

**Bioassay-guided isolation and UHPLC-DAD-ESI-MS/MS quantification of potential anti-inflammatory phenolic compounds from flowers of *Inula montana* L.**

<sup>a</sup> Elnur Garayev , <sup>b</sup> Carole Di Giorgio , <sup>c</sup> Gaëtan Herbette , <sup>a</sup> Fathi Mabrouki , Philippe  
<sup>d</sup> Chiffolleau , <sup>e</sup> David Roux , <sup>e</sup> Huguette Sallanon , <sup>a</sup> Evelyne Ollivier , <sup>a</sup> Riad Elias , <sup>a</sup> Béatrice  
<sup>a</sup> **Baghdikian** \*

<sup>a</sup> Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, FAC PHARM, Marseille, France

<sup>b</sup> Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Laboratoire de Mutagenèse Environnementale, Marseille, France

<sup>c</sup> Aix Marseille Univ, CNRS, Centrale Marseille, FSCM, Spectropole, Service 511, Campus Saint-Jérôme, 13397 Marseille, France.

<sup>d</sup> Parc Naturel Régional du Luberon, 60, place Jean Jaurès, 84400 Apt, France

<sup>e</sup> Avignon Université Qualisud, UMR 95, F-84000, Avignon, France

\*Corresponding author

Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Laboratoire de Pharmacognosie, Faculté de Pharmacie, Marseille, France, 27, bd. Jean Moulin, CS30064, Marseille Cedex 05, France

Tel.:+33-491-83-55-93, E-mail: [beatrice.baghdikian@univ-amu.fr](mailto:beatrice.baghdikian@univ-amu.fr)

*E-mail addresses:*

[elnurgar@mail.ru](mailto:elnurgar@mail.ru) (E. Garayev)

[carole.di-giorgio@univ-amu.fr](mailto:carole.di-giorgio@univ-amu.fr) (C. Di Giorgio)

[gaetan.herbette@univ-amu.fr](mailto:gaetan.herbette@univ-amu.fr) (G. Herbette)

[fathi.mabrouki@univ-amu.fr](mailto:fathi.mabrouki@univ-amu.fr) (F. Mabrouki)

[philippe.chiffolleau@parcduluberon.fr](mailto:philippe.chiffolleau@parcduluberon.fr) (P. Chiffolleau)

[david.roux@univ-avignon.fr](mailto:david.roux@univ-avignon.fr) (D. Roux)

[huguette.sallanon@univ-avignon.fr](mailto:huguette.sallanon@univ-avignon.fr) (H. Sallanon)

[evelyne.ollivier@univ-amu.fr](mailto:evelyne.ollivier@univ-amu.fr) (E. Ollivier)

[riad.elias@univ-amu.fr](mailto:riad.elias@univ-amu.fr) (R. Elias)

[beatrice.baghdikian@univ-amu.fr](mailto:beatrice.baghdikian@univ-amu.fr) (B. Baghdikian)

## ABSTRACT

*Ethnopharmacological relevance:* Flowers of *Inula montana* L. (Asteraceae), commonly known as “Arnica de Provence”, are used in the traditional medicine of Provence in France with the same indication as *Arnica montana*, for the relief of bruises, as an anti-inflammatory agent.

*Aims of the study:* The aim of our study is to evaluate its anti-inflammatory properties and to justify its traditional uses. Its potential valorization is evaluated in order to propose *Inula montana* as an alternative to *Arnica montana*.

*Materials and methods:* Bio-guided fractionation of ethanolic extract allowed the isolation of compounds, responsible of the inhibition of NO production. The fractionation was realized using chromatographic techniques and structure elucidation was conducted by ESI-MS and NMR spectral data. Anti-inflammatory effect of ethanolic extract, different fractions and isolated pure compounds was studied *in vitro* on immortalized mouse macrophages RAW 264.7. An analytical UHPLC-DAD-ESI-MS/MS method was developed for the identification of these compounds in the herbal drug. This UHPLC-DAD method was validated and was used to compare the phenolic profile and content in plant material from the two collection sites: Bonnieux and Merindol.

*Results:* Eleven compounds were identified by UHPLC-MS. Chlorogenic acid (**1**), Luteolin (**2**), Nepetin (**3**), 3,5-*O*-Dicaffeoylquinic acid (**4**), 1,5-*O*-Dicaffeoylquinic acid (**5**), Nepitrin (**6**), Hispiduloside (**7**) and Jaceosid (**8**) were isolated and identified by NMR. Compounds **9**, **10** and **11** were confirmed to be 6-Hydroxykaempferol 3,7-dimethyl ether, Hispidulin and Chrysosplenol C, respectively by comparing retention times and MS/MS data with those of the authentic substances. Six compounds: **1** and **4-8** are reported for the first time in *Inula montana* L. Compounds **2-8** showed promising anti-inflammatory activity with the release of NO with IC<sub>50</sub> value <7 μM. The UHPLC-DAD method of quantification of three major bioactive compounds (**1**, **3** and **5**) was validated.

*Conclusion:* Flowers extracts and isolated compounds present promising anti-inflammatory activity which provides a scientific basis for the traditional use of *Inula montana* and may be proposed in

the same indications as *Arnica montana*. The developed and validated simple, accurate and rapid UHPLC method can be used for the quality control of the herbal drug.

**Keywords:** 1,5-*O*-Dicafeoylquinic acid, Chlorogenic acid, Anti-inflammatory activity, UHPLC-DAD, Accuracy profile

## 1. Introduction

The genus *Inula* is mainly distributed in Africa, Asia and Europe, predominantly in the Mediterranean Area. Flavonoids and terpenes are the major compounds of this genus (Seca et al., 2014; Wu et al., 2015; Zhao et al., 2006).

*Inula montana* L. is a member of *Asteraceae* family. Flowers are used in traditional medicine in France, Spain and Morocco. In France, *I. montana* is called “Arnica of Provence” and is used for the relief of bruises. The flowers are macerated with ethanol at 60-70% (v/v) called “eau de vie” or with olive oil. These preparations are used for cutaneous application with the same indication as *Arnica montana* (Amir, 1998). In Spain, decoction, infusion and maceration in alcohol or olive oil of flowers, leaves and fresh aerial parts are used locally for the relief of bruises, joint pains, rheumatism and orally for stomach ache (Obón et al., 2012). In Morocco, decoction or infusion of leaves are used to treat orally gastrointestinal, otolaryngological and respiratory diseases (Teixidor-Toneu et al., 2016).

Few studies report the phytochemical composition and pharmacological activity of *I. montana*.

Previous phytochemical studies led to the isolation of flavonoid aglycones, sesquiterpene lactones and inositol derivatives (Garayev et al., 2017; Gonzalez Romero M.A. et al., 2001; Reynaud and Lussignol, 1999). Crude extracts and some purified constituents of *I. montana* demonstrated antioxidant, protozoocidal and anti-inflammatory activities (Garayev et al., 2017; Martín et al., 1998; Belyagoubi-Benhammou et al., 2014).

The aim of our study is to validate the traditional use of *Inula montana* by evaluating its potential anti-inflammatory properties and to evaluate the possible use of *I. montana* as an

alternative to *Arnica montana*, a plant species that is endangered and difficult to cultivate (Pasquier and Godin, 2014).

Both species are members of *Asteraceae* family. They contain similar chemical classes of compounds, essentially sesquiterpene lactones, phenolic acids, and flavonoids. Sesquiterpene lactones of *I. montana* are different from those of *A. montana*, however we can find two flavonoid aglycones described in both species, luteolin and hispidulin (Kriplani et al., 2017).

*Inula montana* is a perennial plant showing a rosette (10–40 cm circumference) of thin lancet-shaped sessile leaves (highly variable number). All aerial organs are hairy, leaves and bracts also display numerous glandular trichomes. From May to June, this rhizomatous plant emits a few ≈20 cm floral stems (rarely branched), with a few smaller alternate leaves, holding at the top a solitary capitulum (5–8 cm diameter) of yellow florets (ray florets with long and thin ligules) displaying a discrete odor. *Inula montana* grows from 50 to 1300 m altitude and is frequent from eastern Italy to southern Portugal. This calcicolous and xerophilous plant is locally abundant in Southeast France in the calcareous low scrubland ecoregion named Garrigue (Girerd and Roux, 2011; Gonzalez Romero M.A. et al., 2001; Tela Botanica, 2018). The rare and protected *Arnica montana* is also a rhizomatous herbaceous perennial herb but, in contrast to *Inula montana*, it grows on acid (siliceous) and poor soils mainly in high altitude (600-2700 m) environment such as the alpine mountain. It displays larger oval leaves which are hairy on the upperside. From June to July, it emits a single ≈50 cm floral stem showing one (frequent) to tree opposite pairs of smaller leaves and a bright yellow to orange 7-8 cm capitulum with a pleasant perfume. Ligules of ray florets are far more developed in comparison to *Inula montana* (Rameau et al., 1989; Sugier et al., 2013).

This paper describes the isolation, structure elucidation, quantitative analysis and anti-inflammatory effects of the isolated compounds by measuring their capacity to inhibit nitric oxide production. The identification and the structure elucidation were carried out using NMR and UHPLC-DAD-ESI-MS/MS. This UHPLC-DAD method was validated for the quantification of the three major active compounds. To our knowledge, this is the first time that an analytical method for

the quality control of *I. montana* is reported. This validated UHPLC-DAD method was used to compare the phenolic profile and the content of the two collection sites: Bonnieux and Merindol, in order to observe potential modification of the phenolic composition and to select the plant with the highest content.

## 2. Materials and methods

### 2.1 Plant material

**Table 1** Flowers of *Inula montana* L. from different collection sites

Place of collect	Collect date	Altitude (m)	Voucher specimen
Merindol	03/06/2015	170	IMFLF151
Bonnieux	31/05/2017	680	IMFLFB171
Merindol	31/05/2017	170	IMFLFMe171

The plant material of *Inula montana* was collected in Merindol in 2015 and 2017 and Bonnieux in 2017 located in the Regional Natural Park of Luberon, in the south of France (Table 1). The voucher specimens were deposited in the Laboratory of Pharmacognosy of Aix Marseille University (Marseille, France). Flowers were air-dried and ground.

### 2.2 Instruments and reagents

<sup>1</sup>H 1D and 2D NMR spectra were recorded on Bruker AVANCE III 600 spectrometer (<sup>1</sup>H 600.13MHz) (Bruker, USA) with tetramethylsilane (TMS) as an internal standard at 300K. The chemical shifts ( $\delta$ ) were expressed in ppm with TMS as an internal reference. UHPLC-DAD-ESI-MS/MS analysis were performed using an UHPLC Agilent Infinity 1290 Liquid chromatography system equipped with a binary pump solvent delivery system G4220A and UV photodiode array detector G4212A (Agilent Technologies, Inc., Germany), coupled with Bruker Esquire 2000 (Bruker, USA) which is equipped with an electrospray ionization source (ESI). Preparative HPLC was performed on Gilson PLC2020. Flash chromatography was performed using a CombiFlash Rf 200 (Teledyn Isco, USA). A grinder (Moulinex, France) was used for the homogenization of flowers. The optical density for the evaluation of anti-inflammatory activity was read by fluorescence-luminescence reader Infinite M200 Pro (Tecan, Switzerland).

Extraction solvents, methanol and formic acid of LC-MS quality were purchased from Carlo Erba (Italy).

The standards of Chlorogenic acid, 1,5-*O*-DicaFFEoylquinic acid and Nepetin were purchased from PhytoLab GMBH (Germany).

Reagents for the anti-inflammatory evaluation were purchased from Sigma-Aldrich (USA).

### 2.3 Nitric oxide (NO) production assay

This assay is based on the ability of immortalized mouse macrophages RAW 264.7 to generate a strong inflammatory response leading to NO (Nitric Oxide) release, after the stimulation by antigens of *E. coli*. Mouse macrophages were seeded into 48-well tissue culture plates at the concentration of  $1.10^5$  cells/mL (200 $\mu$ L/well) for 24 hours at 37°C (5% CO<sub>2</sub>). At the end of the incubation period, the culture medium was replaced by 200  $\mu$ L of medium containing appropriate concentrations of evaluated compound and cells were incubated at 37°C (5% CO<sub>2</sub>) for one hour. Experiments were performed in duplicate and dexamethasone was used as a positive control at the concentrations of 1, 5, 10, 50 and 100  $\mu$ M. At the end of the incubation period, pro-inflammatory LPS from *E.coli* were added to the cell cultures (1  $\mu$ g/mL). Then the cells were incubated at 37°C (5% CO<sub>2</sub>) for 18 hours. NO release was measured in the culture supernatant by the Griess reaction: 50  $\mu$ L of the supernatants were transferred into the 96-well tissue culture plate, and 50  $\mu$ L of the Griess modified reagent were added to each well. After a 15 min period at room temperature, Optical Density (OD) of each well was read at 540 nm. Results obtained for wells, treated with the evaluated compound, were compared to the untreated control wells (DMSO, 100% viability) and converted into the percentage values.

Inhibition of NO release was expressed as percentages comparing to the negative controls:

$$\text{Percentage of NO release} = \frac{100 \times (\text{OD of test well} - \text{OD of blank})}{\text{OD of DMSO control} - \text{OD of blank}}$$

The concentrations of the test material causing a 50% decrease of NO release (IC<sub>50</sub>) were calculated through non-linear regression analysis using software Table curve Version 2.0.

## 2.4 UHPLC-DAD-ESI-MS/MS analysis

Chromatographic separation was achieved on Agilent 1290 liquid chromatograph using Zorbax SB-Phenyl (100 × 2.1 mm, 1.8 μm, Agilent, Germany) column, operated at 43 °C. Water acidified with 0.1% of formic acid (v/v) as Solvent A and Methanol as Solvent B were used for the gradient elution at 0.5 mL/min. The gradient program was: 18% B (from 0 to 3.5 min), 18 to 30% B (from 3.5 min to 3.6 min), 30% B (from 3.6 min to 12.0 min), 30 to 40% B (from 12.0 min to 12.1 min), 40% B (from 12.1 min to 16.2 min), 40 to 42% B (from 16.2 min to 16.3 min), 42% B (from 16.3 min to 22.0 min), 42 to 80% B (from 22.0 min to 22.1 min), 80% B (from 22.1 min to 26.0 min) with post time of 5.0 min and injection volume of 0.3 μL. The UV detection wavelength was set at 335 nm. The following MS parameters were employed: on positive ionization mode spray voltage was set at 3.5 kV, sheath gas (N<sub>2</sub>) flow rate was set at 50 psi, auxiliary gas (N<sub>2</sub>) flow rate was set at 12 l/min, capillary exit voltage was set at 102.4 V, tube lens voltage was set at -60 V and capillary temperature was set at 300°C. Fragmentation amplitude was set at 1.0 V.

## 2.5 Extraction and isolation

Powdered flowers (0.2 kg) were macerated with EtOH 95% (v/v) at ambient temperature for 18 h and then extracted by percolation with the same solvent three times (3 x 1.2L). The extracts were combined and evaporated to afford crude extract L2 (23.7 g). The extract was suspended in 500 mL of H<sub>2</sub>O and partitioned successively by 3 x 500 mL of petroleum ether, dichloromethane and ethyl acetate, to afford, after evaporation *in vacuo*, petroleum ether L2P (4.4 g), dichloromethane L2D (0.7 g) and ethyl acetate L2E (3.1 g) dry extracts.

The NO assay led us to select the L2E (2.48 g) extract which was subjected to the reversed phase (C18, 130 g) flash chromatography and eluted with water acidified with 0.1% formic acid (v/v) as Solvent A and methanol acidified with 0.1% (v/v) formic acid as Solvent B (v/v). Elution was performed at a flow rate of 60 mL/min, with fraction collection volume of 18 mL and ~~with~~ the following gradient: 20 to 100% B (from 0 to 25 min), 100% B (from 25 min to 37 min) to afford two fractions (L2E1) and (L2E2) and three compounds **1** (68.4 mg), **2** (19.2 mg) and **3** (20.1 mg).

Fraction L2E1 (221 mg) was subjected to the reversed phase preparative HPLC (Luna C18 column 150 × 21.2 mm, 3 μm, Phenomenex), eluted at a flow rate 21.2 mL/min, using 70% water acidified with 0.1% formic acid (v/v) and 30% methanol. Fractions were collected at 325 nm to give two compounds: **4** (4.5 mg) and **5** (20.8 mg).

Fraction L2E2 (40.2 mg) was also subjected to the reversed phase preparative HPLC (Luna C18 column 150 × 21.2 mm, 3 μm, Phenomenex, USA), eluted at a flow rate 21.2 mL/min using water acidified with 0.1% formic acid (v/v) as Solvent A and methanol as Solvent B. The following gradient: 35% B (from 0 to 33.0 min), 35 to 25% B (from 33.0 min to 33.5 min), 25% B (from 33.5 to 36.0 min), 25 to 35% B (from 36.0 min to 37.0 min), 35% B (from 37.0 min to 50.0 min), with fraction collection at 325 nm, has afforded three compounds **6** (1.8 mg), **7** (2.6 mg) and **8** (1.2 mg).

Compound **1** (Chlorogenic acid) was obtained as an amorphous colorless solid. C<sub>16</sub>H<sub>18</sub>O<sub>9</sub>; ESI-MS m/z: 355 [M + H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.56 (1H, *d*, *J* = 16.0 Hz, H-7'), 7.05 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.96 (1H, *dd*, *J* = 8.1, 2.0 Hz, H-6'), 6.79 (1H, *d*, *J* = 8.1 Hz, H-5'), 6.26 (1H, *d*, *J* = 16.0 Hz, H-8'), 5.33 (1H, *ddd*, *J* = 9.7, 8.7, 4.4 Hz, H-5<sub>ax</sub>), 4.17 (1H, *m*, H-3<sub>eq</sub>), 3.72 (1H, *dd*, *J* = 8.7, 3.3 Hz, H-4<sub>ax</sub>), 2.23 (1H, *brdd*, *J* = 13.5, 4.4 Hz, H-6<sub>eq</sub>), 2.18 (1H, *dd*, *J* = 14.0, 3.1 Hz, H-2<sub>ax</sub>), 2.08 (1H, *dd*, *J* = 13.5, 9.7 Hz, H-6<sub>ax</sub>), 2.05 (1H, *ddd*, *J* = 14.0, 3.3, 2.1 Hz, H-2<sub>eq</sub>); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 177.2 (C-7), 168.7 (C-9'), 149.6 (C-4'), 147.1 (C-7'), 146.8 (C-3'), 127.8 (C-1'), 123.0 (C-6'), 116.5 (C-5'), 115.2 (C-8'), 115.1 (C-2'), 76.2 (C-1), 73.5 (C-4), 72.0 (C-5), 71.3 (C-3), 38.2 (C-2), 36.8 (C-6).

Compound **2** (Luteolin) was obtained as an amorphous yellowish solid. C<sub>15</sub>H<sub>10</sub>O<sub>5</sub>; ESI-MS m/z: 287 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.35 (1H, *m*, H-6'), 7.35 (1H, *m*, H-2'), 6.89 (1H, *d*, *J* = 8.8 Hz, H-5'), 6.52 (1H, *s*, H-3), 6.41 (1H, *d*, *J* = 2.1 Hz, H-8), 6.19 (1H, *d*, *J* = 2.1 Hz, H-6); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 184.3 (C-4), 166.2 (C-2), 166.0 (C-7), 163.2 (C-5), 159.4 (C-9), 151.0 (C-4'), 147.1 (C-3'), 123.8 (C-1'), 120.4 (C-6'), 116.9 (C-5'), 114.3 (C-2'), 105.4 (C-10), 103.5 (C-3), 100.2 (C-6), 95.3 (C-8).



Compound **3** (Nepetin) was obtained as a yellowish powder. C<sub>16</sub>H<sub>12</sub>O<sub>7</sub>; ESI-MS m/z: 317 [M + H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.35 (1H, *dd*, *J* = 8.9, 2.0 Hz, H-6'), 7.35 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.88 (1H, *d*, *J* = 8.9 Hz, H-5'), 6.52 (1H, *s*, H-8), 6.51 (1H, *s*, H-3), 3.88 (3H, *s*, OCH<sub>3</sub>); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 184.2 (C-4), 166.4 (C-2), 154.6 (C-9), 158.7 (C-7), 153.9 (C-5), 151.0 (C-4'), 147.0 (C-3'), 132.8 (C-6), 123.7 (C-1'), 120.3 (C-6'), 116.8 (C-5'), 114.1 (C-2'), 105.7 (C-10), 103.4 (C-3), 95.2 (C-8), 60.9 (OCH<sub>3</sub>).

Compound **4** (3,5-O-Dicaffeoylquinic acid) was obtained as a white powder. C<sub>25</sub>H<sub>24</sub>O<sub>12</sub>; ESI-MS m/z: 517 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.61 (1H, *d*, *J* = 16.0 Hz, H-7''), 7.58 (1H, *d*, *J* = 16.0 Hz, H-7'), 7.07 (2H, *d*, *J* = 2.0 Hz, H-2', H-2''), 6.97 (2H, *dd*, *J* = 8.3, 2.0 Hz, H-6', H-6''), 6.78 (2H, *d*, *J* = 8.3 Hz, H-5', H-5''), 6.36 (1H, *d*, *J* = 16.0 Hz, H-8''), 6.28 (1H, *d*, *J* = 16.0 Hz, H-8'), 5.42 (2H, *m*, H-3<sub>eq</sub>, H-5<sub>ax</sub>), 2.31 (1H, *brd*, *J* = 14.0 Hz, H-6<sub>eq</sub>), 2.20 (2H, *brs*, H-2), 2.14 (1H, *dd*, *J* = 14.0, 5.7 Hz, H-6<sub>ax</sub>); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 176.4 (C-7), 169.0 (C-9''), 168.5 (C-9'), 149.6 (C-4'), 149.6 (C-4''), 147.2 (C-7'), 147.0 (C-7''), 144.5 (C-3'), 144.5 (C-3''), 127.9 (C-1''), 127.8 (C-1'), 122.9 (C-6''), 122.9 (C-6'), 116.4 (C-5'), 116.4 (C-5''), 115.7 (C-8''), 115.2 (C-8'), 115.1 (C-2'), 115.1 (C-2''), 75.2 (C-1), 73.0 (C-5), 72.2 (C-3), 71.2 (C-4), 38.4 (C-2), 36.4 (C-6)

Compound **5** (1,5-O-Dicaffeoylquinic acid) was obtained as a an amorphous solid. C<sub>25</sub>H<sub>24</sub>O<sub>12</sub>; ESI-MS m/z: 517 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.60 (1H, *d*, *J* = 16.0 Hz, H-7'), 7.57 (1H, *d*, *J* = 16.0 Hz, H-7''), 7.06 (2H, *d*, *J* = 2.2 Hz, H-2', H-2''), 6.96 (2H, *dd*, *J* = 8.2, 2.2 Hz, H-6', H-6''), 6.78 (2H, *d*, *J* = 8.2 Hz, H-5', H-5''), 6.30 (1H, *d*, *J* = 16.0 Hz, H-8'), 6.27 (1H, *d*, *J* = 16.0 Hz, H-8''), 5.42 (1H, *brtd*, *J* = 8.9, 4.1 Hz, H-5<sub>ax</sub>), 4.31 (1H, *brq*, *J* = 4.0, H-3<sub>eq</sub>), 3.81 (1H, *dd*, *J* = 8.3, 3.2, H-4<sub>ax</sub>), 2.61 (1H, *dd*, *J* = 13.6, 2.7 Hz, H-6<sub>eq</sub>); 2.52 (1H, *dd*, *J* = 15.1, 5.0, H-2<sub>ax</sub>), 2.44 (1H, *dd*, *J* = 15.1, 2.6, H-2<sub>eq</sub>), 2.08 (1H, *dd*, *J* = 13.6, 9.1 Hz, H-6<sub>ax</sub>); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 175.0 (C-7), 170.5 (C-9''), 168.5 (C-9'), 149.8 (C-4'), 149.8 (C-4''), 147.1 (C-7'), 147.1 (C-7''), 146.5 (C-3'), 146.5 (C-3''), 127.5 (C-1''), 127.5 (C-1'), 122.8 (C-

6'), 122.8 (C-6'), 116.2 (C-5'), 116.2 (C-5''), 115.1 (C-2''), 115.1 (C-2''), 115.1 (C-8'), 114.9 (C-8''), 80.8 (C-1), 72.6 (C-4), 71.2 (C-5), 69.3 (C-3), 36.7 (C-6), 35.5 (C-2).

Compound **6** (Nepitrin) was obtained as an amorphous yellowish solid. C<sub>22</sub>H<sub>22</sub>O<sub>12</sub>; ESI-MS *m/z*: 479 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.43 (1H, *dd*, *J* = 8.3, 2.0 Hz, H-6'), 7.41 (1H, *d*, *J* = 2.0 Hz, H-2'), 6.96 (1H, *s*, H-8), 6.90 (1H, *d*, *J* = 8.3 Hz, H-5'), 6.61 (1H, *s*, H-3), 5.12 (1H, *d*, *J* = 7.6 Hz, H-1''), 3.95 (1H, *dd*, *J* = 12.2, 2.0 Hz, H-6''a), 3.73 (1H, *dd*, *J* = 12.2, 6.1 Hz, H-6''b), 3.57 (1H, *dd*, *J* = 9.1, 7.6 Hz, H-2''<sub>ax</sub>), 3.57 (1H, *m*, H-5''<sub>ax</sub>), 3.52 (1H, *brt*, *J* = 9.1 Hz, H-3''<sub>ax</sub>), 3.42 (1H, *brt*, *J* = 9.1 Hz, H-4''<sub>ax</sub>), 3.89 (3H, *s*, CH<sub>3</sub>O); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 184.0 (C-4), 166.7 (C-2), 157.6 (C-7), 154.5 (C-9), 153.8 (C-5), 150.8 (C-4'), 146.7 (C-3'), 133.7 (C-6), 123.2 (C-1'), 120.1 (C-6'), 116.5 (C-5'), 113.9 (C-2'), 107.2 (C-10), 103.4 (C-3), 101.7 (C-1''), 95.5 (C-8), 78.2 (C-5''), 77.6 (C-3''), 74.4 (C-2''), 71.0 (C-4''), 62.2 (C-6''), 61.2 (CH<sub>3</sub>O).

Compound **7** (Hispiduloside) was obtained as an amorphous yellowish solid. C<sub>22</sub>H<sub>22</sub>O<sub>11</sub>; ESI-MS: *m/z* 463 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.88 (2H, *d*, *J* = 8.7, H-2', H-6'), 6.95 (1H, *s*, H-8), 6.92 (2H, *d*, *J* = 8.7 Hz, H-3', H-5'), 6.64 (1H, *s*, H-3), 5.12 (1H, *d*, *J* = 7.6 Hz, H-1''), 3.96 (1H, *dd*, *J* = 12.1, 2.1 Hz, H-6''a), 3.73 (1H, *dd*, *J* = 12.1, 6.1 Hz, H-6''b), 3.58 (1H, *dd*, *J* = 9.1, 7.6 Hz, H-2''<sub>ax</sub>), 3.58 (1H, *m*, H-5''<sub>ax</sub>), 3.53 (1H, *brt*, *J* = 9.1 Hz, H-3''<sub>ax</sub>), 3.42 (1H, *brt*, *J* = 9.2 Hz, H-4''<sub>ax</sub>), 3.89 (3H, *s*, CH<sub>3</sub>O); <sup>13</sup>C NMR (150.9 MHz, CD<sub>3</sub>OD) δ: 184.0 (C-4), 166.6 (C-2), 162.6 (C-4'), 157.6 (C-7), 154.1 (C-9), 153.8 (C-5), 133.8 (C-6), 129.3 (C-2', C-6'), 122.8 (C-1'), 116.7 (C-3', C-5'), 107.2 (C-10), 103.3 (C-3), 101.7 (C-1''), 95.5 (C-8), 78.3 (C-5''), 77.6 (C-3''), 74.5 (C-2''), 71.0 (C-4''), 62.2 (C-6''), 61.2 (CH<sub>3</sub>O).

Compound **8** (Jaceosid) was obtained as amorphous yellowish solid. C<sub>23</sub>H<sub>24</sub>O<sub>12</sub>; ESI-MS *m/z* : 493 [M+H]<sup>+</sup>. <sup>1</sup>H NMR (600.13 MHz, CD<sub>3</sub>OD) δ: 7.56 (1H, *dd*, *J* = 8.3, 1.8 Hz, H-6'), 7.52 (1H, *d*, *J* = 1.8 Hz, H-2'), 7.02 (1H, *s*, H-8), 6.94 (1H, *d*, *J* = 8.3 Hz, H-5'), 6.71 (1H, *s*, H-3), 5.13 (1H, *d*, *J* = 7.7 Hz, H-1''), 3.97 (3H, *s*, CH<sub>3</sub>O-3'), 3.95 (1H, *dd*, *J* = 12.1, 2.0 Hz, H-6''a), 3.90 (3H, *s*, CH<sub>3</sub>O-6), 3.71 (1H, *dd*, *J* = 12.1, 6.2 Hz, H-6''b), 3.58 (1H, *m*, H-5''<sub>ax</sub>), 3.57 (1H, *ov*, H-2''<sub>ax</sub>), 3.52 (1H, *brt*, *J* = 9.1 Hz, H-3''<sub>ax</sub>), 3.41 (1H, *brt*, *J* = 9.3 Hz, H-4''<sub>ax</sub>); <sup>13</sup>C NMR (150.9 MHz,

CD<sub>3</sub>OD)  $\delta$ : 184.2 (C-4), 166.6 (C-2), 157.7 (C-7), 154.7 (C-5), 154.2 (C-9), 152.3 (C-4'), 149.6 (C-3'), 134.2 (C-6), 123.3 (C-1'), 121.7 (C-6'), 116.6 (C-5'), 110.5 (C-2'), 107.4 (C-10), 103.8 (C-3), 101.8 (C-1''), 95.6 (C-8), 78.3 (C-5''), 77.8 (C-3''), 74.5 (C-2''), 71.1 (C-4''), 62.3 (C-6''), 61.2 (CH<sub>3</sub>O-6), 56.4 (CH<sub>3</sub>O-3').

## 2.6 Validation of UHPLC-DAD method

The recommendations of the “Société Française des Sciences et Techniques Pharmaceutiques” (SFSTP) were respected for the validation of the analytical method in herbal medicinal products (D. Bellenot et al., 2015). Two kinds of samples, calibration and validation standards, were prepared separately. Calibration standards were prepared twice per day over three days. Validation standards were prepared three times per day over three days.

### *Preparation of calibration standards*

Weigh 15.0 mg of Chlorogenic acid (CA) into a volumetric flask of 10.0 mL and dilute to 10.0 mL with MeOH. Weigh 16.3 mg of 1,5-*O*-DicaFFEoylquinic acid (DCQA) into a volumetric flask of 5.0 mL and dilute to 5.0 mL with MeOH. Weigh 12.0 mg of Nepetin (NPT) acid into a volumetric flask of 20.0 mL and dilute to 20.0 mL with MeOH. Place 2.0 mL of each solution into a volumetric flask of 50.0 mL and dilute to 50.0 mL with MeOH. Final concentrations for CA, DCQA and NPT are 60.0  $\mu$ g/mL, 130.4  $\mu$ g/mL and 24.0  $\mu$ g/mL, respectively. Filter 2 mL of this solution through a PVDF filter (0.22  $\mu$ m, Millipore) for UHPLC analysis.

### *Preparation of validation standard*

Put 10 g of powdered flowers into the grinder and homogenize for 5 seconds three times. Weigh 1.000 g of homogenized flowers and mix with 90 mL of MeOH into a round-bottomed flask of 250 mL. Extract under reflux for 30 minutes. Cool for 15 minutes. Filter into a volumetric flask of 100.0 mL through a paper filter, rinse two times with 5 mL of MeOH and dilute to 100.0 mL with MeOH. Filter 2 mL of this solution through a PVDF filter (0.22  $\mu$ m, Millipore) for UHPLC analysis.

## 2.7 Statistical analysis

All statistical analyses were based on two-sided p-values, where  $p < 0.01$  indicates statistical significance. They were performed with SAS version 9.4 (SAS Institute, Cary, NC).

## 3. Results and discussion

### 3.1 UHPLC-DAD-ESI-MS/MS study of L2E

The ethanol crude extract (L2) showed promising anti-inflammatory effect ~~activity~~ with  $IC_{50}$  of NO release of  $0.77 \mu\text{g/mL}$ . In a bio-guided fractionation approach, each extract obtained after solvent partitioning was evaluated for the inhibition of NO release ~~its anti-inflammatory activity~~. The ethyl acetate extract (L2E) has been found to be the most active with  $IC_{50}$  of NO release of  $0.42 \mu\text{g/mL}$  and was selected for the next step ~~study~~. Petroleum ether (L2P) and dichloromethane (L2D) extracts were less active with  $IC_{50}$  of NO release of  $5.06 \mu\text{g/mL}$  and  $0.58 \mu\text{g/mL}$ , respectively.

The UHPLC-DAD-ESI-MS/MS method was used for the separation and identification by analytical chromatography of eleven compounds from the extract L2E. The spectroscopic data are shown in the Table 2.

**Table 2** Spectroscopic data of compounds of the extract L2E analyzed by UHPLC-DAD-ESI-MS/MS

Cmpd n <sup>o</sup>	R <sub>t</sub> (min)	λ <sub>max</sub> (nm)	[M+H] <sup>+</sup> (m/z)	MS/MS (m/z)	Identification
1	2.8	240-326	355	185-215	Chlorogenic acid
4	8.2	242-328	517	-	3,5- <i>O</i> -Dicaffeoylquinic acid <sup>a</sup>
5	9.6	242-328	517	499	1,5- <i>O</i> -Dicaffeoylquinic acid
6	10.6	272-348	479	317	Nepitrin
7	13.6	232-272-334	463	301	Hispiduloside
8	14.4	224-260-346	493	331	Jaceosid
2	15.0	254-348	287	-	Luteolin <sup>a,c</sup>
3	16.1	254-348	317	302	Nepetin <sup>c</sup>
10	19.2	280-338	331	298-316	6-Hydroxykaempferol 3,7-dimethyl ether <sup>b</sup>
9	19.9	222-272-336	301	286	Hispidulin <sup>b,c</sup>
11	20.5	268-344	361	346	Chrysosplenol C <sup>b</sup>

<sup>a</sup>No MS/MS fragments were detected; <sup>b</sup>Garayev et al., 2017; <sup>c</sup>Reynaud and Lussignol, 1999

The compound (**1**) with R<sub>t</sub> 2.8 min and parent ion m/z 355 was suggested to be Chlorogenic acid.

Two compounds (**2** and **3**) with the retention times (R<sub>t</sub>) 15.0 and 16.1 min, UV spectra characteristic of flavonoids and the parent ions m/z 287 and 317 [M + H]<sup>+</sup> were supposed to correspond to Luteolin and Nepetin, respectively.

Two compounds (**4** and **5**) with R<sub>t</sub> 8.2 and 9.6 min and UV spectra characteristic of phenolic acids show the same parent ions m/z 517 [M + H]<sup>+</sup> and suggest that they possess the same caffeoyl chromophore.

Two compounds (**6** and **7**) with R<sub>t</sub> 10.6 and 13.6 min, UV spectra characteristic of flavonoids and the parent ions m/z 479 and 463 [M + H]<sup>+</sup>, could be glucosides of Nepetin and Hispidulin because the fragment ions 317 and 301 can correspond to their aglycones, respectively. The difference between the parent and fragment ion of 162 Da, for both compounds, can suggest the loss of a glucose residue.

The compound (**8**) with R<sub>t</sub> 14.43 min, UV spectra characteristic of flavonoids and parent ion m/z 493 can be also glycosylated.

The chromatogram shows also three compounds (**9**, **10** and **11**) with  $R_t$  19.9, 19.2 and 20.5 min and parent ions  $m/z$  331, 301 and 361  $[M+H]^+$  respectively. These compounds were confirmed to be 6-Hydroxykaempferol 3,7-dimethyl ether, Hispidulin and Chrysosplenol C, respectively by comparing their retention time and MS/MS data with those of the authentic substances (Garayev et al., 2017).

### 3.2 Confirmation by NMR of the structures of isolated compounds from L2E

To confirm the structural assumptions, the compounds have been isolated and identified by NMR  $^1H$ ,  $^{13}C$  and 2D NMR data were compared to the spectroscopic data reported in the literature. The following compounds are present in the flowers of *I. montana* (Figure 1).

Compound **1** shows a molecular formula to be  $C_{16}H_{18}O_9$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  355  $[M+H]^+$ . The  $^{13}C$  NMR and edited-HSQC NMR spectra of **1** displayed 16 carbon signals included six quaternary carbons ( $\delta_C$  177.2, 168.7, 149.6, 146.8, 127.8, 76.2), eight methine ( $\delta_C$  147.1, 123.0, 116.5, 115.2, 115.1, 73.5, 72.0, 71.3) and two methylene groups ( $\delta_C$  38.2, 36.8). Among the four quaternary carbons, one was an acid carbon at  $\delta_C$  177.2 (C-7), one was an ester carbon at  $\delta_C$  168.7 (C-9'), two were an oxygenated aromatic carbon according to its chemical shift at  $\delta_C$  149.6 (C-4'), 146.8 (C-3'). Among the eight methine groups, five corresponded to aromatic carbons according to their chemical shifts at  $\delta_C$  147.1 (C-7'), 123.0 (C-6'), 116.5 (C-5'), 115.2 (C-8'), 115.1 (C-2') and three corresponded to oxymethine carbons according to their chemical shifts at  $\delta_C$  73.5 (C-4), 72.0 (C-5), 71.3 (C-3). All methylene groups are alkane to their chemical shifts at  $\delta_C$  38.2 (C-2) and 36.8 (C-6). The  $^1H$  NMR spectrum of **1** displayed an ABX system at  $\delta_H$  7.05 (1H, *d*,  $J = 2.0$  Hz, H-2'), 6.96 (1H, *dd*,  $J = 8.1, 2.0$  Hz, H-6'), 6.79 (1H, *d*,  $J = 8.1$  Hz, H-5') and two *trans* olefinic protons as doublets ( $J = 16.0$  Hz) at  $\delta_H$  7.56 (H-7'), 6.26 (H-6') giving a crosspeak with an ester function at  $\delta_C$  168.7 (C-9') in HMBC spectrum and a crosspeak with the aromatic carbon at  $\delta_C$  127.8 (C-1'), 123.0 (C-6'), 115.1 (C-2') corresponded to a *trans*-caffeoyl group. The second part of proton at  $\delta_H$  5.33 (H-5), 3.72 (H-4<sub>ax</sub>), 2.23 (H-6<sub>eq</sub>), 2.18 (H-2<sub>ax</sub>), 2.08 (H-6<sub>ax</sub>), 2.05 (H-2<sub>eq</sub>) were characteristic of the quinic acid. The deshielded proton 5 at

$\delta_H$  5.33 and the  $^3J = \text{HMBC}$  correlation with ester carbon at  $\delta_C$  168.7 (C-9') showed that *trans*-caffeoyl moiety was attached on C-3 of the quinic acid. Based on further comparison of the NMR data with those in the literature (Pauli et al., 1999), the structure of **1** was determined to be chlorogenic acid.

The molecular formula of compound **4** was deduced to be  $\text{C}_{25}\text{H}_{24}\text{O}_{12}$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  517  $[\text{M}+\text{H}]^+$ . Compared to compound **1**, the  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra of **4**, indicated the presence of two *trans*-caffeoyl groups and a quinic acid. The position of the two *trans*-caffeoyl groups on quinic acid was established by the downfield shift of the proton signals at  $\delta_H$  5.42 (H-3<sub>eq</sub>) and  $\delta_H$  5.42 (H-5<sub>ax</sub>) and carbon signals at  $\delta_C$  73.0 (C-5) and  $\delta_C$  72.2 (C-3) and by the  $^3J$  HMBC correlation of H-3<sub>eq</sub> at  $\delta_H$  5.42 with ester carbon at  $\delta_C$  168.5 (C-9') and H-5<sub>ax</sub> at  $\delta_H$  5.42 with ester carbon at  $\delta_C$  169.0 (C-9'') showed that two *trans*-caffeoyl moiety were attached on C-3 and on C-5 respectively of the quinic acid. Based on further comparison of the NMR data with those in the literature (Kodoma et al., 1998), the structure of **4** was determined to be 3,5-*O*-Dicaffeoylquinic acid.

Compound **5** presents a molecular formula to be  $\text{C}_{25}\text{H}_{24}\text{O}_{12}$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  517  $[\text{M}+\text{H}]^+$ . The  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra of **5** indicated the presence of two *trans*-caffeoyl groups and a quinic acid and were also similar to those of compound **4**. The major differences were the chemical shift of C-1, C-3 and H-3<sub>eq</sub> at the quinic acid moiety in compound **5**. The C-1 of **5** was deshielded at  $\delta_C$  80.8 and C-3, H-3<sub>eq</sub> were shielded at  $\delta_C$  69.3 and at  $\delta_H$  4.31 respectively, showed that the second *trans*-caffeoyl group was attached on C-1 of the quinic acid. Based on further comparison of the NMR data with those in the literature (Merfort, 1992), the structure of **5** was determined to be 1,5-*O*-Dicaffeoylquinic acid.

The molecular formula of compound **2** was deduced to be  $\text{C}_{15}\text{H}_{10}\text{O}_5$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  287  $[\text{M}+\text{H}]^+$ . The  $^{13}\text{C}$  NMR and edited-HSQC NMR spectra of **2** displayed 15 carbon signals included nine quaternary ( $\delta_C$  184.3, 166.2, 166.0, 163.2, 159.4, 151.0, 147.1, 123.8, 105.4) and six oxymethine carbons ( $\delta_C$  103.5, 120.4, 116.9,

114.3, 100.2, 95.3). Among the nine quaternary carbons, one was an aromatic ketone carbon at  $\delta_C$  184.3 (C-4), six were oxygenated aromatic carbon according to their chemical shifts at  $\delta_C$  166.2 (C-2), 166.0 (C-7), 163.2 (C-5), 159.4 (C-9), 151.0 (C-4'), 147.1 (C-3') and two are aromatic carbon according to their chemical shifts at  $\delta_C$  123.8 (C-1'), 105.4 (C-10). All oxymethine carbons were aromatic carbons according to their chemical shifts at  $\delta_C$  120.4 (C-6'), 116.9 (C-5'), 114.3 (C-2'), 100.2 (C-6), 95.3 (C-8). The  $^1\text{H}$  NMR spectrum of **2** displayed an ABX system at  $\delta_H$  7.35 (1H, *m*, H-2'), 7.35 (1H, *m*, H-6'), 6.89 (1H, *d*,  $J = 8.8$  Hz, H-5') due to a 3',4'-disubstitution pattern of ring B. Moreover, two *meta*-coupled doublets at  $\delta_H$  6.41 (1H,  $J = 2:1$  Hz, H-8) and 6.19 (1H,  $J = 2:1$  Hz, H-6) were consistent with 5,7-dioxygenated ring A. These resonances together with the singlet at  $\delta_H$  6.52 (H-3) and the corresponding carbon signals revealed the presence of a luteolin. Based on further comparison of the NMR data with those in the literature (Azimova and Vinogradova, 2013), the structure of **2** was determined to be luteolin.

Compound **3** shows the molecular formula  $\text{C}_{16}\text{H}_{12}\text{O}_7$  deduced by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  317  $[\text{M}+\text{H}]^+$ . Compared to compound **2**, the  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra of **3** are similar. The major differences were the presence of the chemical shift at  $\delta_C$  60.9 and at  $\delta_H$  3.88 (3H, *s*) corresponding of methoxy group, and a disappeared of H-6 signal. The position of the methoxy group was established by the downfield shift of the carbon signals at  $\delta_C$  132.8 (C-3) and the  $^3\text{J}$  crosspeak in HMBC spectrum between methoxy group at  $\delta_H$  3.88 and carbon signals at  $\delta_C$  132.8 (C-3), showed that the methoxy group was attached on C-3 of the flavone. Based on further comparison of the NMR data with those in the literature (Azimova and Vinogradova, 2013), the structure of **3** was determined to be Nepetin.

Compound **6** presents a molecular formula  $\text{C}_{22}\text{H}_{22}\text{O}_{12}$  deduced by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  479  $[\text{M}+\text{H}]^+$ . Compared to compound **3**, the  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra of **3** are similar. Additionally, the resonances of on anomeric proton, indicative of the presence of one  $\beta$ - linked sugar unit  $\delta_H$  5.12 (1H, *d*,  $J = 7:6$  Hz, H-1''). Assignments for all proton and carbon resonances were achieved by COSY, HSQC and HMBC



experiments, which indicated the presence of  $\beta$ -glucosyl as sugar units. The position of the  $\beta$ -glucosyl as sugar units was established by the  $^3J$  crosspeak in HMBC spectrum between the anomeric proton H-1'' at  $\delta_H$  5.12 and carbon signal at  $\delta_C$  157.6 (C-3), showed that the  $\beta$ -glucosyl moiety was attached on C-7 of the flavone. Based on further comparison of the NMR data with those in the literature (Monsef–Esfahani et al., 2014), the structure of **6** was determined to be Nepitrin.

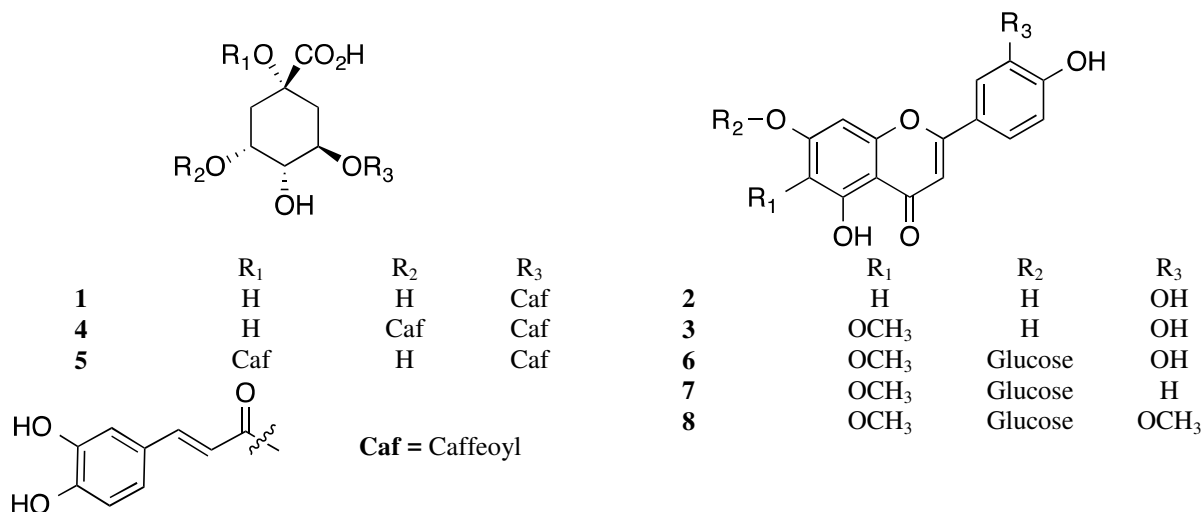
The molecular formula of compound **7** was deduced to be  $C_{22}H_{22}O_{11}$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  463  $[M+H]^+$ . Compared to compound **6**, the  $^{13}C$  and  $^1H$  NMR spectra of **7** are similar, excepted by a presence of an AB system at  $\delta_H$  6.95 (2H,  $d$ ,  $J = 8:7$  Hz, H-2', H-6'),  $\delta_H$  6.95 (2H,  $d$ ,  $J = 8:7$  Hz, H-3', H-5') due to a 4'-substitution pattern of ring B. Based on further comparison of the NMR data with those in the literature (Weng and Wang, 2000), the structure of **7** was determined to be Hispiduloside.

Compound **8** shows a molecular formula  $C_{23}H_{24}O_{12}$  by positive mode electrospray ionization mass spectrometry (ESI-MS) data at  $m/z$  493  $[M+H]^+$ . Compared to compound **6**, the  $^{13}C$  and  $^1H$  NMR spectra of **8** are similar. The major differences were the presence of the chemical shift at  $\delta_C$  56.4 and at  $\delta_H$  3.97 (3H,  $s$ ) corresponding of a second methoxy group. The position of the second methoxy group was established by the  $^3J$  crosspeak in HMBC spectrum between methoxy group at  $\delta_H$  3.97 and carbon signals at  $\delta_C$  149.6 (C-3'), showed that the second methoxy group was attached on C-3' of the flavone ring B. Based on further comparison of the NMR data with those in the literature (Hong, 2008), the structure of **8** was determined to be Jaceosid.

Compounds **1** and **4-8** are reported in *I. montana* for the first time.

Based on literature data, the study of the phenolic composition of *A. montana* shows that five compounds (**1**, **2**, **4**, **5** and **7**) isolated from *I. montana* are also present in *A. montana* flowers (Jaiswal et al., 2011; Merfort, 1992; Merfort and Wendisch, 1987).

**Figure 1.** Isolated phenolic acids and flavonoids from *I. montana*



### 3.3 Evaluation of the anti-inflammatory potential of isolated compounds

Nitric oxide (NO), which is produced by the NO synthase when induced by pro-inflammatory cytokines, has been shown as an important element of the inflammatory mechanisms. During bacterial infection more particularly, a 1000-fold increase of NO production may be observed in neutrophils and macrophages. NO plays two essential roles in cellular-response to bacterial infection: when excreted in the extracellular environment, it acts as a mediator of the inflammatory response, whereas in the lysosomal environment it contributes to microbe destruction with other oxygen radicals. However, although NO is essential in the cellular defense against infectious diseases, its overproduction may lead to adverse effects such as autoimmune reactions and neurodegenerative syndromes. As a consequence, inhibition of NO synthesis or NO scavenging may be considered as possible strategies in the treatment of chronic inflammatory diseases (Sharma et al., 2007).

In the present study, NO-inhibition was assessed in murine macrophages (RAW 267.4 cell line), in which a strong inflammatory response, leading to NO synthesis and secretion in the culture medium, was initiated by LPS of *E. coli* (Gao et al., 2018; Pansanit et al., 2013).

Results are presented in Table 3. All compounds showed inhibitory effects of NO production with IC<sub>50</sub> < 32 μM, however compounds **2**, **3**, **5-8** have been found to be more active than positive control dexamethasone.

**Table 3.** Anti-inflammatory potential of compounds **1-8**

Compounds	NO release. IC <sub>50</sub> (μM)
<b>1</b>	31.46 ± 5.20
<b>2</b>	0.52 ± 0.01
<b>3</b>	3.04 ± 0.27
<b>4</b>	6.94 ± 0.98
<b>5</b>	2.54 ± 0.34
<b>6</b>	1.23 ± 0.09
<b>7</b>	0.48 ± 0.00
<b>8</b>	0.34 ± 0.01
Dexamethasone <sup>a</sup>	3.89 ± 0.94

<sup>a</sup>Positive control

Most of the isolated phenolic compounds ~~in~~ from the flowers of *I. montana* present promising anti-inflammatory.

These results allow to validate scientifically the traditional use of flowers for the relief of bruises.

The study provides evidence that *Inula montana* presents potential anti-inflammatory effect. Further experiments are necessary to confirm these first results in order to propose *Inula montana* as an alternative to *Arnica montana*. Moreover, active compounds **2**, **5** and **7** are present in the both species.

### 3.4 Analytical validation of UHPLC-DAD method

Three major active compounds Chlorogenic acid (**1**), Nepetin (**3**) and 1,5-*O*-Dicafeoylquinic acid (**5**) were retained for their quantification in the plant material, in order to evaluate the quality of *Inula montana*. This UHPLC-DAD method was validated in term of analysis of specificity, response functions, trueness, precision, accuracy, linearity and limits of detection and quantification. The validation summary can be found in Table 4.

The specificity of the method has been investigated. Calibration and validation standards were prepared and analyzed in terms of specificity. No interference and great resolution were observed for each compound which was assessed by comparing the retention time and the UV spectra with the standard. Furthermore, the purity of the investigated peak was confirmed by DAD purity studies.

The calibration curves were based on the through-origin linear regression model which was

fitted with concentration levels ranging from 29.1-94.0 µg/mL, 70.5-221.0 µg/mL and 10.4-32.1 µg/mL for Chlorogenic acid (CA), 1,5-*O*-DicaFFEoylquinic acid (DCQA) and Nepetin (NPT), respectively. Each of three calibration points was prepared in duplicate and each of five validation points was prepared in triplicate over three consecutive days. The coefficients of determination of each-day equation of the regression line were all greater than  $R^2 > 0.999$  for the calibration and validation standards.

The precision of the method was determined by computing the Relative Standard Deviation (RSDs) for repeatability and intermediate precision at each concentration level of the validation standards. The precision of the validation standards did not exceed 5% for each compound, as shown in Table 4. The comparable RSDs between repeatability and intermediate fidelity were mainly due to non-significant intergroup variances, validating the precision of the developed method.

**Table 4** Results of validation of UHPLC-DAD method for the quantitative analysis of Chlorogenic acid (CA), 1,5-*O*-Dicaaffeoylquinic acid (DCQA) and Nepetin (NPT).

Validation criteria	CA		DCQA		NPT	
<b>Response function</b> (m=3; d=3; n=2;) <sup>a</sup> Y=ax						
Day 1	R <sup>2</sup> =0.9999		R <sup>2</sup> =0.9999		R <sup>2</sup> =0.9998	
Day 2	R <sup>2</sup> =0.9999		R <sup>2</sup> =0.9998		R <sup>2</sup> =0.9997	
Day 3	R <sup>2</sup> =0.9999		R <sup>2</sup> =0.9998		R <sup>2</sup> =0.9997	
Linear Range	29.1-94.0 µg/mL		70.5-221.0 µg/mL		10.4-32.1 µg/mL	
<b>Trueness</b> (m=5; d=3; n=3) <sup>a</sup>	Concentration (µg/mL)	Relative bias (%)	Concentration (µg/mL)	Relative bias (%)	Concentration (µg/mL)	Relative bias (%)
Level 1	30.4	0.75	71.9	-0.05	10.5	-0.16
Level 2	45.7	2.72	108.0	0.39	15.8	0.25
Level 3	60.8	0.68	143.9	0.49	21.0	-0.02
Level 4	75.9	-1.70	179.6	-0.26	26.3	0.39
Level 5	91.2	-2.46	215.6	-0.57	31.5	-0.46
<b>Precision</b> (d=3; n=3) <sup>a</sup>	Repeatability RSD (%)	Intermediate precision RSD (%)	Repeatability RSD (%)	Intermediate Precision RSD (%)	Repeatability RSD (%)	Intermediate Precision RSD (%)
Level 1	0.90	0.90	1.96	1.96	1.41	1.58
Level 2	0.90	0.90	1.29	1.38	1.66	1.66
Level 3	1.64	1.64	1.81	1.81	1.95	1.95
Level 4	1.08	1.08	1.74	1.74	1.49	1.71
Level 5	0.94	0.94	1.32	1.32	1.03	1.03
<b>Accuracy</b> (d=3; n=3) <sup>a</sup>	β-expectation lower and upper tolerance limits of the relative errors (%)		β-expectation lower and upper tolerance limits of the relative errors (%)		β-expectation lower and upper tolerance limits of the relative errors (%)	
Level 1	-1.49; 2.98		-4.92; 4.82		-4.31; 3.99	
Level 2	0.48; 4.97		-3.21; 3.99		-3.90; 4.40	
Level 3	-3.40; 4.77		-4.01; 4.99		-4.88; 4.85	
Level 4	-4.40; 1.01		-4.60; 4.07		-4.14; 4.92	
Level 5	-4.90; -0.02		-3.86; 2.72		-3.04; 2.12	
<b>Linearity slope</b>	0.9889		0.9979		0.9993	
R <sup>2</sup>	0.9987		0.9994		0.9997	
<b>LOD<sup>b</sup></b>	0.12 µg/mL		0.17 µg/mL		0.09 µg/mL	
<b>LOQ<sup>b</sup></b>	0.39 µg/mL		0.56 µg/mL		0.31 µg/mL	

<sup>a</sup>m: number of calibration points; d: days; n: replicates. <sup>b</sup>LOD = limit of detection, LOQ = limit of quantification.

Trueness and intermediate fidelity were used for the accuracy profile. The acceptance thresholds were set at  $\pm 5\%$  and the results are acceptable with -4.90 and 4.97% for CA, -4.92 and 4.99% for DCQA and -4.88 – 4.92% for NPT, as it is shown in Table 4.

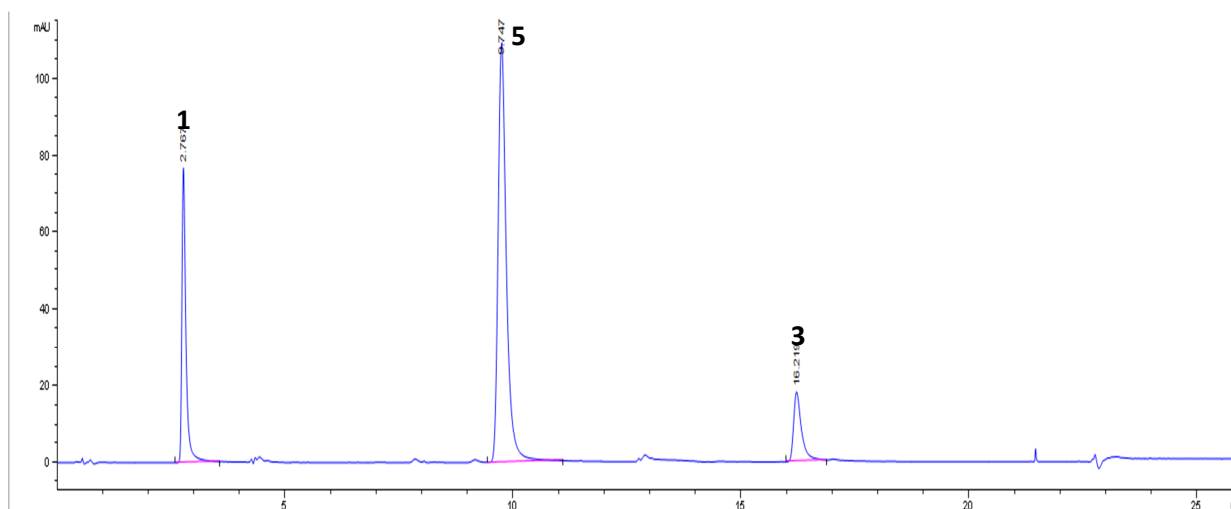
The linearity of this method was confirmed by a linear regression function of estimated concentrations to introduced concentrations calculated all the series of validation standards (N = 45). The following equations of the regression line were determined  $y=0.9889x$  ( $R^2=0.9987$ ),  $y=0.9979x$  ( $R^2=0.9994$ ) and  $y=0.9993x$  ( $R^2=0.9997$ ) for CA, DCQA and NPT, respectively, as it is shown in Table 4.

Limit of Detection (LOD) and Limit of Quantification (LOQ) were determined by calculating LOD and LOQ as 3 and 10 times of intensity of background noise, respectively. LOD and LOQ of CA, DCQA and NPT were calculated to be 0.12 and 0.39  $\mu\text{g/mL}$ , 0.17 and 0.56  $\mu\text{g/mL}$ , 0.09 and 0.31  $\mu\text{g/mL}$ , respectively.

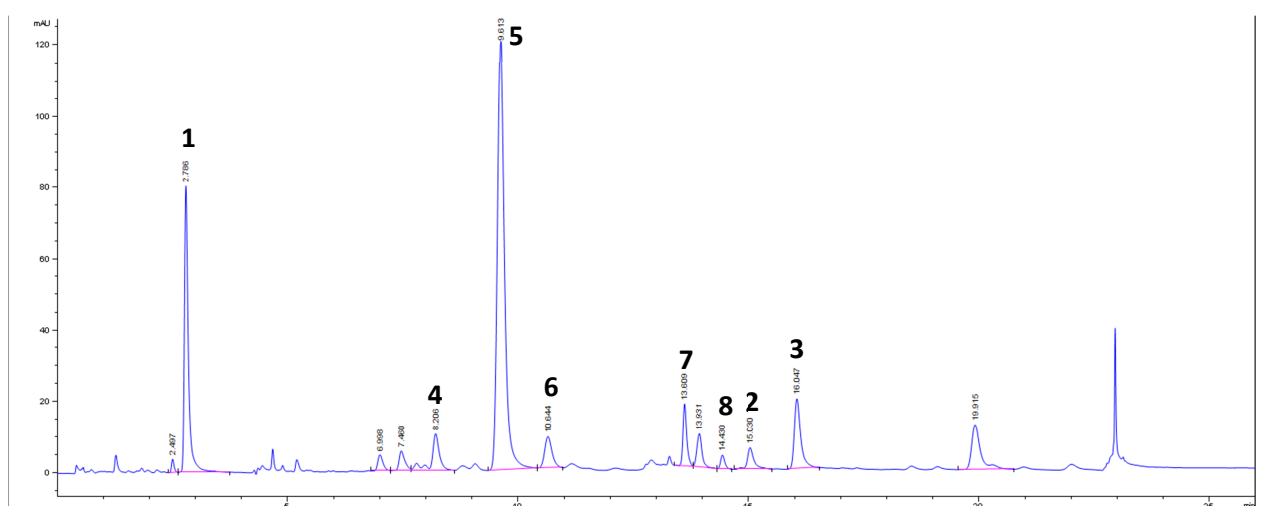
The retention times of CA, DCQA and NPT were 2.79, 9.61 and 16.05 min, respectively. The chromatograms of the calibration and validation standards are presented in Figure 2. These results show that UHPLC-DAD method is validated for the quantification of Chlorogenic acid, 1,5-*O*-Dicafeoylquinic acid and Nepetin in flowers of *Inula montana* L.

**Figure 2.** Chromatograms of Calibration and Validation Standards by UHPLC-DAD at 335 nm

### Calibration standard



### Validation standard



Chlorogenic acid (1), Luteolin (2), Nepetin (3), 3,5-*O*-Dicafeoylquinic acid (4), 1,5-*O*-Dicafeoylquinic acid (5), Nepitrin (6), Hispiduloside (7) and Jaceosid (8)

### 3.5 Comparison of the content of CA, DCQA and NPT in *Inula montana* from different collect sites

The validated method was applied for the quantitative analysis of the three major compounds in flowers: Chlorogenic acid (CA), 1,5-*O*-Dicafeoylquinic acid (DCQA) and Nepetin (NPT), from the two collection sites of Regional Natural Park of Luberon. One-way ANOVA confirms significant results between the mean content of phenolic compounds at  $p < 0.01$  (Table 5).

**Table 5** Quantification of Chlorogenic acid (CA), 1,5-*O*-Dicafeoylquinic acid (DCQA) and Nepetin (NPT) in flowers of *Inula montana* L. from different collection sites

Place of collect	Collect date	Altitude (m)	CA content <sup>a</sup> (%)	DCQA content <sup>a</sup> (%)	NPT content <sup>a</sup> (%)
Merindol	03/06/2015	170	0.61±0.02	1.44±0.03	0.21±0.01
Bonnieux	31/05/2017	680	0.39±0.01	1.02±0.11	0.25±0.01
Merindol	31/05/2017	170	0.39±0.02	0.92±0.05	0.22±0.02

% = g per 100 g of dry plant material. <sup>a</sup>The mean of three independent analysis ± Standard Deviation. The results of one-way ANOVA test are significant at  $p < 0.01$ ;

The content of Chlorogenic acid and 1,5-*O*-Dicafeoylquinic acid in flowers from Merindol was higher in 2015 with 0.61 and 1.44 %, respectively. In order to compare the influence of altitude on the content of phenolic compounds, two collection sites were compared: Merindol (170 m) and Bonnieux (680 m). Despite the fact that the altitude in Merindol (170 m) is four times lower than in Bonnieux (680 m), no significant differences in the content of each phenolic compound were observed between the two collection sites.

#### 4. Conclusion

Bio-guided fractionation of 95% ethanolic crude extract allowed the identification of eleven compounds from *Inula montana*. Chlorogenic acid (**1**), 3,5-*O*-Dicafeoylquinic acid (**4**), 1,5-*O*-Dicafeoylquinic acid (**5**), Nepitrin (**6**), Hispiduloside (**7**) and Jaceosid (**8**) are reported in this species for the first time. Among them, **1**, **4**, **5** and **7** are also present in *Arnica montana*.

A simple, accurate and rapid UHPLC-DAD method was developed as the first report of a simultaneous determination of three main phenolic compounds, chosen as analytical markers of this species, Chlorogenic acid, 1,5-*O*-Dicafeoylquinic acid and Nepetin in *Inula montana*. This validated method allows the quality control of the herbal drug and was applied for the comparison of the phenolic content of the two collection sites: Bonnieux and Merindol. We can conclude that the content of phenolic compounds was close for these collection sites.



The potential anti-inflammatory effect, based on NO-inhibition, of different extracts and compounds from *Inula montana* provides the scientific basis for the traditional use of this plant for the relief of bruises and underlines the ethnopharmacological relevance. Nevertheless this interesting biological activity should be investigated by complementary experiments concerning other mediators of the inflammatory response (TNF $\alpha$ , IL-1) in order to confirm the anti-inflammatory activity and to propose *Inula montana* as an alternative to *Arnica montana*.

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### **Conflict of interest statement**

The authors declare no competing financial interest.

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## SUPPORTING INFORMATION

### **Validation of UHPLC-DAD analysis method of Chlorogenic acid (CA)**

Tab.1 Raw data of Calibration standard

Tab.2 Raw data of Validation standard

Fig.1 Response function of Calibration standard (CS). Two points per day for each concentration over three days

Fig.2 Linearity of Validation standard (VS). Three points per day for each concentration over three days.

Fig.3 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)

### **Validation of UHPLC-DAD analysis method of 1,5-*O*-Dicafeoylquinic acid (DCQA)**

Tab.3 Raw data of Calibration standard

Tab.4 Raw data of Validation standard

Fig.4 Response function of Calibration standard (CS). Two points per day for each concentration over three days.

Fig.5 Linearity of Validation standard (VS). Three points per day for each concentration over three days

Fig.6 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)

### **Validation of UHPLC-DAD analysis method of Nepetin (NPT)**

Tab.5 Raw data of Calibration standard

Tab.6 Raw data of Validation standard

Fig.7 Response function of Calibration standard (CS). Two points per day for each concentration over three days.

Fig.8 Linearity of Validation standard (VS). Three points per day for each concentration over three days

Fig.9 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)

## Validation of UHPLC-DAD analysis method of Chlorogenic acid (CA)

**Tab. 1 Raw data of Calibration standard**

Level / sample / Day	Level	Theoretical concentration (mg/mL)	Introduced concentration (mg/mL)	CA area
				Results
1.1.1	50%	0.0030	0.0296	232
1.2.1		0.0030	0.0296	238
1.1.2		0.0030	0.0296	234
1.2.2		0.0030	0.0296	241
1.1.3		0.0030	0.0296	243
1.2.3		0.0030	0.0296	244
2.1.1	100%	0.0060	0.0592	482
2.2.1		0.0060	0.0592	488
2.1.2		0.0060	0.0592	484
2.2.2		0.0060	0.0592	497
2.1.3		0.0060	0.0592	494
2.2.3		0.0060	0.0592	497
3.1.1	150%	0.0090	0.0888	724
3.2.1		0.0090	0.0888	732
3.1.2		0.0090	0.0888	733
3.2.2		0.0090	0.0888	739
3.1.3		0.0090	0.0888	755
3.2.3		0.0090	0.0888	745

**Tab.2 Raw data of Validation standard**

Level / sample / Day	Level	Introduced concentration equivalent content <sup>[1]</sup> (mg/mL)	Results	
			CA area	Inverse prediction Calculated concentration X = Y/a
1.1.1	50%	0.0312	254	0.0304
1.2.1		0.0312	256	0.0307
1.3.1		0.0314	256	0.0305
1.1.2		0.0307	258	0.0312
1.2.2		0.0308	255	0.0308
1.3.2		0.0309	252	0.0303
1.1.3		0.0293	245	0.0305
1.2.3		0.0292	246	0.0308
1.3.3		0.0291	245	0.0306
2.1.1	75%	0.0473	397	0.0471
2.2.1		0.0470	396	0.0473
2.3.1		0.0469	393	0.0470
2.1.2		0.0461	389	0.0472
2.2.2		0.0463	388	0.0468
2.3.2		0.0461	384	0.0467
2.1.3		0.0437	380	0.0475
2.2.3		0.0438	370	0.0463
2.3.3		0.0438	370	0.0463
3.1.1	100%	0.0626	517	0.0617
3.2.1		0.0625	515	0.0616
3.3.1		0.0625	513	0.0614
3.1.2		0.0616	513	0.0621
3.2.2		0.0614	493	0.0597
3.3.2		0.0616	497	0.0601
3.1.3		0.0584	505	0.0630
3.2.3		0.0583	489	0.0611
3.3.3		0.0586	486	0.0606



Level / sample / Day	Level	Introduced concentration equivalent content <sup>[1]</sup> (mg/mL)	Results	
			CA area	Inverse prediction Calculated concentration X = Y/a
4.1.1	125%	0.0781	635	0.0757
4.2.1		0.0781	615	0.0733
4.3.1		0.0782	626	0.0746
4.1.2		0.0768	626	0.0758
4.2.2		0.0768	615	0.0744
4.3.2		0.0767	616	0.0746
4.1.3		0.0728	594	0.0744
4.2.3		0.0729	602	0.0752
4.3.3		0.0731	592	0.0739
5.1.1	150%	0.0940	749	0.0891
5.2.1		0.0936	747	0.0893
5.3.1		0.0939	733	0.0873
5.1.2		0.0921	740	0.0896
5.2.2		0.0922	735	0.0890
5.3.2		0.0923	732	0.0885
5.1.3		0.0874	721	0.0902
5.2.3		0.0875	704	0.0880
5.3.3		0.0875	713	0.0892

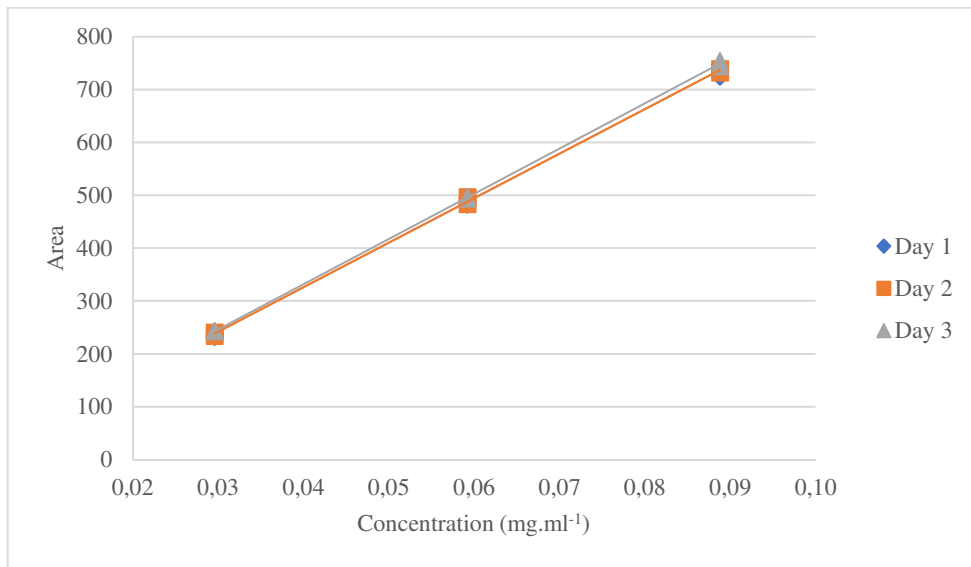
<sup>1</sup>: *Constituent Equivalent (mg/ml)* : calculated using the mean of Chlorogenic acid content calculated on the corresponding day :

Day 1: IMFLF151 contains: 0.624 % Chlorogenic acid,

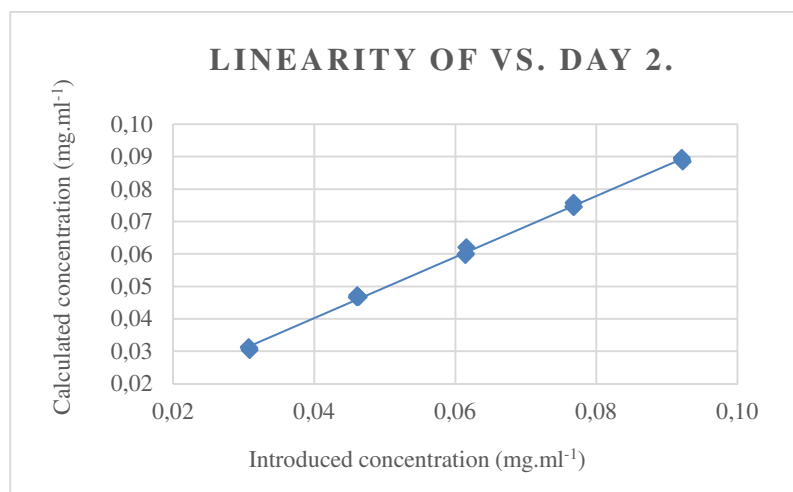
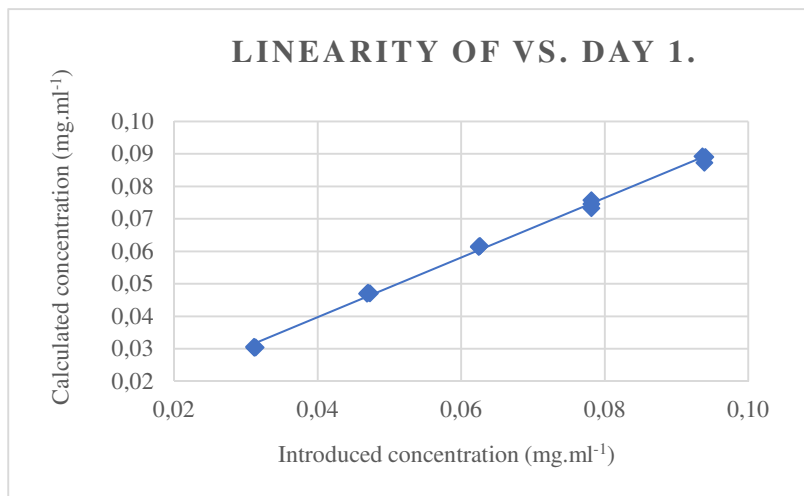
Day 2: IMFLF151 contains: 0.614 % Chlorogenic acid,

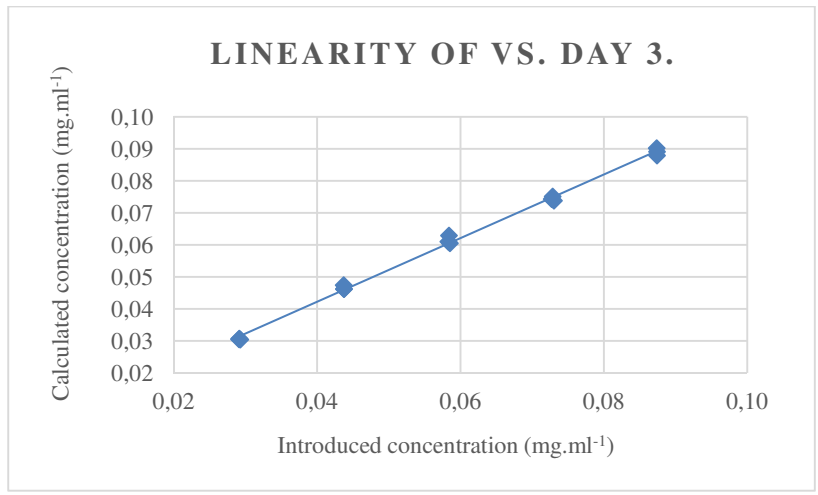
Day 3: IMFLF151 contains: 0.582 % Chlorogenic acid.

**Fig.1 Response function of Calibration standard (CS). Two points per day for each concentration over three days**

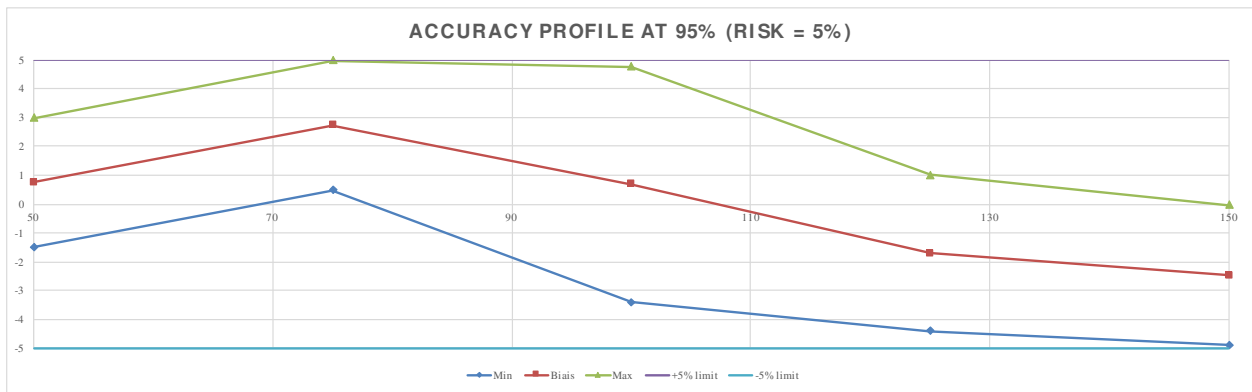


**Fig.2 Linearity of Validation standard (VS). Three points per day for each concentration over three days.**





**Fig.3 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)**



**Validation of UHPLC-DAD analysis method of 1,5-*O*-Dicafeoylquinic acid (DCQA)**

**Tab.3 Raw data of Calibration standard**

Level / sample / Day	Level	Theoretical concentration (mg/mL)	Introduced concentration (mg/mL)	DCQA area
				Results
1.1.1	50%	0.0652	0.0652	600
1.2.1		0.0652	0.0652	593
1.1.2		0.0652	0.0652	569
1.2.2		0.0652	0.0652	568
1.1.3		0.0652	0.0652	597
1.2.3		0.0652	0.0652	567
2.1.1	100%	0.1304	0.1304	1212
2.2.1		0.1304	0.1304	1231
2.1.2		0.1304	0.1304	1196
2.2.2		0.1304	0.1304	1164
2.1.3		0.1304	0.1304	1195
2.2.3		0.1304	0.1304	1170
3.1.1	150%	0.1956	0.1956	1838
3.2.1		0.1956	0.1956	1815
3.1.2		0.1956	0.1956	1784
3.2.2		0.1956	0.1956	1792
3.1.3		0.1956	0.1956	1799
3.2.3		0.1956	0.1956	1756

**Tab.4 Raw data of Validation standard**

Level / sample / Day	Level	Introduced concentration equivalent content <sup>[2]</sup> (mg/mL)	Results	
			DCQA area	Inverse prediction Calculated concentration X = Y/a
1.1.1	50%	0.0734	691	0.0729
1.2.1		0.0732	694	0.0734
1.3.1		0.0737	669	0.0702
1.1.2		0.0715	671	0.0737
1.2.2		0.0717	646	0.0706
1.3.2		0.0718	648	0.0707
1.1.3		0.0709	652	0.0722
1.2.3		0.0707	658	0.0731
1.3.3		0.0705	632	0.0704
2.1.1	75%	0.1112	1051	0.1099
2.2.1		0.1105	1059	0.1114
2.3.1		0.1102	1015	0.1070
2.1.2		0.1072	989	0.1085
2.2.2		0.1077	981	0.1073
2.3.2		0.1071	971	0.1068
2.1.3		0.1059	973	0.1083
2.2.3		0.1060	975	0.1083
2.3.3		0.1061	974	0.1082
3.1.1	100%	0.1472	1376	0.1447
3.2.1		0.1470	1366	0.1439
3.3.1		0.1469	1351	0.1424
3.1.2		0.1432	1362	0.1491
3.2.2		0.1429	1305	0.1433
3.3.2		0.1431	1307	0.1432
3.1.3		0.1416	1325	0.1470
3.2.3		0.1413	1322	0.1469
3.3.3		0.1419	1271	0.1408

Level / sample / Day	Level	Introduced concentration equivalent content <sup>[2]</sup> (mg/mL)	Results	
			DCQA area	Inverse prediction Calculated concentration $X = Y/a$
4.1.1	125%	0.1837	1717	0.1807
4.2.1		0.1837	1712	0.1802
4.3.1		0.1838	1666	0.1751
4.1.2		0.1785	1670	0.1831
4.2.2		0.1787	1641	0.1798
4.3.2		0.1784	1638	0.1798
4.1.3		0.1763	1570	0.1747
4.2.3		0.1766	1648	0.1829
4.3.3		0.1771	1592	0.1763
5.1.1	150%	0.2210	2056	0.2158
5.2.1		0.2201	2035	0.2145
5.3.1		0.2207	2004	0.2106
5.1.2		0.2142	1995	0.2188
5.2.2		0.2143	1924	0.2110
5.3.2		0.2146	1934	0.2119
5.1.3		0.2118	1945	0.2162
5.2.3		0.2119	1925	0.2139
5.3.3		0.2119	1954	0.2169

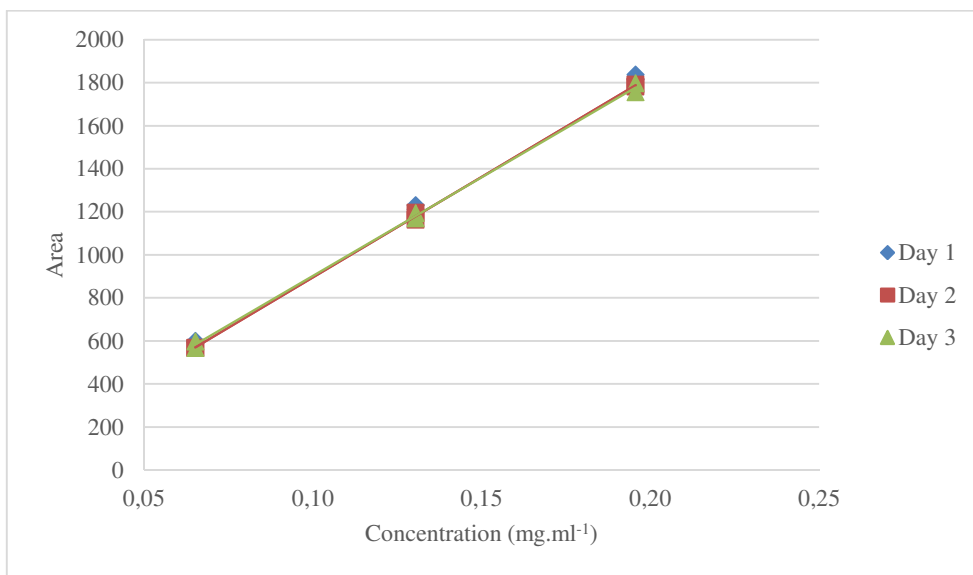
<sup>2</sup>: *Constituent Equivalent (mg/ml)* : calculated using the mean of 1,5-*O*-Dicafeoylquinic acid content calculated on the corresponding day :

Day 1: IMFLF151 contains: 1.468 % 1,5-*O*-Dicafeoylquinic acid,

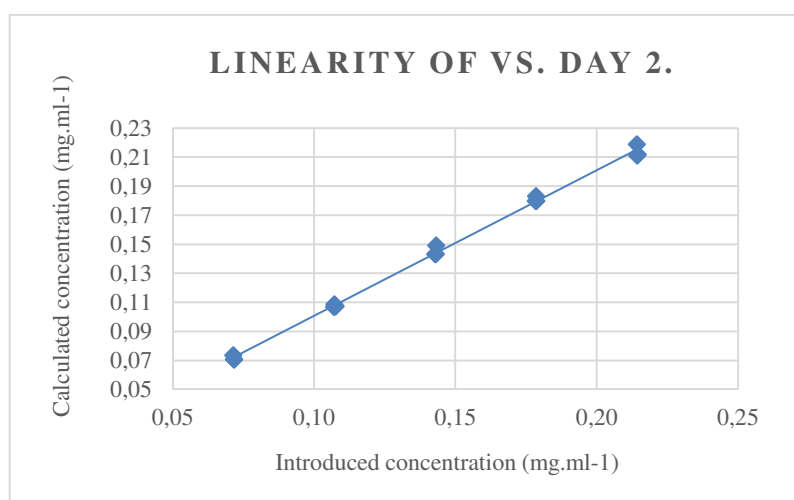
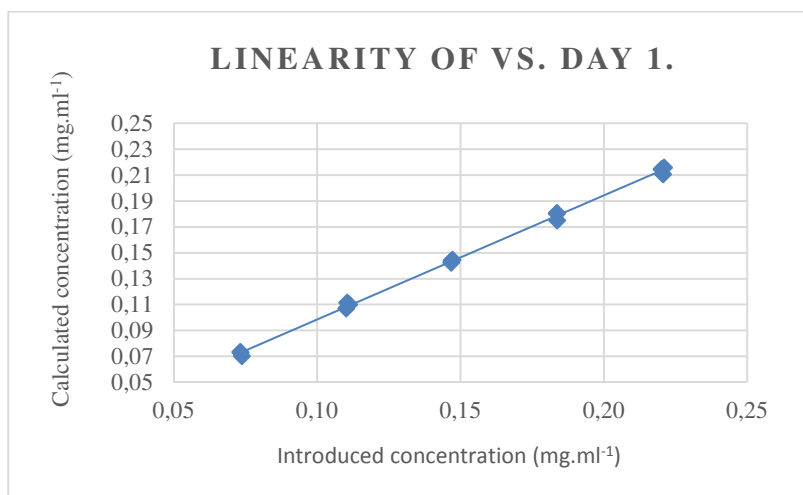
Day 2: IMFLF151 contains: 1.427 % 1,5-*O*-Dicafeoylquinic acid,

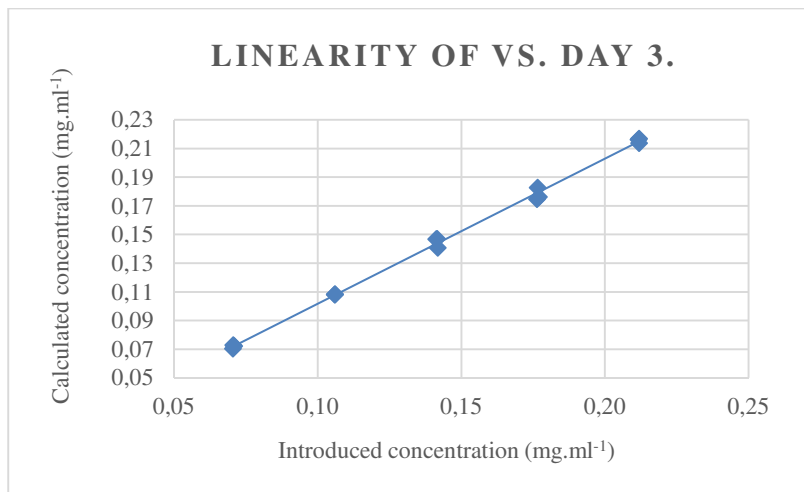
Day 3: IMFLF151 contains: 1.411 % 1,5-*O*-Dicafeoylquinic acid.

**Fig.4 Response function of Calibration standard (CS). Two points per day for each concentration over three days.**

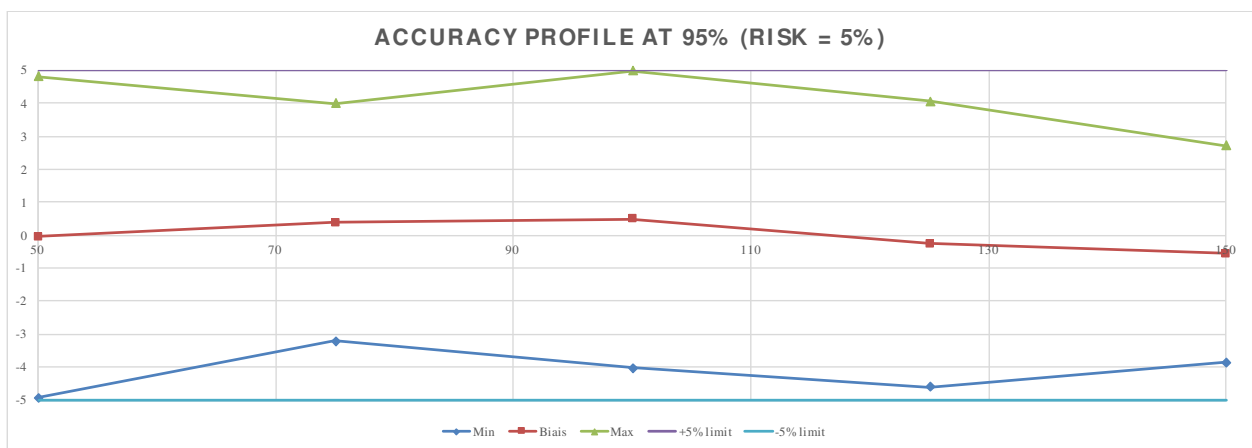


**Fig.5 Linearity of Validation standard (VS). Three points per day for each concentration over three days**





**Fig.6 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)**





## Validation of UHPLC-DAD analysis method of Nepetin (NPT)

**Tab.5 Raw data of Calibration standard**

Level / sample / Day	Level	Theoretical concentration (mg/mL)	Introduced concentration (mg/mL)	NPT area
				Results
1.1.1	50%	0.0120	0.0136	126
1.2.1		0.0120	0.0136	128
1.1.2		0.0120	0.0136	125
1.2.2		0.0120	0.0136	130
1.1.3		0.0120	0.0136	127
1.2.3		0.0120	0.0136	126
2.1.1	100%	0.0240	0.0273	257
2.2.1		0.0240	0.0273	265
2.1.2		0.0240	0.0273	261
2.2.2		0.0240	0.0273	264
2.1.3		0.0240	0.0273	260
2.2.3		0.0240	0.0273	265
3.1.1	150%	0.0360	0.0409	395
3.2.1		0.0360	0.0409	399
3.1.2		0.0360	0.0409	400
3.2.2		0.0360	0.0409	404
3.1.3		0.0360	0.0409	405
3.2.3		0.0360	0.0409	398

**Tab.6 Raw data of Validation standard**

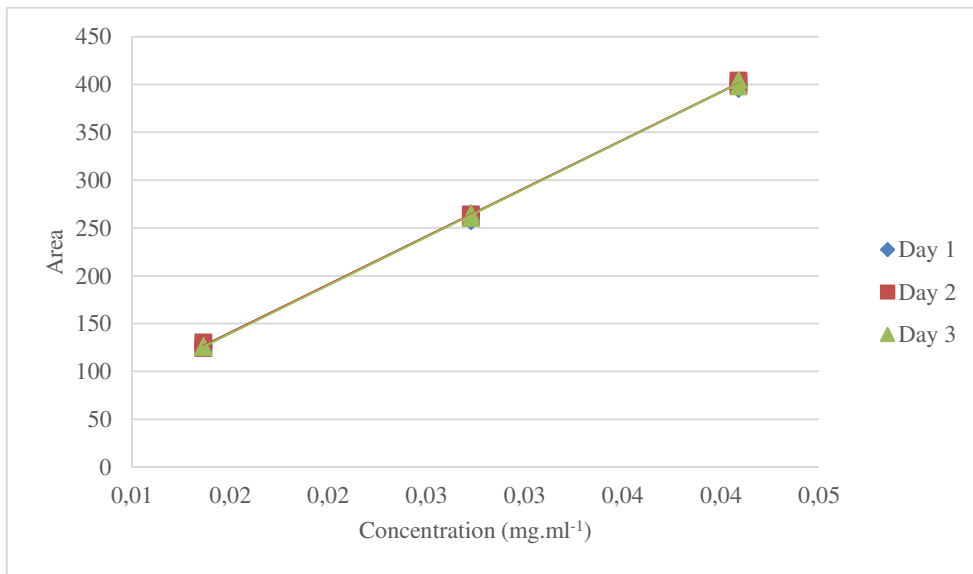
Level / sample / Day	Level	Introduced concentration equivalent content <sup>[3]</sup> (mg/mL)	Results	
			NPT area	Inverse prediction Calculated concentration $X = Y/a$
1.1.1	50%	0.0106	100	0.0105
1.2.1		0.0106	101	0.0106
1.3.1		0.0107	99	0.0103
1.1.2		0.0104	102	0.0108
1.2.2		0.0104	99	0.0105
1.3.2		0.0104	101	0.0106
1.1.3		0.0106	98	0.0103
1.2.3		0.0105	100	0.0105
1.3.3		0.0105	100	0.0105
2.1.1	75%	0.0161	155	0.0161
2.2.1		0.0160	152	0.0158
2.3.1		0.0160	149	0.0156
2.1.2		0.0155	151	0.0160
2.2.2		0.0156	148	0.0156
2.3.2		0.0155	149	0.0158
2.1.3		0.0158	148	0.0155
2.2.3		0.0158	150	0.0157
2.3.3		0.0158	156	0.0163
3.1.1	100%	0.0214	207	0.0216
3.2.1		0.0213	196	0.0205
3.3.1		0.0213	200	0.0209
3.1.2		0.0207	198	0.0210
3.2.2		0.0207	197	0.0210
3.3.2		0.0207	200	0.0212
3.1.3		0.0211	195	0.0204
3.2.3		0.0210	205	0.0215
3.3.3		0.0211	204	0.0212

Level / sample / Day	Level	Introduced concentration equivalent content <sup>[3]</sup> (mg/mL)	Results	
			NPT area	Inverse prediction Calculated concentration X = Y/a
4.1.1	125%	0.0266	253	0.0264
4.2.1		0.0266	255	0.0267
4.3.1		0.0267	250	0.0261
4.1.2		0.0259	247	0.0262
4.2.2		0.0259	245	0.0260
4.3.2		0.0258	244	0.0259
4.1.3		0.0262	248	0.0260
4.2.3		0.0263	258	0.0270
4.3.3		0.0263	259	0.0270
5.1.1	150%	0.0321	304	0.0317
5.2.1		0.0319	303	0.0317
5.3.1		0.0320	299	0.0311
5.1.2		0.0310	300	0.0319
5.2.2		0.0310	294	0.0312
5.3.2		0.0311	292	0.0310
5.1.3		0.0315	300	0.0314
5.2.3		0.0315	296	0.0310
5.3.3		0.0315	300	0.0314

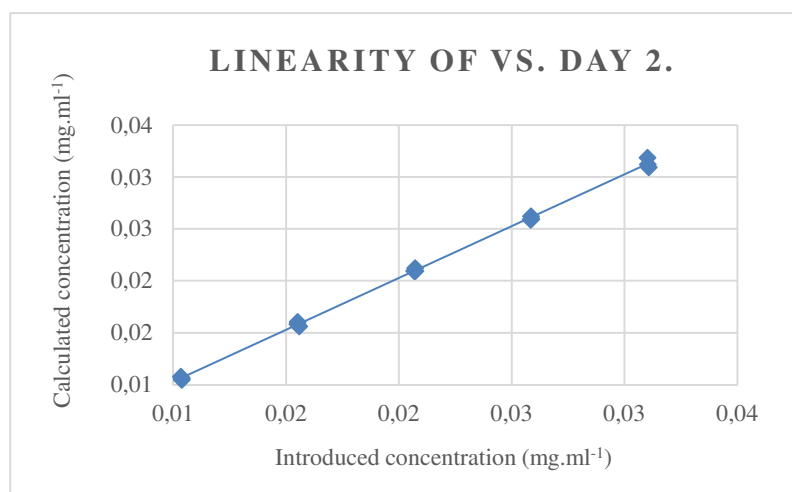
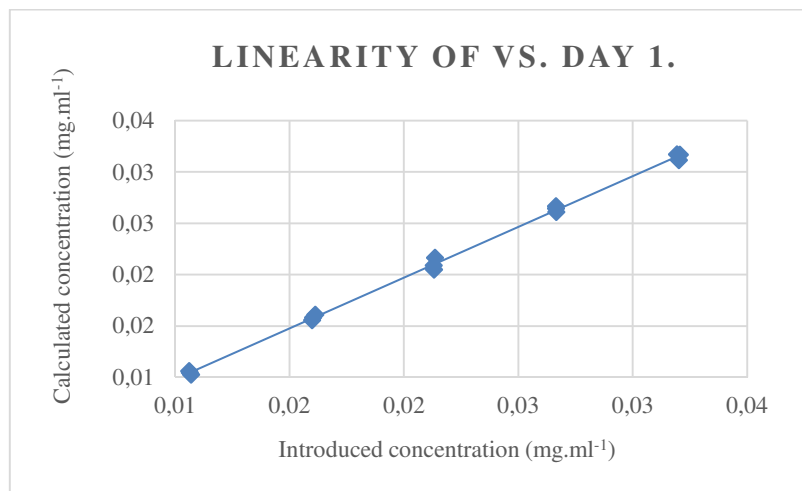
<sup>3</sup>: *Constituent Equivalent (mg/ml)* : calculated using the mean of Nepetin content calculated on the corresponding day :

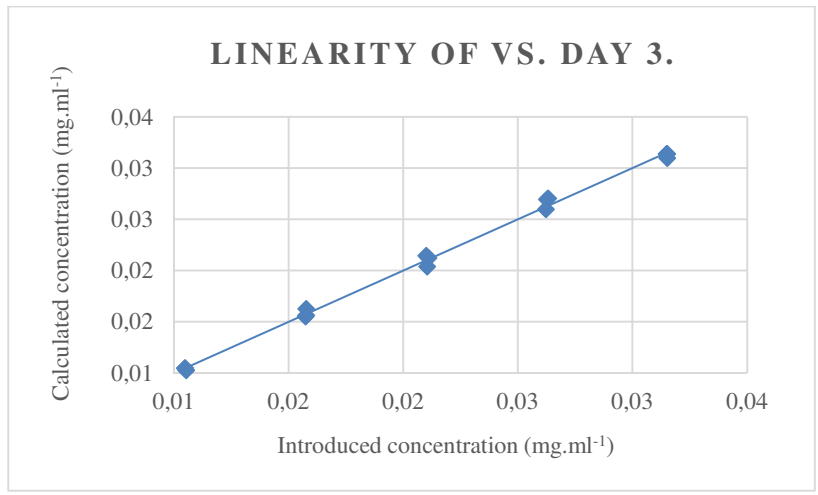
Day 1: IMFLF151 contains: 0.213 % Nepetin,  
Day 2: IMFLF151 contains: 0.207 % Nepetin,  
Day 3: IMFLF151 contains: 0.210 % Nepetin.

**Fig.7 Response function of Calibration standard (CS). Two points per day for each concentration over three days.**



**Fig.8 Linearity of Validation standard (VS). Three points per day for each concentration over three days**





**Fig.9 Accuracy profile of VS at 95% (Risk = 5%) over 5 validation points (50, 75, 100, 125 and 150%)**

