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Phytochemical analysis and antioxidant potential of the phytonutrient-rich decoction of *Cichorium spinosum* and *C. intybus*.

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Abstract

The Cretan diet as the basis of the Mediterranean diet, has provided traditional remedies for the general well-being through the long-established consumption of cooked wild greens and vegetables. The intake of water decoctions of *Cichorium spinosum* and *Cichorium intybus* in the context of the daily dietary regime in Greece, has been long associated with "liver detoxifying" properties. In the current study, we performed an in depth investigation of the water decoctions traditionally prepared from *C. spinosum* and *C. intybus*, through qualitative UHPLC-HRMS profiling and direct quantification of cichoric and caftaric acid, as major antioxidant components of the decoction. In addition, we developed a one-step Countercurrent Chromatography method for the isolation of the two phenolic acids, along with a sulfoconjugate sesquiterpene lactone present only in the Cretan *C. spinosum*. All water decoctions were found not cytotoxic in human fibroblasts, whereas they all significantly reduce the intracellular reactive oxygen species, in consistency with the major presence of strong antioxidant compounds such as cichoric acid. This work demonstrates that the intake of decoction in doses suggested by the Greek traditional use is comparable to the ingestion of a phytomedical preparation of antioxidants. These results contribute to our current knowledge on the beneficial health effect of the Cretan diet.

Keywords

Cichorium spinosum, *Cichorium intybus*, Asteraceae, cichoric acid, caftaric acid, stamnagathi, Cretan and Mediterranean diet, ROS reduction

Introduction

Dietary regional habits have a leading role in the discovery of molecules with important biological effects. From the consumption of garlic (*Allium sativum*, Amarillidaceae) for blood pressure control, to the discovery of sulphoraphane from broccoli (*Brassica oleracea*, Brassicaceae) as a chemoprotective agent, naturally functional foods are in the center of attention for the alleviation of disease cause and symptoms. The Mediterranean diet and its basis, the Cretan diet [1] provides an excellent field for the discovery of new bioactive agents correlated with food consumption and ethnopharmacology. Wild or semi-cultivated leafy greens are an essential part of the Cretan diet, being consumed as raw and cooked salads, in stews or in pies [2,3]. Those greens are collectively referred to as "Chórta" throughout the whole of Greece. Their consumption dates back to antiquity and it is believed that this dietary habit has been inherited also to inhabitants of Calabria, South Italy [4]. Their daily consumption is often supported by traditional knowledge on potential beneficial health effects.

Cichorium sp. (Asteraceae) and specifically *C. intybus* L. and *C. spinosum* L. are included among the most widely consumed Chórta of Greece. *C. intybus* is eaten as a raw or cooked leafy salad, while it is also cultivated for numerous purposes such as the industrial production of inulin and coffee substitute [5]. *C. spinosum* under the common name "Stamnagathi" is widely consumed in the island of Crete as an integral part of the daily salad servings with substantial nutritional value [6]. *C. spinosum*, although distributed throughout the Mediterranean basin (Crete, Sicily, Malta, Spain, Turkey and Libya) has become a culinary trademark of Crete, and an ambassador of the Cretan diet. In Greece, the traditional preparation of a leafy chicory salad involves boiling the raw plant material in water, straining of the cooking water and mixing the boiled leaves with olive oil and lemon. The remaining cooking water is often reserved in closed jars in reduced temperatures and consumed weekly as a remedy for liver disorders [7].

Phytochemical studies of chicories have been mainly focused in the raw or dried plant material and extracts of medium polarity [8,9], with *C. spinosum* being the less studied [10,11]. Even more limited are the phytochemical studies of the bioactive water-soluble decoctions [12], which are often used in the Mediterranean and Greek medical folklore for the treatment of liver disorders and diabetes, and their spasmolytic, anti-cholesterolemic, anti-inflammatory, anti-hypertensive and detoxifying properties [13,14].

One of the main ingredients of the decoction is cichoric acid (**1**), a phenylpropanoid derivative isolated numerous times from *Cichorium* species [15]. Cichoric acid (**1**) has been shown to inhibit both hepatitis B virus [16] and HIV-1 integrase activity [17], and stimulate insulin secretion [18,19]. Recent data suggest that cichoric acid (**1**) is implicated in AMPK pathway activation either by increasing ROS generation [20], or decreasing ROS accumulation [21]. This redox behavior of cichoric acid (**1**) is associated with its potential as a therapeutic agent for the treatment of metabolic and age-related disorders. Variable amount of caftaric (**2**) and/or cichoric acid (**1**) have been reported in different plants from the Asteraceae family such as *Sonchus asper*, *Sonchus oleacerum*, *Taraxacum officinale* [22,23], *Lactuca sativa* [24,25] and *Cichorium intybus* [12,22]. Among them, lettuce with 5±15 mg per 100 g and chicory with twice the concentration, are some of the main dietary sources of cichoric acid (**1**) [26].

The investigation of *Cichorium sp.* water decoctions is limited [12,27], although they are a traditional remedy consumed regularly in many Mediterranean countries including Greece. In this context, the present study is aiming at investigating the water decoctions of *C. spinosum* and *C. intybus* traditionally consumed in Greece for liver detoxification including the identification of their phytonutrients, the isolation of compounds of interest as well as the quantification of the major bioactive constituents. Moreover, the cellular antioxidant potential of the decoctions was assessed and compared with the activity of cichoric acid, which was also found to be the predominant phenolic constituent of the decoctions. Focus is particularly

given in the Cretan Stamnagathi (*C. spinosum*) due to the limited information concerning the composition of the decoction, and the pivotal role as a home remedy under the Cretan diet.

Results and discussion

The first step of the present work was the phytochemical analysis of the decoction prepared from *C. spinosum*, due to the limited phytochemical studies of this species. The isolation of the main components as well as compounds that were present uniquely in *C. spinosum*, was achieved by fractionation of the extract using Countercurrent Chromatography (CCC) in step-gradient elution mode. Gradient elution has been chosen as an established and the most effective method which has been successfully applied for the direct separation of compounds with wide range of polarities from complex plant extracts [28-30].

In order to select a biphasic solvent system for efficient separation, we studied the partition behavior of the main components of the extract (cichoric and caftaric acid), in two simple binary systems: one of medium polarity composed of ethyl acetate:water in ratio 1:1 (v/v) and a second one of high polarity composed of n-butanol:water 1:1 (v/v). The analysis showed an important increase of distribution of the target compounds in the upper phase of the highly polar system (n-butanol/water) compared to this of middle polarity biphasic system (ethyl-acetate/water). Based on these results and taking into account previous CCC separation for cichoric acid (**1**) [31,32] and caftaric acid (**2**) [33] we created and tested two series of biphasic systems: the ternary ethyl-acetate/n-butanol/acidified water (10% acetic acid) in ratios x/y/15 (Series A) and the tertiary ethyl-acetate/n-butanol/ethanol/acidified water (10% acetic acid) in ratios x/y/1/14 (Series B) (Table S1, supporting information). In total, 9 major peaks in the HPLC-DAD chromatogram were chosen as target compounds, and the corresponding distribution coefficients (K_D) were calculated. The study of K_D values and separation factor α of the target peaks revealed that the systems of series B, had the greatest potential to better separate the compounds of the analyzed mixture. We chose to run the step-gradient

fractionation with the first three biphasic systems of series B (*B1*, *B2* and *B3*) in order to reduce the solvent consumption and analysis time. The step-gradient CPC fractionation was performed as described in Materials and Methods, and resulted to the isolation of cichoric acid (**1**) from fraction B, caftaric acid (**2**) (fr. G) [34] and 8-deacetylmatricarin-8-*O*-sulphate (**8**) (fr. J) (Figure 1; Figure S2, Supporting Information). It is important to note that these compounds have been recovered in high purity, simultaneously and in one-step procedure (Figure S3, Supporting Information).

For the structure elucidation of the purified compounds **1**, **2** and **8**, spectrometric and spectroscopic methods were employed. More attention was given to the 8-deacetylmatricarin-8-*O*-sulphate. ^{1}H, ^{13}C and 2D NMR data and optical rotation of compound **8** were studied and found are in agreement with the literature [35,36], while HRMS was decisive for verifying the presence of sulfur in the molecule. The HRMS spectrum presented a pseudomolecular ion $[M-H]^-$ with a *m/z* of 341.0694 yielding in a prominent fragment ion characteristic of sulfoconjugates at *m/z* 97. Indeed, it has been shown that cleavage promoted by charge from alicyclic sulfates generates such diagnostic ion (*m/z* 97, $[HSO_4]^-$) in CID spectra in the negative ESI mode [37]. Additionally, as presented in Figure 2, spectral data for positive ESI mode were investigated showing an $[M+H]^+$ at *m/z* 343 and fragment ions of *m/z* 245 $[M+H-H_2SO_4]^+$ and of *m/z* 263 $[M+H-SO_3]^+$. Furthermore, experimental isotopic distribution showed consistency with the theoretical one from the proposed formula ($C_{15}H_{17}O_7S$) and this compound was identified as deacetylmatricarin-8-*O*-sulfate (**8**), a guaianolide sulfate conjugate that has been previously isolated in *Taraxacum alpinum* [35] and *Lactuca sativa* var. *capitata* [36,38]

Further analysis of CCC combined fractions C, D, F, M and N led to the identification of ten more secondary metabolites, however in low purity. The structure elucidation of these compounds was based on 1D/2D-NMR and HRMS analysis as well as comparison of

experimental findings with bibliographic data (See Materials and Methods). More specifically, the above analysis of the selected CPC fractions, resulted to characterization of chlorogenic acid (**3**) (fr.F), caffeoylmalic acid (**4**) (fr. C), caffeic acid (**5**) (fr. D), kaempferol-3-*O*-β-D-glucuronide (**6**) (fr.C), quercetin-3-*O*-β-D-glucuronide (**7**) (fr.D), quinic acid (**9**) (fr.N), tartaric acid (**10**) (fr.N), malic acid (**11**) (fr.M) and pyroglutamic acid (**12**) (fr.M) (Figure 1). It is noticeable that among the compound isolated, three of them (**1**, **2**, **8**) were isolated in high purity in a one step procedure.

Isolated compounds were a useful guide for the in-depth qualitative investigation of *C. spinosum* and *C. intybus* decoctions, in order to identify as many compounds as possible and compare the profiles of the different species. To this end, UHPLC-HRMS technology was used. HRMS profiles were performed in both ESI positive and negative mode. In the analysis conditions, ESI(+) mode did not provided significant observable peaks. As a consequence, results presented here are based on ESI(-) base peak chromatograms (Figure S4, Supporting Information). Characterization of the detected metabolites was achieved considering *m/z* values with a 5 ppm tolerance from the proposed theoretical mass, as well as RDB values and MS2 fragmentation. These results are presented in Table 1. A total of 40 compounds were identified in the *C. spinosum* decoction, belonging to different chemical classes including small organic acids, cinnamic acid and flavonoid derivatives, coumarins and sesquiterpene lactones.

Small organic acids of high polarity can be observed within the first part of the chromatograms of both extracts. Specifically, the $[M-H]^-$ of tartaric acid (*m/z* 149) was monitored along with molecular ions of polar di- and tri- carboxylic acids such as succinic (*m/z* 117) and citric (*m/z* 191), hydroxylated acids such as gluconic (*m/z* 195), quinic (*m/z* 191) and malic (*m/z* 133), and amino acids such as glutamic (*m/z* 146) and pyroglutamic (*m/z* 128), all eluted between 0.65 and 1.41 minutes. Malic acid was characterized by a signal

corresponding to $[M-H]^-$ at *m/z* 133 with a RDB of 2.5 which could be observed only in *C. spinosum* decoction while the amino-acid pyroglutamic acid with a $[M-H]^-$ at *m/z* 128 and a RDB of 3.5 was detected in both species.

Another important feature of both chromatograms was the presence of caffeoylquinic acids. Caffeoylquinic acid isomers differ in their esterification position that could occur on carbons 1, 3, 4 or 5 of the quinic acid moiety. Clifford *et al.* [39,40] suggested assignment of structural caffeoylquinic acid isomers based characteristic fragmentation patterns from the cleavage of caffeoyl and quinic groups as well as from their further dehydrated and decarboxylated product ions. Parveen *et al.* [41] also implemented such a strategy for the determination of the position isomers in *Miscanthis x giganteus* extracts. On the basis of these previous works and given the fact that 1-caffeoylquinic acid is rare in higher plants, 3-caffeoylquinic acid (chlorogenic acid, **3**), 5-caffeoylquinic acid (neochlorogenic acid) and 4-caffeoylquinic acid (cryptochlorogenic acid), were assigned to peaks at retention time 6.21, 7.78 and 7.93 minutes respectively. All caffeoylquinic acids presented a $[M-H]^-$ ion at *m/z* 353 associated with a major fragment corresponding to quinate ion at *m/z* 191 followed by the caffeate ion at *m/z* 179 which could also generate the *m/z* 135 after the loss of CO_2. 3,4-dicaffeoylquinic acid eluted at 10.85 minutes yielding in product ions at *m/z* 353 $[M-H\text{-caffeoyl-}H]^-$ and 335 $[M-H\text{-caffeoyl-}H_2O\text{-}H]^-$ [40,41]. Peak at 11.60 minutes with MS2 fragments of *m/z* 353, 317, 203 and 299 could be attributed to 4,5-dicaffeoylquinic acid whereas peak at 11.04 minutes is likely to be 3,5-dicaffeoylquinic acid although it can not be confirmed due to the absence of MS2 data.

The most characteristic property of both decoctions is the presence of caffeoyltartaric acid derivatives such as cichoric acid (**1**) and caftaric acid (**2**). Chiroric acid (**1**) pseudomolecular ion $[M-H]^-$ at *m/z* 473 yielded in the characteristic product ion *m/z* 311 $[M-H\text{-caffeoyl-}H]^-$ which further dehydration stands for the *m/z* 293. Caftaric acid (**2**) dissociation yielded in

MS2 fragments [caffeic acid-H]$^-$ at *m/z* 179 and [tartaric acid-H]$^-$ at *m/z* 149. Several other cinnamic acids were identified including ferulic and coumaric acid derivatives. Among them, *p*-coumaroyltartaric acid (*m/z* 295), di-*p*-coumaroyltartaric acid (*m/z* 441), *p*-coumaroylcaffeoyltartaric acid (*m/z* 457), *p*-coumaroylferuloyltartaric acid (*m/z* 471), feruloylcaffeoyltartaric acid (*m/z* 487) and *p*-coumaroylsinapoyltartaric acid (*m/z* 501) which all showed characteristic fragmentation with at least one residue resulting from the loss of either 194 or 164 u corresponding respectively to the ferulic and coumaric acids moieties. Interestingly, three of them, namely *p*-coumaroyltartaric, di-*p*-coumaroyltartaric, and *p*-coumaroylferuloyltartaric acids were only monitored in *C. spinosum*.

Flavanol and flavone glucuronides were observed in both extracts. All of them displayed an RBD value of 13.5 and yielded in a single fragment [M-H-glucuronide]$^-$ at *m/z* -176 u. More specifically, apigenin glucuronide (*m/z* 445) and luteolin-7-*O*-glucuronide (*m/z* 461) were assigned to peaks at 11.80 and 10.85 minutes. Another compound with similar features to luteolin-7-*O*-glucuronide was observed in *C. spinosum* extract at 11.74 minutes and was attributed to kaempferol-3-*O*-glucuronide based on C18 retention time described in the literature [42]. Moreover, quercetin-3-*O*-glucuronide (*m/z* 477) was characterized in *C. spinosum* whereas the *m/z* 639 at 8.51 minutes observed in *C. intybus* extract is believed to be quercetin hexose glucuronide because of the coexistence of MS2 product ions *m/z* 477 ([M-H-glucoside]$^-$), 463 ([M-H-glucuronide]$^-$), and 301 ([M-glucuronide-glucoside-H]$^-$) [43].

Finally, the feature *m/z* 341 at 12.18 minutes identified as deacetylmatricarin-8-*O*-sulfate (**8**) was only observed in *C. spinosum* decoction. Sesquiterpene lactone 8-sulfate derivatives were introduced by Sessa *et al.* [44] after the isolation of lactucopicrin-15-sulfate, and 15-deoxy-8-lactucin sulfate from *Lactuca sativa*. A recent publication reported the presence of deacetylmatricatin-8-*O*-sulfate (**8**) in different subspecies of *L. sativa* and *C. endivia* as well

as in *C. intybus* var. *foliosum* whereas it was not observed in the common chicory, *C. intybus* [38]

According to the isolation and chemical profiling of *Cichorium intybus* and *C. spinosum* as described above, cichoric (**1**) and caftaric acid (**2**) were identified as key components, with major presence in the decoctions prepared. Due to the biological importance of those molecules in combination with the regular consumption of the decoctions as a home remedy, we proceeded to the direct quantification of **1** and **2**. Four samples of *C. spinosum* and one sample of *C. intybus* were selected, from different vendors, in order to compare the variability in the levels of cichoric (**1**) and caftaric acid (**2**) especially among samples of the Cretan stamnagathi. A total of 21 points were considered to draw the calibration curves (7 levels analyzed in triplicate). Calibration models were then analyzed by observation of R^2 and plots of residuals. Coefficients of determination for caftaric (**2**) and cichoric (**1**) acid were respectively of 0.996 and 0.998 and plots of residuals displayed homogenous distributions. None on the measured compounds could be detected in blank samples and instrumental repeatability was demonstrated to be satisfactory as area ratio (Standard/Internal Standard) relative standard deviations in the QC samples (n=10) were of 4% for caftaric acid (**2**) and of 3% for cichoric acid (**1**). Instrumental limits of quantification were evaluated following a signal to noise approach (S/N>10) and were found to be of 1.2 ng injected for cichoric acid (**1**) and of 1.0 ng injected for caftaric acid (**2**). Observed relative standard deviation on area ratios (n=3) at these levels were inferior to 7% for both compounds.

Table 2 displays the average concentrations of caftaric (**2**) and cichoric acid (**1**) measured in *C. spinosum* and *C. intybus* decoctions together with their extraction yields regarding the fresh and the dry weight of the leaves. Standard deviation (σ_C) of the measured concentration for all the analyzed samples (n=6) ranged from 14 to 24% for caftaric acid (**2**) and from 4 to 11% for cichoric acid (**1**). Caftaric (**2**) and cichoric acid (**1**) levels were found to be similar in

the four *C. spinosum* extracts. Average caftaric acid (**2**) concentrations ranged between 11.7±2.3 and 14.2±2.7 mg g^{-1} of extracts. Cichoric acid (**1**) levels were found to be two to three times higher than those of caftaric acid (**2**) and the minimum concentration was of 28.4±3.1 mg g^{-1} of extract for *C. spinosum* 3 whereas the maximum concentration was of 38.3±2.7 mg g^{-1} of extract for *C. spinosum* 2. Comparable results were observed in *C. intybus* decoction even though individual levels of both targeted analyte were found to be higher with 19.1±2.0 and 54.6±7.4 mg g^{-1} of extracts for caftaric (**2**) and cichoric acid (**1**) respectively.

Comparison of the herein reported results with those available in the literature is difficult for several reasons. Firstly, qualitative characterization of such decoctions has not been described neither for *C. spinosum* or *C. intybus*, individually or simultaneously. In addition, in cases where the levels of cichoric and caftaric acid needed to be determined in a given plant extract or matrice, the quantification method is indirect and is sometimes expressed as another standard equivalent. For instance cichoric (**1**) and caftatic acid (**2**) concentration in fresh leaves of several varieties of *C. intybus* has been reported before but were determined as caffeic acid equivalent [12]. Lastly, results can be expressed either per g or per 100 g on an extract, on a fresh-weight or dry-weight basis. Some of these methodological considerations were already mentioned by Nuissier *et al.* [45] before the difficulty to compare the cichoric acid (**1**) levels of methanolic extracts from *Syringodium filiforme* with those in other plants. In this context, reporting extraction yields in both fresh and dry weight basis along with quantitative results in a given extract appears as a simple way to improve comparability.

The nutritional significance and chemical profile of *C. spinosum* and *C. intybus* decoctions, led us to investigate both their cytotoxicity (samples *C. spinosum* 4 and *C. intybus*) and their antioxidant potential, in terms of both basal ROS production and after induction of oxidative stress with hydrogen peroxide (Figure 3). In the cell viability assay, the decoctions were not cytotoxic for human skin fibroblasts at the concentration range tested (up to 100 µg mL^{-1}),

based on the widely used MTT assay. Hence, both extracts were further tested for their antioxidant activity at 100 μg mL^{-1}. As shown in Figure 3, they were found to significantly reduce the intracellular reactive oxygen species (ROS) levels of human skin fibroblasts compared to the control culture. The ROS reductions achieved by both extracts were comparable to those induced by cichoric acid (**1**) and trolox used as positive controls. No statistically significant difference was observed between the antioxidant activity of the two extracts. Beyond the reduction of basal ROS levels, both extracts were further capable to significantly attenuate the ROS induction caused by a moderate concentration (100 μM) of hydrogen peroxide, an established oxidative agent. Although the water extract of *C. intybus* was more active than the one of *C. spinosum*, the difference was marginally non-significant (p=0.0575; see supporting Information).

The aforementioned results are consistent with both the observed levels of cichoric (**1**) and caftaric acid (**2**) in the *Cichorium sp*. decoctions, and with the Greek traditional use of those extracts as "liver detoxifying" agents, that actually could be attributed to their strong antioxidant capacity. Indeed, the famous antioxidant *Echinacea purpurea* that affords some of the best-selling phytomedicines worldwide, is considered as the main source of cichoric acid (**1**) [46]. Pellati *et al.* [47] reported concentrations of caftaric (**2**) and cichoric acid (**1**) of respectively 3.97 and 19.27 mg g^{-1} d.w. in *E. purpurea* roots, while analysis of the water extract of the aerial parts showed levels of 4.4 and 5.7 % (w/w) for caftaric (**2**) and cichoric acid (**1**) respectively [48].

When comparing our results with available data form the literature, it is observed that *C. spinosum* and *C. intybus* water decoctions might be among the most valuable dietary sources of caftaric (**2**) and cichoric acid (**1**). Indeed, considering an average concentration of 12 and 30 mg g^{-1} of caftaric (**2**) and cichoric acid (**1**) respectively together with an average extraction yield of 2% (f.w.), a rough estimation based on the traditional habits of consumption in

Greece suggest that drinking the boiling water of 500 g of *C. spinosum* leaves within a week is equivalent to a daily intake in the order of 20 mg of caftaric acid (**2**) and of 40 mg of cichoric acid (**1**). As shown from the qualitative analysis of the decoctions, a range of phytonutrients is present except the major antioxidants. In the case of the Cretan *C. spinosum* it is worth noticing that it is the only one affording sulfoconjugated lactone analogues (**8**), thus presenting an interesting diversity. The antioxidant potential and chemical diversity of *C. spinosum* and *C. intybus* decoctions in combination with their regular consumption contributes to our knowledge on the Cretan diet and its significance in healthy living.

Materials and methods

Materials and chemicals

For the preparation of the decoctions distilled water was used. All analytical grade solvents used for extraction and CPC separation (ethyl acetate, n-butanol, ethanol 96%), were purchased from Sigma-Aldrich. HPLC and LC-MS grade solvents used for the HPLC-DAD and UHPLC-MS analyses (acetonitrile, methanol, water) were purchased from Merck Chemicals. Acetic and formic acid were obtained from Lach-Ner. NMR solvents (MeOH-d_4, pyridine-d_5) for were purchased from Eurisotop. Analytical standards of cichoric acid (≥98.0%), caftaric acid (≥98.0%) and syringaldehyde (≥97.0%), dichloro-dihydro-fluorescein diacetate (DCFH-DA), methylthiazolyldiphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Fetal Bovine Serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Biochrom.

Plant material and extract preparation

All edible greens (*Cichorium spinosum* samples 1-4*, Cichorium intybus*) were purchased from local markets in Athens, Greece on April 2015, and all samples were transferred immediately to the laboratory. The samples were botanically characterized and a voucher specimen

(KL011_1-4 for the four samples of *C. spinosum* and KL698 for the sample of *C. intybus*) was deposited in the herbarium of the Laboratory of Pharmacognosy, University of Athens, Greece. In total, one sample of *C. intybus* and four samples of *C. spinosum* were selected. The edible parts (healthy, clean leaves) were separated and used for extraction. In specific, a decoction was prepared for each sample by boiling 500g of fresh plant material in 1.5 L of water for 30 minutes. The resulting mixture was left to cool (40°C) and was filtered through paper. The resulting filtrate was evaporated under reduced pressure and the viscous residue was lyophilized in order to obtain the dry water extract. All dry decoctions (*C. spinosum* 1-4, *C. intybus*) were forwarded for qualitative profiling and quantification studies with the aid of UHPLC-HRMS, while *C. spinosum* 4 was also used for CPC fractionation and isolation of major compounds.

Selection of solvent system and step-gradient CCC fractionation of C. spinosum

For the solvent system trials, CCC fractionation and separation, *C. spinosum* 4 sample was used. The suitability of two-phase solvent systems was first evaluated by TLC, and then the solvent systems giving the best apparent partition, was further analyzed by HPLC-DAD for the determination of partition coefficient values (*K*) of selected compounds. Analysis was carried out on a Thermo Finnigan HPLC instrument equipped with a SpectraSystem P4000 pump, a SpectraSystem 1000 degasser, a SpectraSystem AS3000 automated injector, and a UV SpectraSystem UV6000LP detector. The samples were analyzed in a Discovery HS C18, (250 x 4.6 mm, 5μm) column (Supelco) using the following method: solvent A, 2% (v/v) acetic acid in water; solvent B, 1% (v/v) acetic acid and 2% (v/v) methanol in acetonitrile; 0–10min (0–15% B), 10-25 min (15–25% B), 25–40 min (25–40% B), 40-45 min (40-95% B), 45-47 min (95-0% B), 45-50 min (0-0% B) in a flow rate of 1 mL min^{-1}. The UV-DAD detector was set at 260, 320 and 366 nm (Figure S1, Supporting information).

For the evaluation of potential CCC biphasic systems, 10 mg of dry *C. spinosum* extract (*C. spinosum* 4) were weighted into a 10 mL test tube, while 2 mL of each phase of the pre-

equilibrated biphasic solvent system were added to the sample and shaken vigorously. Following the equilibration and separation of phases, 1 mL of each layer was removed and evaporated to dryness. The residues were diluted in 1 mL of methanol and analyzed by HPLC-UV/DAD. The *K* values were expressed as the peak area of the target compounds in the stationary phase divided by the one in the mobile phase. In total, eight biphasic solvent systems were tested (Table S1, Supporting Information).

The step-gradient CPC fractionation was carried out on a Kromaton FCPC200® instrument equipped with a rotor of 200 mL. The solvents were pumped through the system *via* a preparative LabAlliance pump, the sample injected *via* a 10 mL sample loop and fractions were collected with a Büchi B-684 fraction collector. Chromatograms were recorded at 260 and 325 nm using a UV detector SPECTRASYSTEM UV 2000.

The series of three biphasic systems consisting of the solvents ethyl acetate, n-butanol, ethanol and 10% acetic acid in water in proportions 15/0/1/14, 11/4/1/14 and 7/8/1/14 (v/v/v/v) were selected as the most promising (systems *B1*, *B2* and *B3* respectively; Table S1, supporting information). The solvents were thoroughly mixed in a separating funnel at room temperature prior to use, and the two phases of each system were separated after equilibration of the mixture. The column was filled with the lower phase of *B1* (stationary phase) in ascending mode (10 mL min^{-1}, 200 rpm). Afterwards, the rotation speed of the column was increased to 1000 rpm and the upper phase (first mobile phase) was pumped through the column (10 mL min^{-1}). When equilibrium of two phases was established (Sf = 75%) 700 mg of the extract, dissolved in a mixture 1:1, v/v of lower and upper phase of *B1*, was injected via a 10 mL sample loop. For the gradient elution, three mobile phases were used: the upper phases of *B1*, *B2* and *B3*. The passing of 100 mL of *B1*-upper, 100 mL of *B2*-upper and 120 mL of *B3*-upper was followed with the extrusion step with 250 mL of *B3*-lower. The initial stationary phase retention was calculated at 75% while the retention of the stationary phase in the end of elution step was 43%. This lost of stationary phase is a common phenomenon

during the gradient CPC analysis and is primarily due to the affection of the different mobile phases, and especially the increase of n-butanol ratio, in stationary phase composition [49]. The flow rate was stable at 10 mL min^{-1} during the separation while fractions were collected every 1 min (total of 60 fractions of 10 mL). The fractions were combined based on HPLC-DAD analysis to give finally 15 combined fractions (A 1.5 mg, B 25.5 mg, C 5.3 mg, D 4.9 mg, E 3.4 mg, F 3.9 mg, G 6.6 mg, H 6.9 mg, I 3.4 mg, J 2.8 mg, K 4.2 mg, L 14.8 mg, M 22.0 mg, N 536 mg, O 39.6 mg) (Figure S3, Supporting Information)

Fractions B, G and J contain chichoric acid **(1)**, caftaric acid **(2)** and 8-deacetylmatricarin-8-*O*-sulphate **(8)** respectively, in pure form. Fraction C is a mixture of cafeoylmalic acid **(4)** and kaempferol-3-*O*-β-D-glucuronide **(6)**, fraction D contain mainly caffeic acid **(5)** and quercetin-3-*O*-β-D-glucuronide **(7)**, fraction F chlorogenic acid **(3)** and impurities while fraction M is a mixture of malic acid **(11)** and pyroglutamic acid **(12)**. Finally, in fraction N, which constitutes the bigger part of the extract, quinic acid **(9)** and tartaric acid **(10)** have been identified. Structure elucidation of the isolated compounds was achieved with the aid of NMR and HRMS analysis. 1D and 2D NMR experiments (^{1}H, ^{13}C, COSY, COSY-LR, HSQC-DEPT, HMBC) were performed on a 600 MHz Bruker Avance spectrometer equipped with an autosampler.

UHPLC-HRMS analysis

Liquid chromatography analysis was performed on an Accela® High-Speed LC System (Thermo Scientific) and detection was carried out on a LTQ-Orbitrap® XL hybrid mass spectrometer equipped with an ESI source (Thermo Scientific).

For qualitative analyses, separation was achieved on an Acquity® BEH C18 (Waters) column (150 mm x 2.1mm, 1.7µm) using a gradient of water (A) and acetonitrile (B) containing 0.1% (v/v) formic acid each. The column was maintained at 40°C and the flow rate was set at 0.4 mL min^{-1}. Elution started with 95% A for 3 minutes and decreased to 0% A in 21 minutes. These conditions were kept for 2 minutes before getting back to initial conditions for a 5

minutes re-equilibration. 5 µL of water extracts at approximately 300 µg g^{-1} were injected. MS data were acquired in both negative and positive mode, in the full scan *m/z* range of 113–1000, with a resolution of 30000. Data dependent acquisition was simultaneously performed using a CID value of 30% and a mass resolution of 7500. Capillary temperature was set at 350°C in both polarities whereas source voltage was of 3.5 kV in ESI+ and of 2.7 kV in ESI-. Tube lens and capillary voltage were respectively tuned at 80 V and 35 V in positive mode and at -40 V and -10 V in negative mode. Finally, nitrogen was used as sheath gas (40 arbitrary units) and auxiliary gas (10 arbitrary units).

A Fortis® C18 (Fortis Technologies) column (100 mm x 2.1mm, 1.7 µm) was used for the quantitative determination of cichoric (**1**) and caftaric acid (**2**) in the investigated extracts. Each of them was prepared independently in duplicate at 3 different concentrations: low (50 µg g^{-1}), medium (100 µg g^{-1}) and high (200 µg g^{-1}) in H_2O-MeOH (1:1, v/v) whereas internal standard (syringaldehyde) concentration was of 5 µg g^{-1} in every samples. Injection volume was of 5 µL and mobile phases consisted in 2mM ammonium formate in water (A) and acetonitrile (B) both supplemented with 0.1% formic acid. Column temperature was triggered at 25°C and mobile phases flow rate was of 0.3 mL min^{-1}. Elution gradient started from 80% A to 50% A during 5 minutes before decreasing to 10% A for the next 3 minutes. Finally, theses conditions were hold for 2 minutes before the final 5 minutes re-equilibration step. MS data were acquired in negative mode using selected ion monitoring scans (SIM) for *m/z* 311.04 (caftaric acid, **2**), 473.06 (cichoric acid, **1**) and 181.05 (IS) with an isolation width of 2 amu and a resolution of 30000. Tune method was similar to the one used for qualitative purposes. 7 levels calibration curves were prepared between 0.2 to 10 µg g^{-1} in H_2O-MeOH (1:1, v/v) for both targeted compounds, while internal standard concentration was fixed at 5 µg g^{-1}. Each calibration curve point was analyzed in triplicate and was injected randomly throughout the sequence. Moreover, in order to monitor any eventual cross contaminations or carryover effects, a procedural blank was analyzed every 5 injections along with a QC sample

that consisted of both analytical and internal standards at 5 μg g^{-1} in H_2O-MeOH (1:1, v/v). All analytical and QC samples were elaborated under gravimetric control and prepared on the day of the analysis.

Determination of intracellular anti-oxidant activity

Cells and Cell Culture Conditions

A commercially available normal human neonatal foreskin fibroblast strain was used (AG01523, Coriell Institute for Medical Research). Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics (100 IU mL^{-1} penicillin; 100 μg mL^{-1} streptomycin) and 15% fetal bovine serum in an environment of 5%CO_2, 85% humidity, and 37°C, and subcultured twice a week at a 1:2 split ratio, using a trypsin-citrate solution (0.25–0.3%, respectively). Cell counting after trypsinization was performed using a Z1 Coulter counter (Beckman Coulter International SA). Cells were tested periodically and found to be mycoplasma free.

Assessment of cytotoxicity

The cells were plated in 96-well flat-bottomed microplates at a density of 7,000 cells/well in DMEM 15% FBS and were left to adhere for 18 hours. Then, the test extracts were added appropriately diluted with DMSO in serum-free DMEM. After a 72-h incubation, the medium was replaced with MTT dissolved at a final concentration of 1 mg mL^{-1} in serum-free, phenol-red-free DMEM for a further 4-h incubation. Then, the MTT formazan was solubilized in 2-propanol and the optical density was measured using a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH) at a wavelength of 550 nm (reference wavelength 660 nm).

Intracellular ROS assay

The cells were plated in 96-well clear-bottomed black microplates at a density of 7,000 cells/well in DMEM 15% FBS and were left to adhere for 18 hours. Then, the test extracts

were added appropriately diluted with DMSO in serum-free, phenol-red-free DMEM. After a 24 h incubation, DCFH-DA was added at a 10 μM final concentration for further 45 minutes. Then, the medium was replaced by phosphate buffered saline (PBS) or PBS supplemented with 100 μM H_2O_2, and, after further 15 minutes, fluorescence emission was determined at 520 nm following excitation at 485 nm in a FLUOstar OPTIMA microplate reader (BMG Labtech). Three independent experiments were performed, and the results are presented here as means (± standard deviation). Differences from the control (DMSO-treated) culture were assessed with Student's *t*-test using the Statgraphics Centurion XV software (StatPoint Technologies Inc).

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Supporting information

HPLC chromatograms of *C. spinosum* decoction and CPC fractions B, G and J, Biphasic solvent systems for the CPC separation and calculation of partition coefficients (K_D), 1H-NMR spectra of cichoric acid and caftaric acid and ^{1}H-, ^{13}C-NMR data and 2D spectra for 8-deacetylmatricarin-8-*O*-sulphate, are provided as Supporting Information.

Conflict of interest

The authors declare no conflict of interest.

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Legends to tables and figures

Table 1 Retention time (R_t), mass spectral data and proposed identification of detected features in *C. spinosum* and *C. intybus* water decoctions by RP-UHPLC-ESI(-)-HRMS

Table 2 Average concentrations (n=6) of caftaric and cichoric acids (expressed as mg per g of extract) measured in *C. spinosum* and *C. intybus* decoctions

Figure 1: Chemical structures of compounds isolated from *C. spinosum* decoctions

Figure 2: MS and MS/MS data for the identification of 8-deacetylmatricarin-8-*O*-sulfate

Figure 3: Intracellular anti-oxidant activity of *C. spinosum* and *C. intybus* extracts; control; *C. spinosum* 4; *C. intybus*; cichoric acid analytical standard (100 µg mL^{-1}); trolox (20 µg mL^{-1}). * $p<0.01$ compared to control basal levels; * $p<0.01$ compared to stimulated control levels (Student's *t*-test).

Table 1

R_t (min)	*Observed m/z* ([M-H]$^-$)	MS/MS fragment*m/z* (relative intensity)	Proposed formula	RDB	Δ (ppm)	Proposed compound	Ref.	Detected in	
								C. spinosum	*C. intybus* L.
0.65	132.0299	-	$C_4H_6O_4N$	2.5	-2.583	aspartic acid		+	+
0.65	146.0463	128 (100), 102 (9)	$C_5H_8O_4N$	2.5	2.732	glutamic acid		+	+
0.65	225.0618	179 (100)	$C_7H_{13}O_8$	1.5	0.930	heptonic acid		+	+
0.69	195.0514	129 (100), 177 (37), 159 (17), 99 (5)	$C_6H_{11}O_7$	1.5	1.867	gluconic acid	[50]	+	+
0.71	387.1142	341 (100)	$C_{13}H_{23}O_{13}$	2.5	-0.475	-		+	+
0.71	165.0408	129 (100), 147 (68)	$C_5H_9O_6$	1.5	0.359	pentonic acid		-	+
0.74	191.0565	-	$C_7H_{11}O_6$	2.5	0.419	quinic acid	[9]	+	+
0.76	149.0086	103 (100), 131 (77), 87 (60)	$C_4H_5O_6$	2.5	-3.900	tartaric acid [b]		+	+
0.86	133.0146	-	$C_4H_5O_5$	2.5	0.393	malic acid	[9,50,51]	+	-
1.07	135.0303	89 (100)	$C_4H_7O_5$	1.5	2.987	threonic acid		-	+
1.11	191.0199	111 (100), 173 (25)	$C_6H_7O_7$	3.5	0.755	citric acid	[50,51]	+	+
1.17	128.0350	-	$C_5H_6O_3N$	3.5	-2.705	pyroglutamic acid[b]		+	+
1.37	117.0190	-	$C_4H_5O_4$	2.5	-2.837	succinic acid[b]	[50,51]	+	+
1.41	147.0301	129 (100), 85 (83), 87 (62)	$C_5H_7O_5$	2.5	0.233	hydroxyglutaric acid		+	-
4.51	153.0196	109 (100)	$C_7H_5O_4$	5.5	0.278	dihydrobenzoic acid	[51,52]	+	-
6.17	311.0407	149 (100), 179 (63)	$C_{13}H_{11}O_9$	8.5	-0.563	caftaric acid[a,b]	[9,42]	+	+
6.21	353.0872	191 (100), 179 (43), 135 (10)	$C_{16}H_{17}O_9$	8.5	-0.615	3-caffeoylquinic acid[b]	[9,41]	+	+
6.80	339.0716	177 (100)	$C_{15}H_{15}O_9$	8.5	-1.579	cichoriin	[51]	+	+
7.38	295.0456	-	$C_{13}H_{11}O_8$	8.5	-0.381	*cisp*-coumaroyltartaric acid	[52]	+	+
7.54	295.0456	163 (100), 119 (3), 149 (3)	$C_{13}H_{11}O_8$	8.5	-0.381	*transp*-coumaroyltartaric acid	[52]	+	+
7.78	353.0873	191 (100), 179 (6), 135 (1)	$C_{16}H_{17}O_9$	8.5	-0.465	5-caffeoylquinic acid[b]	[9,40,41]	+	+
7.93	353.0873	173 (100), 179 (53), 191 (28), 135 (5)	$C_{16}H_{17}O_9$	8.5	-0.525	4-caffeoylquinic acid[b,*]	[9,40,41]	+	+
7.97	485.1655	439 (100), 421 (21)	$C_{22}H_{29}O_{12}$	8.5	-0.979	-		+	-
8.15	639.1180	463 (100)	$C_{27}H_{27}O_{18}$	14.5	-2.307	quercetin hexose glucuronide*	[51]	+	+
8.23	325.0559	193 (100), 113 (3)	$C_{14}H_{13}O_9$	8.5	-1.739	feruloyltartaric acid	[52]	+	+
8.28	491.0794	329 (100), 293 (78), 311 (11)	$C_{22}H_{19}O_{13}$	13.5	-3.155	caffeoyl-dihydroxyphenyllactoyl-tartaric acid	[53]	-	+
8.51	639.1185	477 (100), 463 (89), 301 (26), 519 (20)	$C_{27}H_{27}O_{18}$	14.5	-2.173	quercetin hexose glucuronide	[51]	-	+
9.02	295.0455	179 (100), 133 (49)	$C_{13}H_{11}O_8$	8.5	-0.401	caffeoylmalic acid	[42]	+	+
10.43	473.0715	311 (100), 293 (87), 149 (6)	$C_{22}H_{17}O_{12}$	14.5	-2.154	cichoricacid[a,b]	[9,42]	+	+

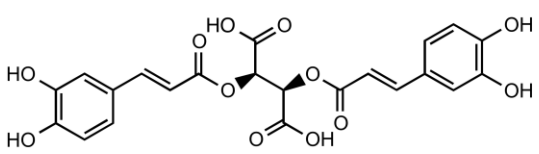
10.85	461.0716	285 (100)	$C_{21}H_{17}O_{12}$	13.5	-2.080	luteolin-7-*O*-glucuronide	[42]	+	+
10.85	515.1184	353 (100), 335 (8), 255 (5), 299 (4), 179 (4), 173 (3), 191 (2)	$C_{25}H_{23}O_{12}$	14.5	-2.231	3.4 dicaffeoylquinic acid	[9,40,41]	+	+
10.89	477.0664	301 (100)	$C_{21}H_{17}O_{13}$	13.5	-1.084	quercetin-3-*O*-glucuronide[b]	[42,52]	+	-
11.04	515.1184	-	$C_{25}H_{23}O_{12}$	14.5	-2.231	3.5 dicaffeoylquinic acid**	[9,40,41]	+	+
11.37	457.0763	295 (100), 293 (65), 277 (45)	$C_{22}H_{17}O_{11}$	14.5	-0.766	*p*-coumaroylcaffeoyltartaric acid	[52]	+	+
11.60	515.1184	353 (100), 317 (8), 299 (8), 203 (8), 255 (4), 179 (3), 173 (3)	$C_{25}H_{23}O_{12}$	14.5	-2.231	4.5 dicaffeoylquinic acid	[9,40,41]	+	+
11.66	487.0870	325 (100), 293 (77), 307 (50)	$C_{23}H_{19}O_{12}$	14.5	-2.503	feruloylcaffeoyltartaric acid	[52]	+	+
11.74	461.0716	285 (100)	$C_{21}H_{17}O_{12}$	13.5	-2.080	kaempferol 3-*O*-glucuronide[b]	[42]	+	-
11.80	445.0767	269 (100), 175 (7)	$C_{21}H_{17}O_{11}$	13.5	-2.055	apigenin glucuronide	[52,53]	+	+
12.18	341.0694	97 (100), 217 (3), 297 (3)	$C_{15}H_{17}O_7S$	7.5	-1.896	8-deacetylmatricarin-8-*O*-sulfate	[51]	+	-
12.30	441.0821	277 (100), 295 (13)	$C_{22}H_{17}O_{10}$	14.5	-0.660	di-*p*-coumaroyltartaric acid *(isomer)*	[52]	+	-
12.59	471.0891	277 (100), 307 (89), 203 (4)	$C_{30}H_{15}O_6$	23.5	1.729	*p*-coumaroylferuloyltartaric acid *(isomer)*	[52]	+	-
12.73	441.0821	277 (100), 295 (41), 203 (2)	$C_{22}H_{17}O_{10}$	14.5	-0.660	di-*p*-coumaroyltartaric acid *(isomer)*	[52]	+	-
12.89	501.1024	307 (100), 233 (5)	$C_{24}H_{21}O_{12}$	14.5	-1.429	*p*-coumaroylsinapoyltartaric acid *(isomer)*	[52]	+	+
13.05	471.0891	307 (100), 277 (63), 325 (18), 295 (9)	$C_{30}H_{15}O_6$	23.5	1.729	*p*-coumaroylferuloyltartaric acid *(isomer)*	[52]	+	-

a: identification with analytical standard, b: identification by NMR from isolated fraction, *MS^2 fragmentation established from isolated fraction, ** no MS^2 date available for this compound, putative annotation

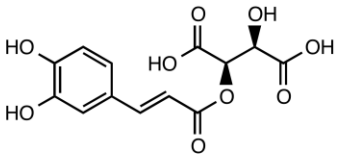
Table 2

Extract	caftaric acid $C \pm \sigma_C$ (mg g^{-1}) (n=6)			cichoric acid $C \pm \sigma_C$ (mg g^{-1}) (n=6)			extraction yields (%)	
							fresh weight	*dry weight*
C. spinosum 1	11.7	±	2.3	36.2	±	1.6	1.7	8.0
C. spinosum 2	12.6	±	2.5	38.3	±	2.7	2.1	9.3
C. spinosum 3	12.5	±	3.0	28.4	±	3.1	2.2	8.5
C. spinosum 4	14.2	±	2.7	34.6	±	2.7	2.2	13.0
C. intybus	19.1	±	2.0	54.6	±	7.4	2.5	14.0

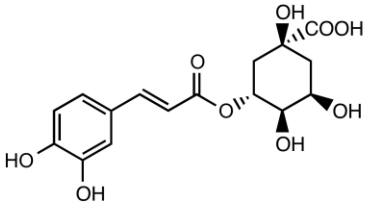
Figure 1



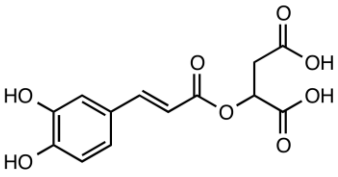
Cichoric acid **(1)**



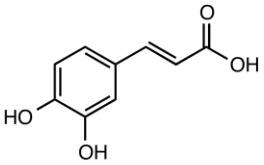
Caftaric acid **(2)**



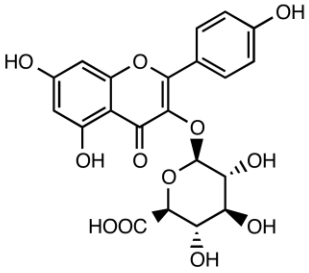
Chlorogenic acid **(3)**



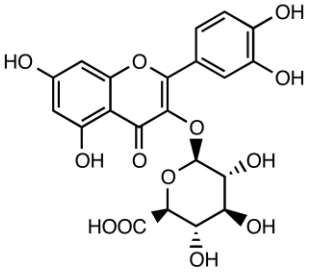
Caffeoylmalic acid **(4)**



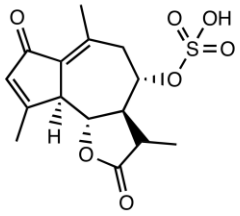
Caffeic acid **(5)**



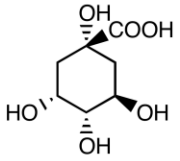
Kaempferol-3-*O*-glucuronide **(6)**



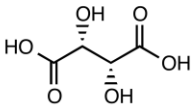
Quercetin-3-*O*-glucuronide **(7)**



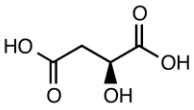
8-deacetylmatricarin-8-*O*-sulfate **(8)**



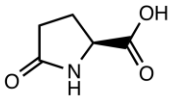
Quinic acid **(9)**



Tartaric acid **(10)**

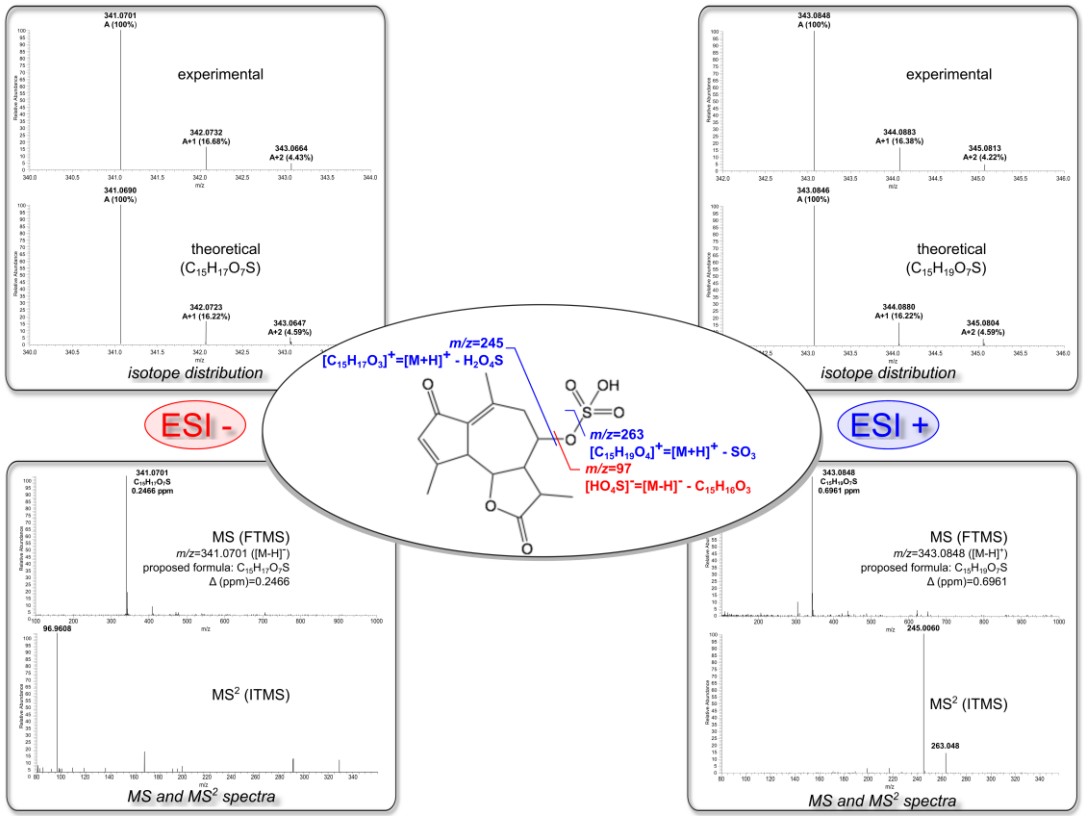


Malic acid **(11)**



Pyroglutamic acid **(12)**

Figure 2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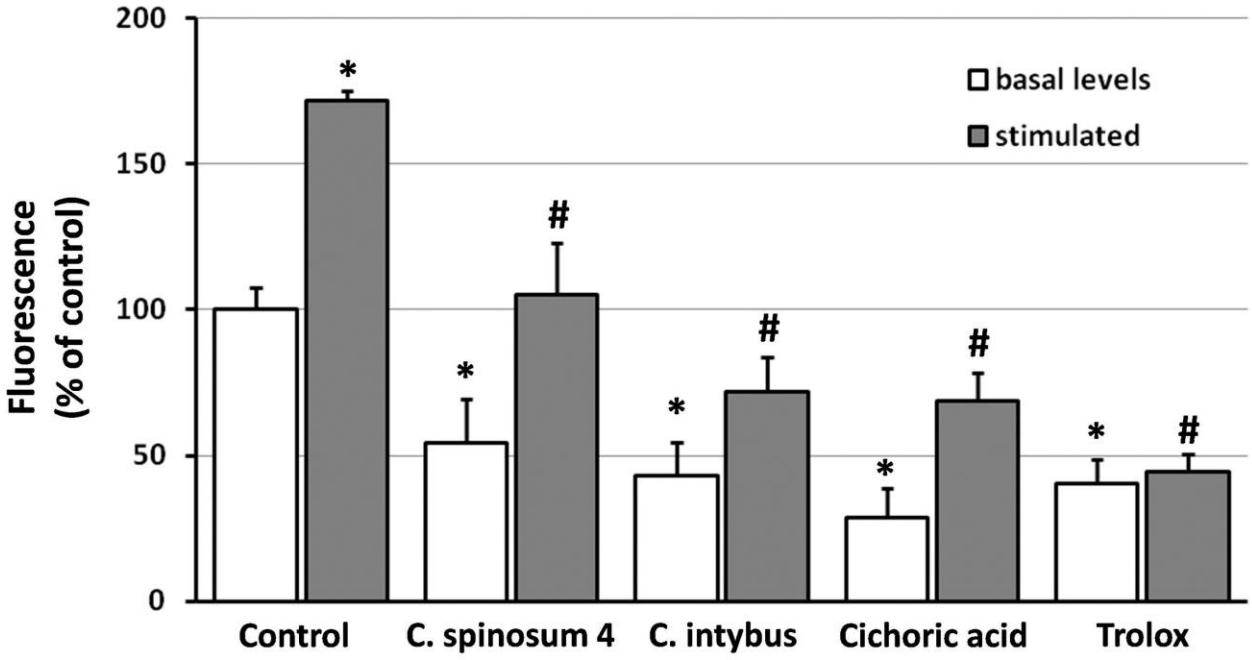
A (100%)
experimental
342.0732
A+1 (16.68%)
343.0664
A+2 (4.43%)
341.0690
A (100%)
theoretical
(C15H17O7S)
342.0723
A+1 (16.22%)
343.0647
A+2 (4.59%)
isotope distribution
343.0848
A (100%)
experimental
344.0883
A+1 (16.38%)
345.0813
A+2 (4.22%)
343.0846
A (100%)
theoretical
(C15H19O7S)
344.0880
A+1 (16.22%)
345.0804
A+2 (4.59%)
isotope distribution
ESI -
ESI +
m/z=245
[C15H17O3]+=[M+H]+ - H2O4S
m/z=263
[C15H19O4]+=[M+H]+ - SO3
m/z=97
[HO4S]-=[M-H]- - C15H16O3
341.0701
C15H17O7S
0.2466 ppm
MS (FTMS)
m/z=341.0701 ([M-H]-)
proposed formula: C15H17O7S
Δ (ppm)=0.2466
96.9608
MS2 (ITMS)
MS and MS2 spectra
343.0848
C15H19O7S
0.6961 ppm
MS (FTMS)
m/z=343.0848 ([M-H]-)
proposed formula: C15H19O7S
Δ (ppm)=0.6961
245.0060
MS2 (ITMS)
263.048
MS and MS2 spectra

Figure 3


200
150
100
50
0
Fluorescence (% of control)
basal levels
stimulated
*
#
Control
C. spinosum 4
C. intybus
Cichoric acid
Trolox