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Analysis and stability of the constituents of *Curcuma longa* and *Harpagophytum procumbens* tinctures by HPLC-DAD and HPLC–ESI-MS

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ABSTRACT

In the present study two methods based on liquid chromatography with diode array detection (HPLC-DAD) coupled to an electrospray ionisation (ESI-MS) interface were developed for the determination of constituents in the tinctures (60%, v/v, DER 1:5) of turmeric (*Curcuma longa* L.) and Devil's claw (*Harpagophytum procumbens* L.). The developed simple and effective assays permitted the quality control of both tinctures. The aim of this work was to assess the qualitative and quantitative profile of the constituents of two widely marketed commercial preparations and to evaluate chemical stability of their marker constituents during accelerated thermal stability test by HPLC analysis. Characteristic constituents of *C. longa* rhizomes are the curcuminoids, whereas characteristic constituents of *H. procumbens* are acylated iridoid glycosides and phenylethylalcohols. Constituents of Devil's claw tincture (mainly iridoids) were more stable than curcuminoids of turmeric.

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1. Introduction

Tinctures are liquid preparations obtained by maceration or percolation, using as extraction solvent ethanol of a suitable concentration. According to the monograph of European Pharmacopoeia drug-extract ratio (DER) is 1:5 or 1:10 [1]. Although they represent the most used pharmaceutical forms of plant origin being both Herbal Drug Preparation (HDP) and Herbal Medicinal Product (HMP), little is known about their stability (the European Pharmacopoeia general monograph on tinctures only suggest their protection from light) and analytical procedures for their quality control. According to the ICH guidelines referred to other pharmaceutical preparations, a maximum limit of the loss of the active or marker constituents at 5–10% is acceptable to guarantee their safety and efficacy, and a validated stability-indicating testing method is required in order to perform such estimation [2].

In continuing our studies [3–5] on the constituents and stability of herbal drug preparations, two commercial widely used tinctures were evaluated for their qualitative and quantitative control and thermal stability testing: *Curcuma longa* L. and *Harpagophytum procumbens* L. Tinctures based on *C. longa* are used for the symptomatic relief of dyspepsia [6]. Characteristic constituents responsible for the therapeutic effect are the curcuminoids, namely curcumin, desmethoxycurcumin and bisdesmethoxycurcumin [7]. Tinctures of *H. procumbens* (Devil's claw) are prescribed for loss of appetite [8] and mainly for the relief of low back pain [9] (ESCOP). The pharmacologically active components of *H. procumbens* are considered to be the iridoid glycosides, other constituents, such as phenylethanoid glycosides and flavonoids are considered to contribute to the pharmacological action [10].

To the best of our knowledge this is the first report on the analysis and stability of both these two widely used tinctures.

2. Material and methods

2.1. Plant materials, tincture and sample preparation

C. longa L. (dried ground rhizomes, lot no. D-270607230707), *H. procumbens* L. (dried roots, lot no. D-150306160306), were purchased by A. Minardi & Figli s.r.l. Bagnacavallo (RA), Italy. Tinctures were commercial preparations prepared in the Officina Plantarum laboratories, Impruneta (FI)-Tuscany, and were obtained according to the European Pharmacopoeia [1]. A 100 g of dried plant material were macerated for a minimum period of 21 days, using (60%, v/v) hydro-alcoholic solutions to obtain 500 ml of tincture. The herbal drug was separated by the tincture by filtration and was mechanically pressed. The tincture was filtered and adjusted according to European Pharmacopoeia to a ratio of 1:5 (dry plant material:tincture).

Stability testing was performed according to ICH guidelines. Batches (about 10 ml) of each tincture were stored in brown glass in an oven. They were analyzed on the day of preparation and fort-

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Table 1
Mobile-phase composition used for the HPLC-DAD and HPLC–MS analysis of *Curcuma longa* and *Harpagophytum procumbens* tinctures.

C. longa			*H. procumbens*		
Time (min)	H_2O/HCOOH (A)	CH_3CN (B)	Time (min)	H_2O/HCOOH (A)	CH_3CN (B)
0	82%	18%	0	5%	95%
21	73%	27%	5	15%	85%
25	56%	44%	8	24%	76%
27	52%	48%	15	25%	75%
32	20%	80%	19	27%	73%
42	15%	85%	24	29%	71%
			29	50%	50%

nightly during a 6-month period for accelerated testing at 40 °C. HPLC determinations were performed in triplicate on each sample after centrifugation. *C. longa* tinctures were analyzed as such. After a preliminary HPLC-DAD analysis the tincture of *H. procumbens* showed to contain extremely high amounts of analytes and therefore all analyses were conducted in diluted samples (1:5) in methanol.

2.2. Chemicals

All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were purchased from Merck (Darmstadt, Germany). Formic acid (85%, v/v) was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Qplus system from Millipore (Milford, MA, USA). For the filtration of samples 0.45 μm PTFE membrane filters were used which were purchased from Waters Co. (Milford, MA). All laboratory chemicals used in this study were of reagent grade.

2.3. Standards

For the quantitative analysis the following standards were used: verbascoside was an isolated commercial compound from IRB (Istituto di Ricerche Biotecnologiche) of Altavilla Vicentina further purified by HPLC in our lab and its purity was estimated to be over 98% by HPLC and NMR. Curcumin and harpagoside were purchased from Extrasynthèse (purity more than 98%, checked by HPLC and NMR). For the qualitative analysis the following standards used: 8-O-feruloyl-harpagide was an isolate from the tincture as described in Section 2.5; leucosceptoside A was an isolate from *Marrubium velutinum* [11].

2.4. Stability study

The thermal stability testing was carried out in triplicate for accelerated testing at temperature conditions: at +40 ± 2 °C (75 ± 5% RH). The climatic chamber employed was a CC700 model (Piardi, Italia).

2.5. Isolation of the characteristic constituents

Part of the *H. procumbens* tincture (100 ml) was concentrated under reduced pressure to eliminate the EtOH and then partitioned (in triplicate) with equal volumes (50 ml) of chloroform and ethylacetate to obtain three major fractions: HP_1 (chloroform), HP_2 (ethylacetate) and HP_3 (aqueous). The chloroform fraction contained mainly the caffeic acid ethylester (ethyl caffeate) which was further subjected to size exclusion chromatography over a Sephadex LH-20 (Sigma–Aldrich, Amersham, Sweden) column (20 cm × 1.5 cm), using a mixture of MeOH:H_2O 50:50 to afford pure caffeic acid ethylester. NMR (^{1}H NMR, COSY) spectroscopic analysis confirmed the structure [12]. Fraction HP_2 contained mainly harpagoside, as confirmed by HPLC-DAD–ESI-MS analysis and co-chromatography with the reference sample. Part of the aqueous phase (fraction HP_3, 2 g) was also subjected to Sephadex LH-20 column chromatography using a gradient elution system which consisted of mixtures H_2O:MeOH (90:10 → 60:40) and led to the isolation of three iridoid derivatives, namely harpagide [13] (6.3 mg), harpagoside (12.7 mg) [13], 8-*E*-p-coumaroyl-harpagide (9.6 mg) [14], the iridoid derivative, pagoside (4.3 mg) [15], a mixture of pagoside and methoxy-pagoside (see Table 3 and Section 3.2.1), and 3 phenylethylalcohols, decaffeoylverbascoside (2.4 mg) [16], verbascoside (15.2 mg) [17] and isoverbascoside (23.5 mg) [18]. A flowchart of the separation is demonstrated in Supplementary material. All fractionations were monitored by TLC analysis and complemented by HPLC-UV-DAD or HPLC-UV-DAD–MS analysis where necessary. The structural identification of the constituents was carried out by ^{1}H- and 2D NMR (where necessary, see Table 3) and comparison of their spectral data with those reported in the literature.

2.6. Other analytical methods

2.6.1. TLC analysis

TLC analysis of the fractionation of the *H. procumbens* tincture was carried out on Merck plates pre-coated with silica gel 60 F_{254} (Art.5554) using EtOAc–HCOOH–H_2O (8:2:2) as elution system. Detection was done by using vanillin/H_2SO_4 spray reagent consisting of equal volumes of 5%, (w/v) solution of vanillin (Fluka) in ethanol (A) and 5% (v/v) solution of H_2SO_4 in methanol (B) [19].

2.6.2. NMR analysis

Final checking of the purity of the isolated compounds which were used as standards was carried out by both LC-DAD–MS according to the method reported in Section 2.6 and by NMR spectroscopy. NMR spectroscopy was also used to elucidate the structure of all isolated constituents by means of 1D and 2D NMR (COSY, HSQC, HMBC, and ROESY) experiments. NMR spectra were recorded in CD_3OD on a Bruker DRX-400 instrument at 295 K. Chemical shifts are given in ppm (δ) and were referenced to the solvent signals at 3.31 and 49.5 ppm for ^{1}H and ^{13}C NMR, respectively.

2.7. HPLC apparatus

2.7.1. HPLC-DAD analysis instrumentation

The HPLC system consisted of a HP 1100 L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Agilent Techologies, Palo Alto, CA, USA). The column was a Luna RP-C18 Prepacked column (150 mm × 3 mm) with a particle size of 5 mm (Phenomenex) maintained at 26 °C. The eluents were H_2O at pH 3.2 by formic acid (A) and acetonitrile (B) with a flow rate of 0.4 ml/min. Timetables are reported in Table 1. Injected volume of the samples was 2 μl solution. The UV–vis spectra were recorded between 220 and 500 nm in both cases. For the *C. longa* tincture the chromatographic profiles were registered at 240, 280 and 420 nm, while for the *H. procumbens* tincture chromatographic profiles were registered at 240, 280, 310 and 330 nm.

Table 2
Positive and negative ion mode MS fragmentation (*m*/*z* values) and UV–vis absorption data of the compounds (C1–C11) detected in the tincture of *Curcuma longa*.

No	Rt (min)	UV (nm)	Negative	Positive	Identification	Mode of identification
C1	7.2	220, 284	**121** $[M-H]^-$	**123** $[M+H]^+$	p-OH-benzaldehyde	UV/MS + std
C2	7.7	226, 290sh, 310	**163** $[M-H]^-$	**165** $[M+H]^+$, 147	*E*-Coumaric acid	UV/MS + std
C3	7.9	230, 282, 310	**151** $[M-H]^-$	**153** $[M+H]^+$	Vanillin	UV/MS + std
C4	8.6	218, 236, 296sh, 324	**193** $[M-H]^-$	**195** $[M+H]^+$, 177	*E*-Ferulic acid	UV/MS + std
C5	27.9	248, 416	**307** $[M-H]^-$	**309** $[M+H]^+$	Bisdemethoxycurcumin	UV/MS
C6	28.8	252, 422	**337** $[M-H]^-$	**339** $[M+H]^+$	Demethoxycurcumin	UV/MS
C7	29.8	264, 428	**367** $[M-H]^-$	**369** $[M+H]^+$	Curcumin	UV/MS + std
C8	37.1	220, 238	–	**217** $[M+H]^+$, 119	ar-Turmerone	UV/MS
C9	39.2	236	–	**219** $[M+H]^+$, 121	α-Turmerone	UV/MS
C10	39.4	240, 268sh	–	**219** $[M+H]^+$, 121	β-Turmerone	UV/MS
C11	41.0	270	–	**219** $[M+H]^+$, 125	Curlone	UV/MS

std: standard; characters in bold correspond to pseudomolecular ions.

2.7.2. HPLC–MS analysis instrumentation

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, CA, USA). The same column, elution gradient and flow rate were used during the HPLC–MS analyses. Mass spectrometry operating conditions were optimised in order to achieve maximum sensitivity values: negative and positive ionisation mode, scan spectra from *m*/*z* 100 to 800, was used with a gas temperature of 350 °C, nitrogen flow rate of 10 l/min, nebulizer pressure 30 psi, gain 1.0, threshold 50, stepsize 0.1 and capillary voltage 3500 V. The applied fragmentors were in the range of 60–180 V.

2.8. Identification of peaks and peak purity

Identification of all constituents was performed by HPLC-DAD and MS analysis by comparing the retention time, the UV and MS spectra of the peaks in the samples with those of authentic reference samples or isolated compounds and in some cases data reported in the literature. A detailed presentation of the mode of identification is provided in Tables 2 and 3. The purity of peaks was checked by a Diode Array Detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic references samples and/or by examination of the MS spectra.

2.9. Linearity LOD, LOQ, precision and accuracy

The linearity range of responses of the standards was determined on ten concentration levels with three injections for each level. The method of external standard was used in both cases. For the preparation of the three stock solutions 1.18 mg of curcumin, 1.46 mg of verbascoside and 2.02 mg of harpagoside were exactly weighed and transferred to a volumetric flask of 2 ml to give stock solutions of 0.59, 0.73 and 1.01 mg/ml, respectively. Calibration graphs for HPLC were recorded using different dilutions of these stock solutions of standards (1:2 and 1:5 dilution for curcumin; 1:5 and 1:10 dilution for verbascoside; 1:5 dilution for harpagoside) with different injection volumes varying from 2 to 6 μl; LOQ were included in the calibration curves. For curcumin the linearity range was from 0.295×10^{-3} to 0.59 mg/ml, for harpagoside was 0.42×10^{-3} to 1.01 mg/ml and for verbascoside from 0.584×10^{-3} to 0.146 mg/ml. The limit of detection (LOD) and quantification (LOQ) under the chromatographic conditions were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD and 10 for LOQ. To evaluate the repeatability, six samples of each tincture were analyzed by HPLC. The contents of each constituent were evaluated to calculate the relative standard deviation (RSD). For the intra-day variability test, freshly prepared standard samples in the range of the calibration curve (final concentrations: curcumin standard solution 0.1193 mg/ml; harpagoside and verbascoside standard solution 0.3525 and 0.1195 mg/ml, respectively) were analyzed in six replicates within 1 day. For the inter-day variability test, the solutions were examined in triplicates for 3 consecutive days. For the accuracy test, the spiking method was applied. A known amount of freshly prepared standard solutions in three different concentration levels (in the range of the calibration curve) was added into a certain amount of sample. The samples were measured in triplicates and the amount of the standards added was calculated by subtraction (in total 12 injections). More precisely, 200 μl of three consecutive solutions of harpagoside (1.93, 0.965 and 0.4825 mg/ml) were mixed with 200 μl of consecutive solutions of verbascoside (1.685, 0.8425 and 0.42125 mg/ml) and equal volume of the tincture. The amount of the added analytes in a 2 μl injection was calculated. Similarly 200 μl of three solutions of curcumin (0.472, 0.236, and 0.118 mg/ml) were mixed with equal volume of tincture and the amount of the standard added was calculated on the basis of a 2 μl injection.

2.10. Quantitative determination of constituents

The method of external standard was applied to quantify each compound. Quantification of individual constituents was performed using a regression curve, each point in triplicate. Measurements were performed at 420 nm for the curcuminoids, at 280 nm for harpagoside, at 310 nm for the acylated derivatives of iridoids (8-O-*E*-p-coumaroyl-harpagide and 8-O-feruloyl-harpagide) and at 330 nm for verbascoside and isoverbascoside.

3. Results and discussion

In continuing our studies [3–5] on the constituents and stability of herbal drug preparations, the present work reports the qualitative and quantitative profiles of the constituents of commercial tinctures of *C. longa* and *H. procumbens*. Their chemical stability was also evaluated according to ICH guidelines (fully validated and stability indicating analytical procedures). The constituents of the tinctures were identified by UV and MS spectral data and further confirmed by comparison with reference data or authentic samples. In Figs. 1 and 2 the HPLC-DAD chromatograms of the tinctures are presented. Data concerning identification of the peaks are shown in Tables 2 and 3, where the retention time, UV–vis absorptions and electrospray ionisation mass spectrometry in both positive and negative ion mode of all the compounds detected in the tinctures are reported.

In the chromatogram of the *C. longa* tincture (Fig. 1) eleven peaks were identified, four of which simple phenolic acids, three curcuminoids and four sesquiterpenoids. In the chromatogram of the *H. procumbens* tincture (Fig. 2) fifteen peaks were identified, five of which belonged to the group of iridoids, two unusual iridan derivatives, seven phenylethyl alcohols and one simple phenolic ester.

Table 3
Positive and negative ion mode MS fragmentation (*m/z* values) and UV–vis absorption data of the compounds (H1–H15) detected in the tincture of *Harpagophytum procumbens*.

No	Rt (min)	UV (nm)	Negative	Positive	Identification	Mode of identification
H1	6.8	257	**363** [M−H]⁻, 727 [2M−H]⁻	**387** [M+Na]⁺, **403** [M+K]⁺	Harpagide	MS + 2D NMR
H2	8.3	220, 280	**461** [M−H]⁻	**485** [M+Na]⁺, **501** [M+K]⁺	Decaffeoylverbascoside	UV/MS + NMR
H3a, 3b	11.9, 12.3	289, 322	**639** [M−H]⁻, 621 [M−H_2O-H]⁻	**663** [M+Na]⁺	β-OH-acteoside diastereomer	UV/MS
H4	13.2	290, 330	**623** [M−H]⁻	**647** [M+Na]⁺	Verbascoside	UV/MS + std
H5	13.6	294, 316	**507** [M−H]⁻	–	p-Coumaroyl-procumbide	UV/MS
H6	14.0	289, 327	**623** [M−H]⁻	**625** [M+H]⁺	Isoverbascoside	UV/MS + NMR
H7	15.5	292sh, 312	**509** [M−H]⁻	**533** [M+Na]⁺	8-O-p-coumaroyl-harpagide	UV/MS + NMR
H8	16.5	296, 324	**539** [M−H]⁻, 509, 193	**563** [M+Na]⁺	8-O-feruloyl-harpagide	UV/MS + std
H9	17.0	288, 322	**637** [M−H]⁻	–	Leucosceptoside A	UV/MS + std
H10	18.5		**665** [M−H]⁻	–	Acetylacteoside	UV/MS
H11	19.5	212sh, 230, 294sh, 314	**491** [M−H]⁻, 983 [2M−H]⁻, 311, 163 [coumaric]⁻	**515** [M+Na]⁺, 313 [M−glucose]⁻, 149	Pagoside	UV/MS + 2D NMR
H12	20.7	220sh, 234, 296sh, 328	**521** [M−H]⁻, 341, 193 [ferulic]⁻, 147	**545** [M+Na]⁺, **561** [M+K]⁺, 343 [M−glucose]⁻, 177	Methoxy-pagoside	UV/MS + NMR
H13	22.1	289, 327	**651** [M−H]⁻	**675** [M+Na]⁺	Martynoside	UV/MS
H14	23.5	280	**493** [M−H]⁻, 539 [M+HCOO]⁻, 987 [2M−H]⁻	**517** [M+Na]⁺	Harpagoside	UV/MS + NMR + std
H15	26.9	296, 326	**207** [M−H]⁻	**209** [M+H]	Caffeic acid ethylester	UV/MS + NMR

std: standard; characters in bold correspond to pseudomolecular ions.

3.1. Chromatographic conditions

The use of the RP-C18 column provided good separation for the constituents of both tinctures under the described conditions. Peaks had satisfactory shape and the asymmetry factor ranged between 1.3 and 1.4 for the *C. longa* tincture, whereas for the *H. procumbens* tincture ranged between 1.0 and 1.2. The purity of each constituent was investigated by inspecting the UV-spectra in the beginning at the apex and at the end of the peaks. No deviations were seen.

3.2. Identification of constituents

3.2.1. C. longa

The presence of the simple phenolics (peaks C2–C4), including vanillin, *p*-coumaric and ferulic acid has been reported previously in the literature [20,21] however, their presence in the tincture could be a result of the degradation of curcuminoids in the tincture [22]. *E*-Coumaric and *E*-ferulic acids gave characteristic UV absorptions (at 310 and 324 nm, respectively) [23]. The retention time of the first group of constituents (C1–C4), the UV absorption

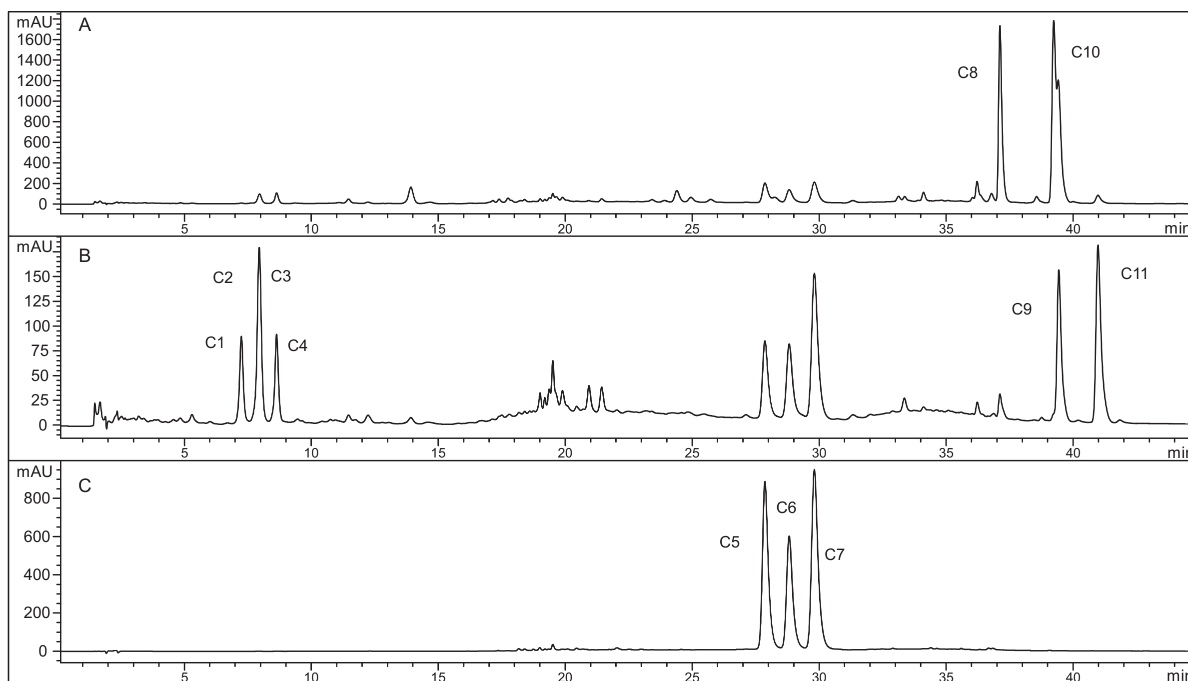

Fig. 1. Chromatogram of the *Curcuma longa* tincture at 240 nm (A), 280 nm (B) and 420 nm (C).

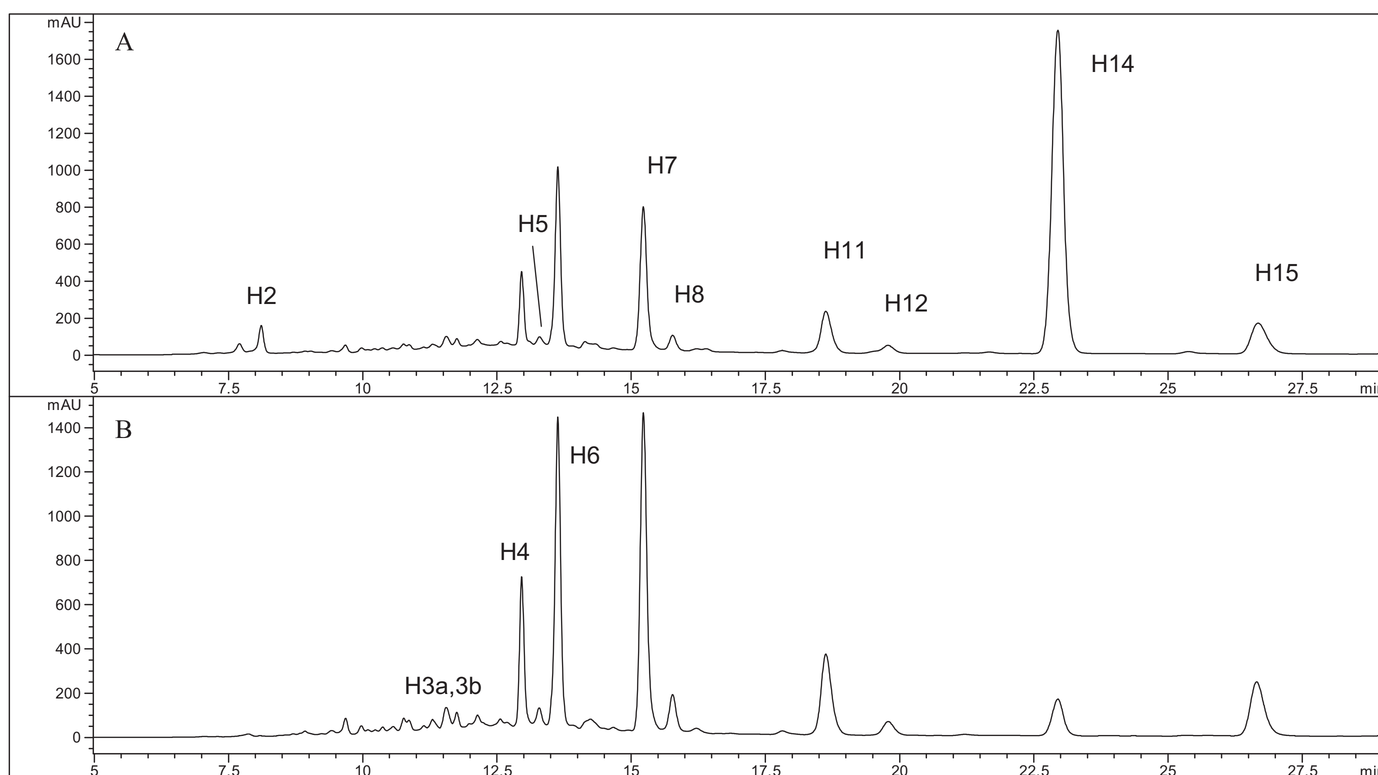

Fig. 2. Chromatogram of the *Harpagophytum procumbens* tincture at 280 and 330 nm. Peak H1 was observed with the help of the mass spectra.

and the lack of pseudomolecular ions in higher fragmentor of 120 (positive or negative) suggested they belonged to small metabolites. Indeed by the use of low fragmentors of 60 or 80 in both positive and negative ionisation modes their pseudomolecular ions were evidenced (Table 2). In order to further confirm their presence reference standards were used. Curcuminoids, the typical constituents of *C. longa* (C5–C7), presented characteristic absorption band at approximately 420 nm [24]. Negative ion mode gave the best results. Sesquiterpenoids (peaks C8–C11) such as curlones and turmerones were also identified based on the UV spectra and their mass data. In general, phenolic constituents gave better results in the negative ion mode, whereas terpenoids were observed in the positive mode.

3.2.2. *H. procumbens*

The identification of the peaks in the *H. procumbens* tincture was based mainly on the UV and MS spectral data except for the peaks H2, H11, H12 whose spectral data differed considerably from those of the characteristic constituents of the plant, and peak H15 which seemed to be a small molecule not previously identified in the *H. procumbens*. For this reason phytochemical isolations were considered as necessary in order to confirm their identity. Phytochemical investigations and NMR experiments revealed the presence of decaffeoylverbascoside (H1) reported for the first time in *H. procumbens* and pagoside (H11), an unusual iridoid derivative previously isolated from the same plant [15]. A minor peak with retention time at 20.7 min having similar spectral data to pagoside (234 and 328 nm) and a pseudomolecular ion at $m/z = 521$ (30 units bigger than pagoside) was tentatively identified as methoxy-pagoside which is a newly reported natural compound. Its fragmentation pattern was similar to that of pagoside. It presented a peak at $m/z = 341$ $[M-180]^-$, due to the loss of glucose, while a peak at $m/z = 193$ was attributed to the presence of a feruloyl unit in the molecule. Its retention time (is eluted after pagoside) supports this hypothesis. Phytochemical investigations did not permit its isolation in pure form due to its very low quantity, allowed however, the isolation of a mixture of this compound together with pagoside (ratio 8:1). 1D and 2D NMR (HMBC, ROESY) spectra showed the presence of a feruloyl moiety in the aromatic area which confirmed the presence of this constituent in the tincture (see supplementary data). To our knowledge this is the first time that this constituent is reported in *H. procumbens*, but also as a new naturally occuring compound. Finally, peak H15 at 26.9 min was identified by ^{1}H NMR as caffeic acid ethylester. It is a constituent previously reported in plants, but its presence in the samples could be the result of the preparation procedure of the tincture (artefact).

3.3. Validation data

3.3.1. Linearity, repeatability of the standards and samples, LOD, LOQ, precision and accuracy

All compounds showed good linearity. The following r^2 values were obtained: curcumin $r^2 = 0.9990$; verbascoside $r^2 = 0.9997$; harpagoside $r^2 = 0.9998$. LOD for curcumin was 0.35×10^{-3} μg (0.118 μg/ml, 3 μl of injection) and LOQ was 0.88×10^{-3} μg (0.29 μg/ml, 3 μl of injection). For verbascoside LOD was calculated as 0.39×10^{-3} μg (0.195 μg/ml, 2 μl of injection) and LOQ was 1.17 μg (0.584 μg/ml, 2 μl of injection), whereas for harpagoside LOD was 0.25×10^{-3} μg (0.084 μg/ml, 3 μl of injection) and LOQ was 0.8×10^{-3} μg (0.4 μg/ml, 2 μl of injection) (Figs. 3 and 4).

The repeatability, based on six samples of each tincture was analyzed by HPLC and the relative standard deviation (RSD %) of the contents of the major constituents was calculated (Table 4). The following values were obtained: 0.43% (curcumin), 0.46% (demethoxycurcumin), 0.42% (bisdemethoxycurcumin), 2.17% (verbascoside), 1.37% (isoverbascoside), 2.17% (harpagoside), 1.98% (8-O-p-coumaroyl-harpagide), 1.12% (pago-

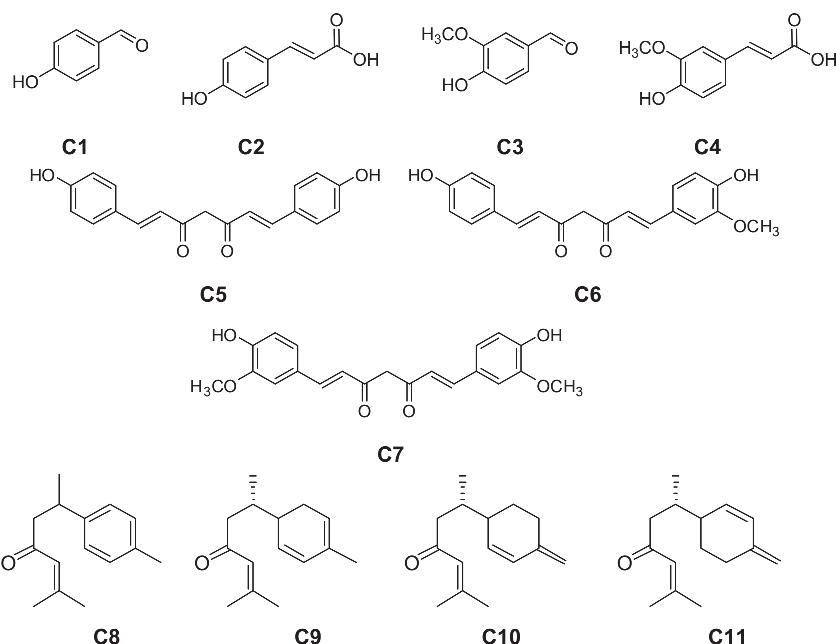

Fig. 3. Structures of the constituents identified in the tincture of *Curcuma longa*: p-OH-benzaldehyde (C1), *E*-coumaric acid (C2), vanillin (C3), *E*-ferulic acid (C4), bis-demethoxycurcumin (C5), demethoxycurcumin (C6), curcumin (C7), ar-turmerone (C8), α-turmerone (C9), β-turmerone (C10), and curlone (C11).

side). The overall intra- and inter-day time variations of the standards were less than 0.14% and 0.69% for curcumin, less than 1.75% and 1.32% for harpagoside and less than 1.49% and 1.35% for verbascoside, respectively. Results are displayed in Tables 5 and 6.

3.4. Quantitative analysis and stability studies

The quantification of individual constituents in both tinctures in the initial galenic preparation (time = 0), as well as samples taken during the accelerated stability testing was performed using

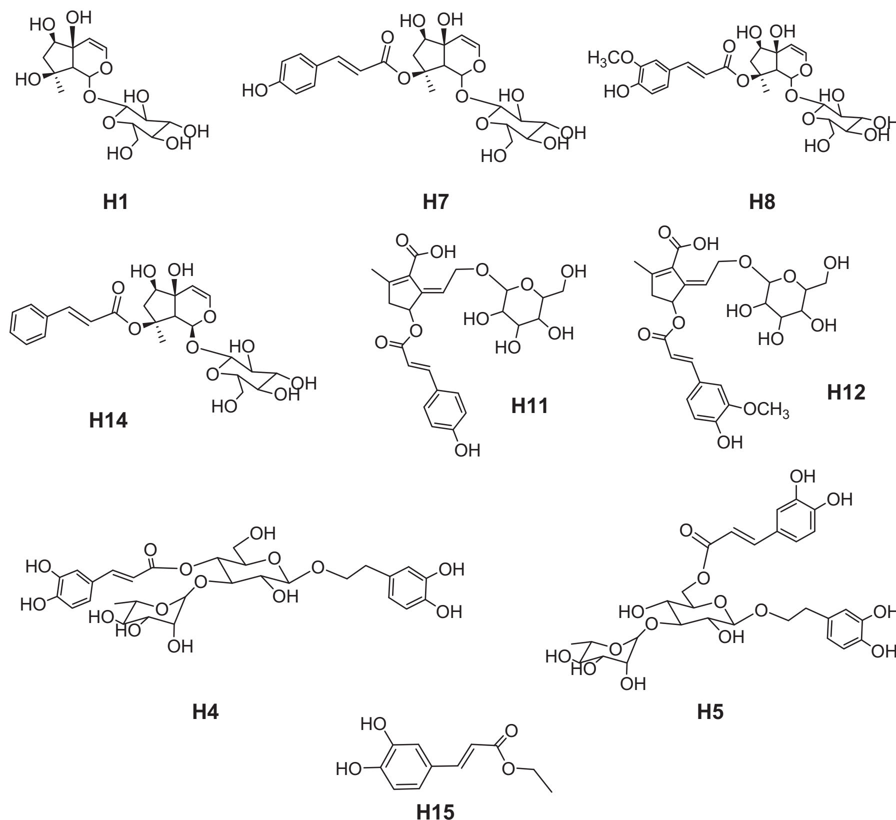

Fig. 4. Structures of the some of the main constituents identified in the tincture of *Harpagophytum procumbens*: harpagide (H1), 8-*E*-p-coumaroyl-harpagide (H7), 8-*E*-p-feruloyl-harpagide (H8), pagoside (H11), methoxypagoside (H12), verbascoside (H4), isoverbascoside (H5) and caffeic acid ethylester (H15).

Table 4
Contents of the active constituents in each tincture expressed as mg/ml of tincture ($n = 6$).

Constituents	Concentration (mg/ml) ± SD	RSD %
Curcuma longa		
Bisdemethoxycurcumin (C5)	0.38 ± 0.00	0.91
Demethoxycurcumin (C6)	0.27 ± 0.00	1.27
Curcumin (C7)	0.46 ± 0.01	1.50
Total curcuminoids	**1.11 ± 0.01**	**1.21**
Harpagophytum procumbens		
8-*E*-p-Coumaroyl-harpagide (H7)	0.63 ± 0.01	1.98
Pagoside (H11)	0.32 ± 0.01	1.12
Harpagoside (H14)	2.12 ± 0.05	2.17
Total iridoids	**3.07 ± 0.02**	**1.96**
Verbascoside (H4)	0.84 ± 0.02	2.17
Isoverbascoside (H5)	1.93 ± 0.03	1.37
Total phenylethylalcohols	**2.77 ± 0.02**	**0.63**

the DAD detector. Measurements were performed at 420 nm for the curcuminoids, at 280 nm for harpagoside, at 310 nm for the acylated derivatives of iridoids (8-O-*E*-p-coumaroyl-harpagide and 8-O-feruloyl-harpagide) and at 330 nm for verbascoside and isoverbascoside.

3.4.1. *C. longa tincture*

Since the biological activity of the *C. longa* tincture and preparations is attributed to its content in curcuminoids only curcumin and its derivatives were considered for the quality control studies. Curcuminoids are expressed as curcumin. From Fig. 5 it is clearly observed that the content of the active principles decreases during time in a linear mode. As it can be observed, the content of the curcuminoids present in the tincture of *C. longa* decreases approximately up to 34% of the initial concentration in 5 months of accelerated stability at 40 °C. No further analyses after this period were performed because significant physical changes appeared

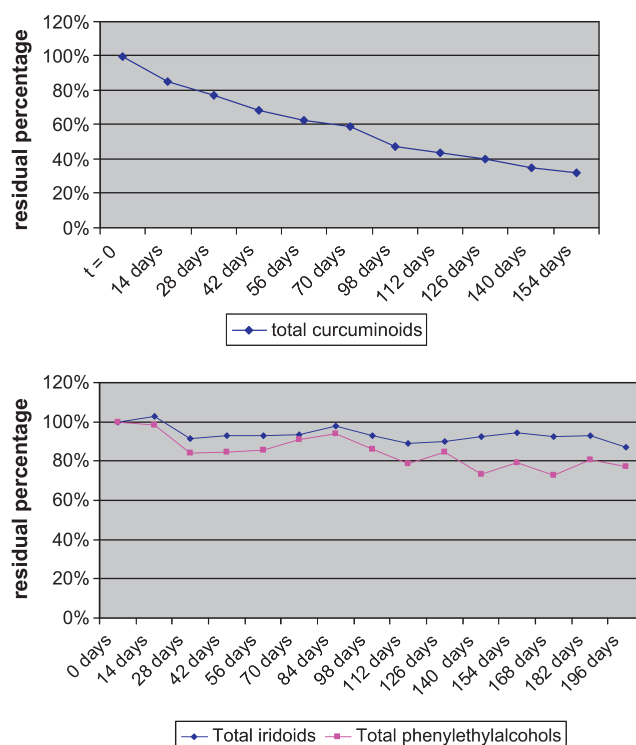

Fig. 5. Residual percentage of the active principles of each tincture in different batches obtained during the accelerated thermostability test. For the *Curcuma longa* tincture the measurements during the 6th month of thermostability test were not considered due to the fast degradation of its active principles.

Table 5
Precision data of the 3 analytes, expressed as RSD (%).

	Intra-day precision			Inter-day precision ($n = 3$)
	Day 1 ($n = 6$)	Day 2 ($n = 3$)	Day 3 ($n = 3$)	
Curcumin	0.14	0.0	0.30	0.69
Harpagoside	1.75	2.10	0.49	1.32
Verbascoside	1.49	1.70	0.94	1.35

Table 6
Recoveries of the 3 marker compounds of the tinctures.

	Added	Calculated	Recovery (%)	RSD %
Curcumin	0.118	0.115	97.4	1.15
	0.236	0.231	97.9	0.45
	0.472	0.468	99.1	0.55
Harpagoside	0.322	0.329	102.2	1.76
	0.643	0.658	102.3	0.17
	1.287	1.280	99.5	0.35
Verbascoside	0.281	0.285	101.4	0.88
	0.562	0.567	100.9	0.11
	1.123	1.126	100.3	0.75
Curcumin	0.118	0.115	97.4	1.15
	0.236	0.231	97.9	0.45
	0.472	0.468	99.1	0.55
Harpagoside	0.322	0.329	102.2	1.76
	0.643	0.658	102.3	0.17
	1.288	1.280	99.4	0.35
Verbascoside	0.281	0.285	101.4	0.88
	0.562	0.567	100.9	0.11
	1.124	1.126	100.2	0.75

in the tincture. It has been proposed that the degradation products of the curcuminoids are simple phenolics, such as vanillin, p-hydroxybenzaldehyde, coumaric and ferulic acids [22]. Although the scope of this quality control was not to quantify the simple phenolics, a clear increase in the content of this group of constituents in the tinctures was observed with time. Instead, turmerones and curlone showed a small decrease in their content which is expected due to their volatile character. Results show that this preparation is not stable in elevated temperatures and that the active principles degrade in a percentage over than the limit of 5% which is acceptable for pharmaceutical products.

3.4.2. H. procumbens tincture

Iridoids are considered to be the constituents responsible for the antiinflammatory properties of *H. procumbens* [25]. However, since verbascoside and its derivatives have an important anti-inflammatory activity [26] they were chosen for quantitation as well. Iridoids were expressed as harpagoside, whereas phenylethylalcohols (isoverbascoside and verbascoside) were expressed as verbascoside. Quantitation results are depicted in Fig. 5, where it can be seen that this tincture was more stable and the iridoids do not degrade below 90% of their initial concentration in a 6 months period under the described conditions. Instead, phenylethylalcohols degrade approximately at 80% of their initial concentration.

4. Conclusions

In the present study, commercial tinctures of *C. longa* rhizomes and *H. procumbens* roots were evaluated using validated analytical methods. To the best of our knowledge this is the first report on the analysis and evaluation of stability of these widely marketed tinctures. The analytical methods provided good separations of different classes of constituents. Good linearity of the calibration curves was achieved between 0.8×10^{-3} μg and 4 μg ($r^2 > 0.999$). Furthermore, the two assays were validated for limits of detection and quantification, precision, including time precision, and accuracy. All validation criteria were fulfilled.

In addition to the known constituents, the analysis of *H. procumbens* revealed the presence of a minor new constituent, methoxypagoside. Its presence in the tincture was further proved by 2D NMR spectra reported in the supplemenatry data.

The proposed methods could be used for the direct quality control and stability testing of *C. longa* and *H. procumbens* tinctures and other HDPs.

H. procumbens tincture resulted to be quite stable while the tincture of *C. longa*, succumbs an elevated degradation of its active principles during the same period. These results show that in particular turmeric tincture should be prepared immediately before consumption by the patient who should also take good care of storing them in order to retain the efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.02.029.

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