 (*19*, **Table 1**). Thus, signals of H-3 of structures A

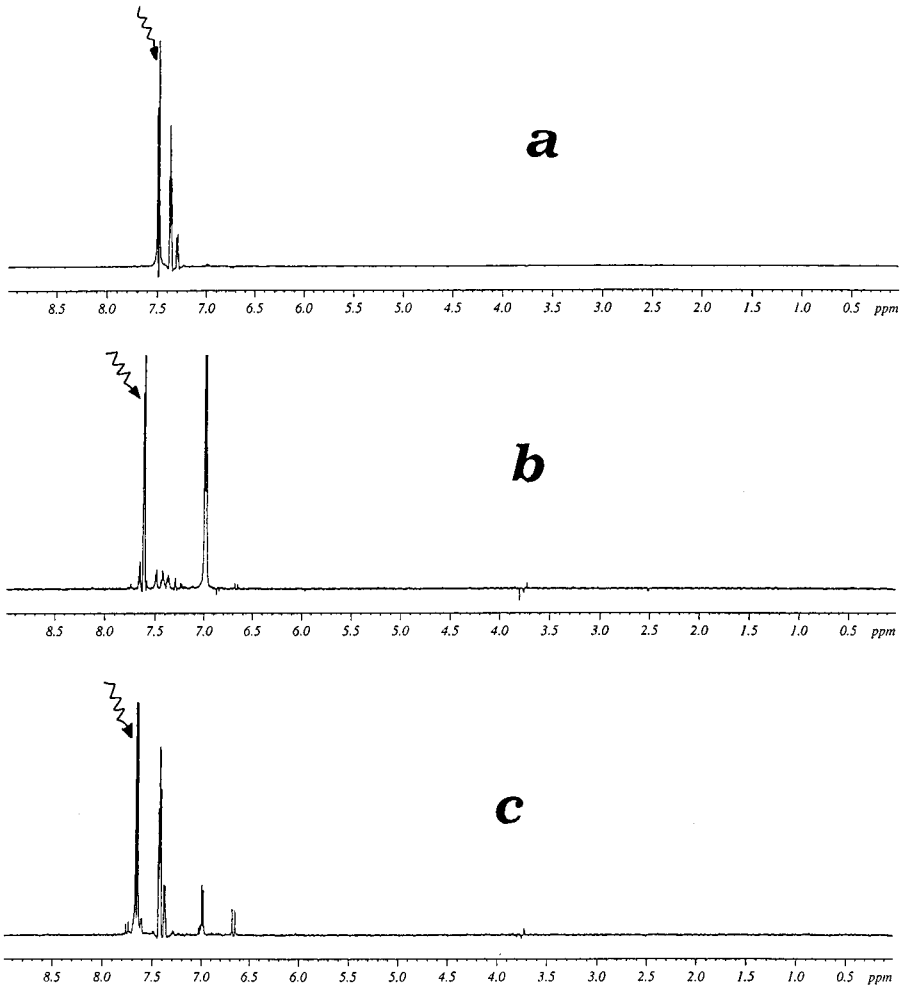


Figure 6. 1D-TOCSY obtained from selective irradiation of signal at (a) 7.50 ppm, (b) 7.61 ppm, and (c) 7.67 ppm. corresponding to aromatic protons.

and C correlated with the signals at 91.8, 92.0, and 92.2 ppm, respectively. Thus, signals of H-3 of ring B correlated with the resonances 88.3 and 89.6 ppm. The H-5 resonances of systems A and C showed cross-peaks with the signals at 33.8, 33.6, and 32.0, respectively. The H-5 resonances of system B showed cross-peaks with the signals at 102.0 and 102.5 ppm. The carbon resonances of the OMe were at about 57.5 ppm. H-6 of system A showed a cross-peak at 75.8 ppm, while H-6 of system C showed cross peaks at 76.3 and 76.4 ppm (**Table 1**).

Signal Assignment in the Styryl Moiety. The signals of 7,8 dihydro moieties were indicated by 1D-TOCSY experiments (**Figure 5a**). Thus, by selective irradiation of signal H-6 of system A at 4.35 ppm, the signal of H-3 was confirmed at 5.16 ppm; in addition, the signals of H-7a,b were identified at 1.95 ppm, and the signals at 2.71 and 2.77 ppm were attributed to H-8a and H-8b.

These data were confirmed by their couplings in the COSY experiments and the carbon resonances obtained by HMQC experiments (**Table 1**).

In the B and C systems, the degree of unsaturation of the pyrone moiety affected the chemical shift of H-7. Its value was less than 6.50 ppm in the 5,6 dihydro-derivatives (moiety C) and more than 6.50 ppm in the conjugated systems (moiety B) (*20*−*22*) (**Table 1**). Thus, the signal at 5.07 ppm (H-6 of C moiety) correlated in the TOCSY experiments with the resonances at 6.28 ppm (dd, H-7 of C moiety, $J_{6,7} = 6.4$ Hz; $J_{7,8} = 16.1$ Hz) and 6.64 ppm (d, H-8 of C moiety). The signal at 5.11 ppm (H-6 of a second C moiety) correlated with the signals at 6.42 ppm (dd, H-7, $J_{6,7} = 6.4$ Hz; $J_{7,8} = 16.9$ Hz) and 6.74 ppm (d, H-8) (**Table 1**). The data were confirmed by 1D-TOCSY experiments (**Figures 4d**, **5b** and **5c**).

In the styryl moiety of the B system, the H-8 protons are distinguished by their higher deshielded signals (7.27 and 7.32 ppm) due to their being in the therminal- rather than the penultimate-position of a triply conjugated, unsaturated system (**Table 1**). The H-7 protons are then identified (6.84 and 6.90 ppm) by their COSY correlations with the H-8 (**Table 1**).

These data were confirmed by HMQC experiments in which the resonances of H-7 correlated with the signals at 126.4 and 128.3 ppm, respectively, and the resonances of H-8 correlated with the signal at 134.0 ppm (**Table 1**).

1D-TOCSY experiments were also useful to attibute the substitution pattern of aromatic rings. Thus, the presence of two nonsubstituted aromatic rings was evidenced (**Figure 6a**, **6c**) by irradiating doublets at 7.50 ($J = 7.3$ Hz) and 7.67 ppm ($J = 7.8$ Hz) that showed the other aromatic signals between 7.30 and 7.45 ppm.

The presence of one *p*-substituted aromatic ring was observed by 1D-TOCSY by irradiation of the doublet at 7.61 ppm ($J = 8.8$ Hz) which gave the signal at 6.99 ppm (**Figure 6b**).

At the third aromatic moiety, thus the oxymethylene derivatives, was exhibited by comparing the NMR data with pure methysticin and dihydromethysticin, isolated from the extract by the authors. The ABX system was evidenced at 6.68 ppm, which correlated with signal at 6.80 ppm (**Table 1**). In the same

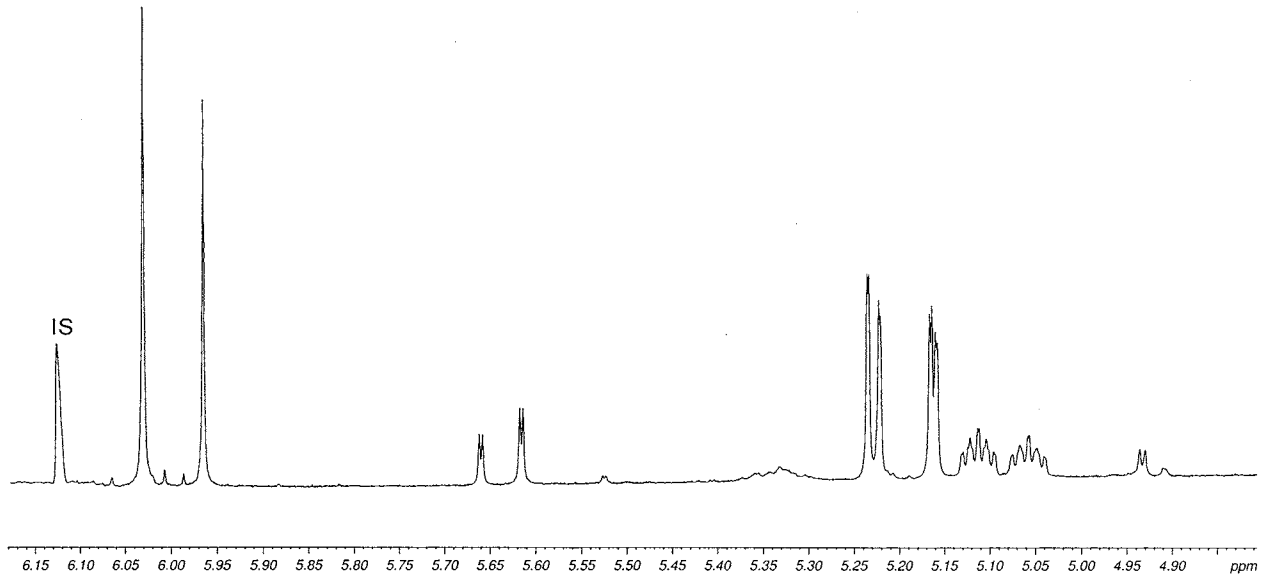


Figure 7. Expanded region of the ^{1}H NMR spectrum of kava-kava commercial extract; IS= internal standard-maleic acid.

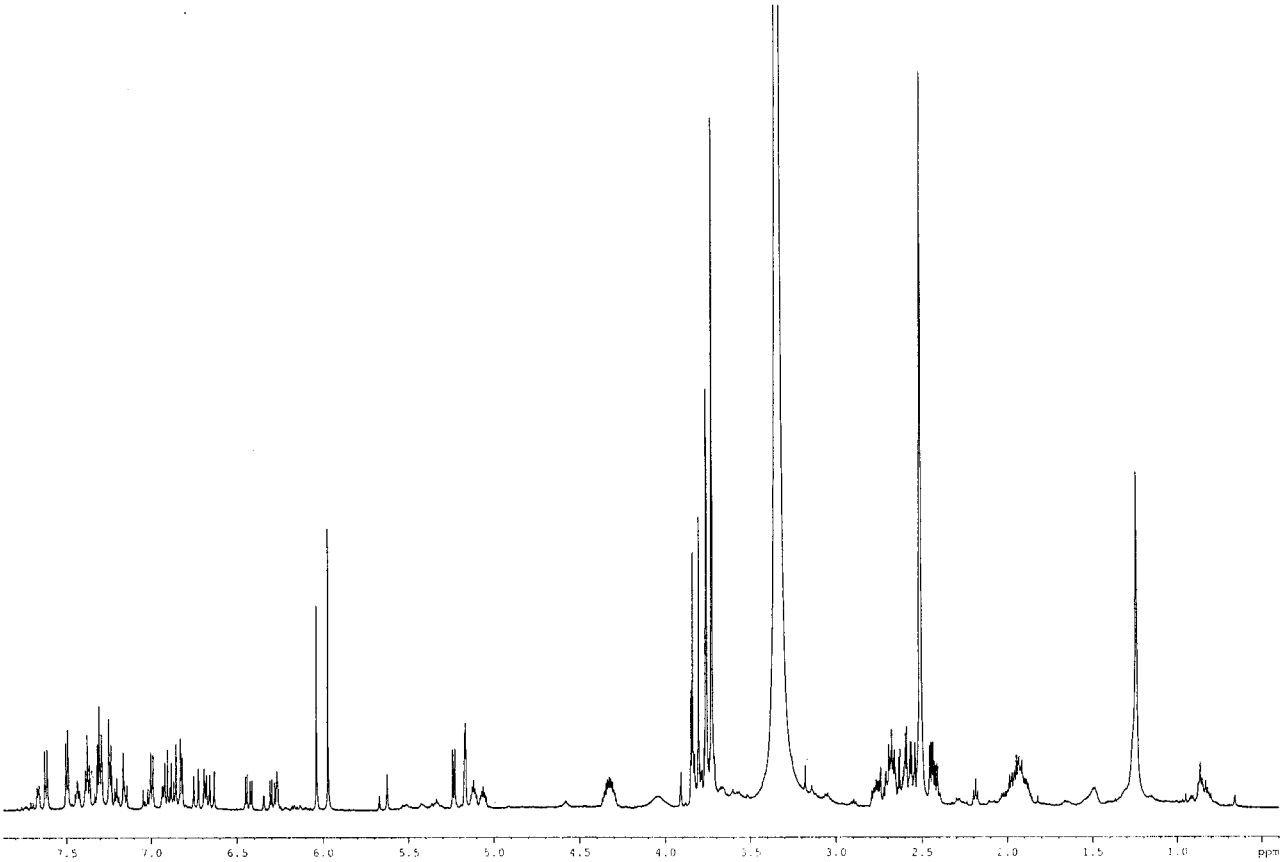


Figure 8. 600 MHz ^{1}H NMR spectrum of kava-kava finely powdered HD.

region were also present the signals of the ABX system of dihydromethysticin.

The presence of at least two ABX oximethylene derivatives was identified by two signals of oximethylenes at 6.03 ppm, probably attributed to methysticin, and at 5.96 ppm attributed to dihydromethysticin (**Table 1**).

From the above results were found the presence of at least one derivative with the A system, two derivatives of the B system, and two C systems.

The presence of at least two oxymethylene derivatives, two nonsubstituted aromatic rings, and one *p*-oxy aromatic system were evidenced by the NMR analysis.

By comparing all these data, in the dried extract of kava-kava were present at least the following kavalactones: kavain, methysticin (C system with nonsubstituted aromatic moiety and oxymethylene aromatic moiety respectively); 7,8-dihydromethysticin (A system with oxymethylene aromatic system); and dehydrokavain and yangonin (B system with nonsubstituted and *p*-methoxy aromatic ring).

Other Constituents. Fats were indicated by characteristic signals at 1.22, 1.24, and 1.28 ppm in the proton spectra which correlated in ^{13}C with the signals at 29.1, 29.2, and 29.4 ppm (*1*) (**Table 1**). Signals of residual solvents (probably acetone and others) were observed at 1.84 and 2.25 ppm in the proton

spectra. The presence of methanol arose by the signal at 3.09 which correlated with the signal at 54.0 ppm in the HMQC experiment. Sucrose was identified by the signals at 5.18 and 4.26 which correlated with the resonances at 91.8 and 95.6, respectively, in the HMQC experiment (*1*). Traces of chalcones were evidenced by the signals 6.08−6.20 (protons H-3′ and H-5′) and 7.50−7.90 (protons H-2, H-6, and H-α and H-*β*) (*23*).

As a second step in our investigation, we have applied the NMR analysis to quantify the content of kavalactones in the lyophilised extract related to A−C systems.

A solution of maleic acid in DMSO-d_6 was used as internal standard. The resonance of two protons of the maleic acid were identified at 6.12 ppm, not overlapped by the kavalactone protons (**Figure 7**).

The integration of the peaks in the ^{1}H NMR spectrum allowed quantification of the different structures of kavalactones, by comparison with of the areas corresponding to the signal of protons of maleic acid and signals of H-3 of A system, B systems, and C systems. Thus, the percentages were 6.9% for kavalactones having A system, 4.6% for kavalactones having B system, and 8.6% for kavalactones having C system. The total amount of kavalactones was 20.1%. These data were consistent with those found by HPLC−DAD analysis, i.e., 7.0, 4.3, and 8.9%, respectively. The total amount of kavalactones by HPLC was 20.2%.

The reproducibility of the different steps of the NMR quantitative method was determined. The average variation due to the integration process alone, expressed as standard deviation (SD), was approximately 0.096. The values obtained for the combined process of recording and integration were 0.106 for system A, 0.539 for system B, and 0.198 for system C.

As a final step of our investigation, the suitability of NMR analysis in the evaluation of quality and identity of HDs was investigated through the analysis of the sample of a commercial finely powdered HD.

The HD was directly treated with DMSO-d_6 (50 mg/0.8 mL), and, after filtration of the powder, the DMSO-d_6 solution was directly analyzed by NMR. By comparing the two spectra (**Figures 2** and **8**), the NMR profiles were really similar. By the analysis of the low-field region between 7.8 and 5.9 ppm, no significant qualitative variations were noted. Only the relative ratio of a few signals was changed, such as oxymethylenes that were in reverse order (**Figure 2**). The mid−low field region (5.7−4.1 ppm) of both samples was similar too, with the presence of two new signals at 5.30 and 4.52 ppm probably due to olefinic moieties of long-chain fats.

The upfield signals from 3.6 to 3.0 ppm were submerged by the signal of water that was extracted from the herbal drug during the treatment with DMSO. Finally, the resonances between 2.8 and 1.8 ppm resulted superimposed with those of the dried extract, with a higher amount of fats.

The presence of the kavalactones observed by NMR experiments was also confirmed by HPLC−DAD−MS analysis (not reported) using a method developed by the authors.

CONCLUSIONS

As previously reported, NMR experiments can represent a generally applicable technique for rapid screening and a complementary technique to the classic high-performance thin-layer chromatography, high-performance liquid chromatography, and capillary gas chromatography analyses. NMR spectroscopy is a suitable analytical tool for HDPs and HMPs composition without requiring fractionation or isolation steps, as previously demonstrated, but it can be also used to analyze HDs. This is the first report of its use in the direct analysis of an herbal drug. DMSO is a suitable and general solvent to completly dissolve metabolites from the herbal drugs, as demonstrated by the absence of metabolites in the chromatograms obtained by HPLC analysis of the residual powder.

In both kava-kava extract and herbal drug it is also possible to evaluate the relative ratio among the different derivatives (A−C) which are related to different activities, and by using an external standard (such as maleic acid), it was also possible to perform a quantitative analysis of the crude extract, and data were in agreement with those obtained by HPLC.

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