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Phytochemical analysis of a herbal tea from *Artemisia annua* L.

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ABSTRACT

Strategies to control diffusion of malaria needs to account for the increase of resistance of the parasite to the conventional antimalarial drugs. It has been proposed that a traditional aqueous preparation from *Artemisia annua*, with a low content of the active compound, artemisinin, may reduce the risk of resistance of the protozoa and be relatively more effective in the treatment of the disease. The solubility properties of the molecule have been the matter of concern about the therapeutic usefulness of herbal teas from *A. annua*. The present study aimed at analysing the chemical profile of a tea infusion from *A. annua*. Tea from *A. annua* was prepared through infusion of the plant aerial parts in water for 1, 24 and 48 h. Content of artemisinin was determined by HPLC-ELSD. Overall chemical characterization of the extracts was carried out by a combination of metabolomic techniques. The artemisinin content varied only slightly in the three different extracts (about 0.12%). A series of mono-caffeoyl- and mono-feruloyl-quinic acids, di-caffeoyl- and di-feruloyl-quinic acids was identified as main components of the tea infusion, together with some flavonoids. Reconstitution of the same extracts in less polar or apolar solvents resulted in a different composition with no phenolics and a much lower concentration of artemisinin.

1. Introduction

Artemisia annua (annual wormowood), is an annual herb belonging to the *Asteraceae* family. It is native to Asia, but now worldwide naturalized and cultivated in many countries in Europe, including Italy where it has been proposed as a new economic crop to replace traditional cultivation of tobacco [1,2].

The chemistry of the plant has been extensively studied leading to the identification of more than hundred secondary metabolites until now [2–6]. They mainly consist of sesquiterpenoid constituents including a large number of sesquiterpene lactones which are unique for *A. annua* [3]. Among these, particular attention has been directed to artemisinin, the unusual endoperoxide sesquiterpene lactone contained in the glandular trichomes of the aerial organs of the plant [3,7] and recognized as the bioactive ingredient of the drug. Presently this compound is reputed as the most potent and rapidly acting antimalarial drug. Moreover, based on its pharmacological properties and therapeutic applications, a series of artemisinin-based molecules have been developed and they are currently used to treat mild and severe forms of malaria [3,8–10].

Malaria is the world's most important parasitic disease in terms of morbidity and mortality [9,10] and it is caused by *Plasmodium* sp. protozoa of which *P. falciparum* is the most common species responsible for the infection. Strategies to control diffusion of this disease have to take into account the increase of resistance of the parasite to the conventional antimalarial drugs [3,10]. Multi-drug resistant strains of the *Plasmodium* have in fact developed and recently the emergence of parasites resistant to artemisinin has also been reported [11].

It has been proposed that a traditional aqueous preparation from the herb, with a lower concentration of artemisinin, may reduce the risk of resistance of the protozoa and even be relatively more effective in the treatment of the disease [9,11–14]. Clinical efficacy of infusion and decoction from *A. annua* has been evaluated and results found consistent with their therapeutic effect against malaria in humans [12,13].

Artemisinin is poorly soluble in water and it has been reported as labile in acid and basic conditions, but fairly stable in neutral solvents even when heated up to 150 °C [3,15,16]. These chemical properties have been the matter of concern about the therapeutic usefulness of herbal teas from *A. annua*.

Besides the peculiar chemical properties of the molecule, also the very low (0.1–1% dw) amount of artemisinin in the plant has represented an important feature to be investigated. During the recent years several studies have been carried out, for

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example, to establish an appropriate analytical method to detect artemisinin [15,17]. From classical gas-chromatographic analyses which evaluate indirectly artemisinin content through its degradation derivatives, more recently the use of sensitive detectors hyphenated to HPLC has gained importance. One of these is the Evaporative Light Scattering Detector, ELSD.

Some studies have been carried out [1,12,13] primarily to understand whether artemisinin was present in the water extracts and not finalized to fully characterize the composition of such extracts. Moreover, chemical determinations have been made after re-extraction with an organic solvent.

In the present paper, we aimed to investigate the phytochemical profile of three aqueous extracts of *A. annua* prepared by infusion of the plant aerial parts for 1, 24 and 48 h, respectively. The water extraction efficiency over the time of infusion was determined through artemisinin quantification by HPLC-ELSD. An appropriate protocol for artemisinin detection with ELSD in our tea extract was developed and reported. Content of the bioactive artemisinin in the aqueous extracts was also confirmed by ESI-MS/MS and NMR. In addition to artemisinin detection, our investigation also includes the study of the compositional profile of the water extracts as such through the identification of the other metabolites extracted by water infusion and data are reported here. Methods used in this investigation of artemisia water infusion allowed to detect a wide range of constituents, mainly phenolics, which deserve to be further studied in order to disclose, if any, their contribution to the therapeutic effect of *A. annua*.

2. Materials and methods

2.1. Plant material

A. annua was cultivated at CRA-Istituto Sperimentale per i Tabacchi, Monteroni (Lecce). The aerial parts were collected at the flowering stage during September 2008. Reference specimens of the plants are available from the authors. The harvested plant material was air dried at temperatures below 40 °C.

2.2. Tea preparation

Herbal teas were prepared from *A. annua* dried leaves. Boiling water (13 ml) was added to about 500 mg of plant material and the mixture left to cool, manually stirred from time to time and then filtered. Three different preparations were made leaving the dried aerial parts in infusion for 1, 24 and 48 h, respectively. Water extracts were freeze-dried and stored until used.

2.3. HPLC

The freeze dried extracts of *A. annua* obtained as above (1 h, 24 h, 48 h water infusion) were dissolved (15 mg/ml) in water or in organic solvents (acetonitrile and hexane) and analyzed by HPLC. An aliquot of 20 μl was injected for the HPLC runs.

HPLC analyses were performed with a Waters HPLC 600 Liquid Chromatograph equipped with a Diode-Array-Detector, DAD 2998 Waters and an Evaporative Light Scattering Detector, ELSD 2424 Waters. Data were processed with Empower™ 2 Waters Software. ELSD parameters were set to optimize the detection of artemisinin and were as follows: drift tube temperature, 55 °C; gain, 1000. Final value for this parameter was gradually obtained over 55 min of run, starting from gain 10. Nitrogen was used as the driving gas for nebulization at 60 psi. Helium was used as the degassing solvent. Extracts were separated with a Gemini C18 (Phenomenex) column (250 mm × 4.60 mm, 5 μm particle size) equipped with a Security Guard C18 Cartridge (4 mm × 3 mm, Phenomenex). The following elution system was used with DAD detection: solvent A, H_2O–HCOOH 0.1%, pH 4 with NaOH; solvent B, CH_3CN–HCOOH 0.1%. The elution gradient was: 12% B in A increasing to reach 25% B at 60 min, 60% B at 80 min and 100% B at 85 min. When ELSD was used for detection, the elution system did not include NaOH and run chromatographic conditions were as follows: 12% B increasing to reach 20% B at 30 min, 25% B at 46 min and 100% B at 66 min. The flow rate was 1 ml/min. Removal of NaOH from the elution system, in case of ELSD use, was due to its low vaporization. UV spectra of each extract were conventionally recorded at 210, 270, 310 and 350 nm.

2.4. Calibration curves, limit of detection (LOD) and limit of quantification (LOQ)

Quantification of artemisinin by HPLC-ELSD analyses was made against a calibration curve obtained with six reference solutions of artemisinin (Sigma) at the concentration of 12.3, 24.5, 48.9, 78.9, 98.6 and 118.8 μg/ml. Correlation coefficient (r^2) of the standard curve in the linear plot of log-transformed data was $r^2 = 0.9987$ ($y = 1.7382x + 3.4295$) indicating a good linearity between the log-transformed area and log-transformed concentrations within the tested concentration range.

The limit of detection (LOD) and limit of quantification (LOQ) were evaluated on the basis of signal-to-noise ratios (S/N) of 3:1 and on 10:1, respectively. The LOD for artemisinin in the adopted analytical conditions was 2.2 μg/ml; LOQ was 7.9 μg/ml.

For phenolics, linear calibration curves were constructed with six solutions of different concentrations, in the linear range of 1.5–335.0 μg/ml, of the following chemical markers: caffeic acid, chlorogenic acid and 3,4-dicaffeoylquinic acid. The established calibration curves showed good linear regression with a high correlation coefficient within the tested range of concentrations, that is: for caffeic acid, $r^2 = 0.9997$ ($y = 91846x - 14685$); for chlorogenic acid, $r^2 = 0.9999$ ($y = 32063x + 31946$); for 3,4-dicaffeoylquinic acid, $r^2 = 0.9996$ ($y = 59885x + 72921$). Calculated LOQ and LOD in the adopted analytical conditions were, respectively, for caffeic acid, 0.05 and 0.125 μg/ml, for chlorogenic acid 0.50 and 0.18 μg/ml, for 3,4-dicaffeoylquinic acid, 0.76 and 1.09 μg/ml. All the HPLC analyses were run in triplicate.

2.5. Precision, accuracy and recovery

Intra-day and inter-day variations were recorded to determine the precision of the developed analytical method. For intra-day variability test, reference standard solutions of artemisinin or phenolics chemical markers (caffeic, chlorogenic or 3,4-dicaffeoylquinic acids) were analyzed in three replicates, respectively, within the same day, while for inter-day precision test, analyses were run in three replicates as above on three consecutive days. The relative standard deviation (RSD) value of retention times and peak areas were adopted to evaluate precision. The intra-day and inter-day variability of the same compounds in the drug extract were also evaluated as above.

Efficiency of the extraction procedure was determined by the standard addition method. Accurate amounts of artemisinin or the phenolic acids used as markers (80, 100 and 120% of their content in 0.5 g plant sample, respectively) were added to 0.5 g of air-dried plant material and then extracted and processed for HPLC analyses and quantification. The total recovery of either artemisinin or marker phenolic acids was calculated from the corresponding calibration curves (see above). Analyses were run in triplicate. The average recovery of each compound was obtained using the following formula: recovery (%) = (amount found − original amount)/amount spiked × 100.

2.6. Synthesis of caffeoylquinic acids

Isomers of chlorogenic acid (5-caffeoylquinic acid, 5-CQA) were prepared starting from commercial chlorogenic acid (Sigma) according to Ossipov et al. [18]. The obtained mixture of isomers, 3-CQA, 5-CQA and 4-CQA, was purified through a Sep Pak Vac C18 cartridges (Waters) and then separated by HPLC.

2.7. LC–ESI-MS/MS

Flow injection MS analysis were performed on a 1100 Series Agilent LC/MSD Trap-System VL. An Agilent Chemstation (LC/MSD TrapSoftware 4.1) was used for the acquisition and processing of the data. The same HPLC chromatographic conditions as described in Section 2.3 have been adopted. All the analyses were carried out using a ESI ion source both in the positive and negative mode with the following settings: capillary voltage, 4000 V; nebulizer gas (N_2), 15 psi; drying gas (N_2), 350 °C, 5 l/min. Full scan spectra were acquired over the range of 100–2200 m/z with a scan time of 13,000 m/z/s. Automated MS/MS was performed by isolating the base peaks (molecular ions) using an isolation width of 4.0 m/z, threshold set at 100 and ion charge control on, with max acquire time set at 300 ms. Different collision energies were conventionally used (1.0, 10 and 30 V) for MS/MS fragmentation. Samples were dissolved in MeOH:H_2O (9:1) at the concentration of 20–30 ppm and injected at a flow rate of 10 μl/min (KD Scientific Syringe Pump).

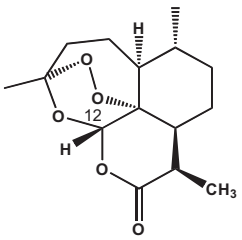
2.8. NMR

Proton (^{1}H NMR) nuclear magnetic resonances were recorded on a Bruker Avance III 400 spectrometer equipped with probes for inverse detection and with z gradient-accelerated spectroscopy. Standard ^{1}H NMR analysis was first performed on 20 mg aliquots of the freeze-dried total extract, dissolved in 500 μL D_2O, containing 1 mg/ml DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as chemical shift reference. Artemisinin presence was assessed on the basis of comparison of the chemical shift of the 12-H methine proton (δ 6.20 ppm in D_2O saturated solution for the authentic standard). The presence of artemisinin in the extract was confirmed by further experiments. Briefly, the lyophilized infuse was extracted with hexane, the solvent dried under vacuum, and the residual redissolved in 500 μL $CDCl_3$ to acquire NMR spectra. Residual ^{1}H peaks of the solvent were used as internal standards to calculate chemical shifts referred to tetramethylsilane.

3. Results

Artemisinin identification in the aqueous extracts was made by comparison of its retention time (R_t = 68.3) with that of the artemisinin peak from a reference standard analyzed by HPLC-ELSD in the same analytical conditions. Presence of artemisinin in the aqueous extracts was also confirmed by ESI-MS/MS analyses. The ion observed in the MS^1 at m/z 305, corresponding to the sodium adduct [M+Na]$^+$ of artemisinin (MW 282), was further fragmented and the MS^2 gave additional peaks at m/z (rel. int.) 277.0 [M+Na−CO]$^+$ (30%), 263.0 [M+Na−CH_2CO]$^+$ (100%) and 259.1 [M+Na−CO−H_2O]$^+$ (27%), consistent with the fragmentation of the active molecule (Fig. 1). Artemisinin in the tea extract was further detected by ^{1}H NMR and unequivocally identified through the characteristic chemical shift of the proton H-12 at δ 6.20 (in D_2O) or at δ 5.87 (in $CDCl_3$) [19].

The reproducibility of the HPLC-ELSD analytical method for artemisinin (see above) in terms of retention times and peak areas gave RSDs values less than 0.24% (retention times) and 2.76% (peak areas) showing good precision of the instrument. Recovery of artemisinin by the standard addition method was



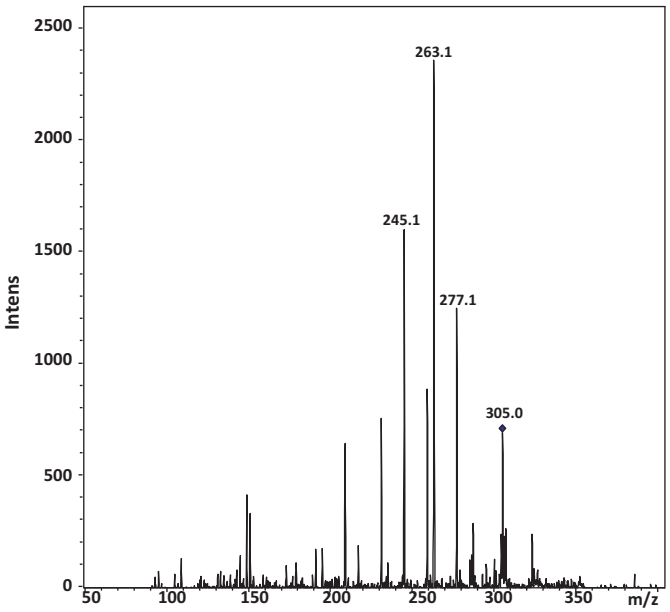


Fig. 1. Positive ion MS^2 spectrum of artemisinin from *A. annua* water extract.

97.25% ± 2.40 (80% addition), 105% ± 2.66 (100% addition) and 98.30% ± 0.11 (120% addition) indicating a good accuracy of the extraction method.

As shown in Table 1 and Fig. 2, the content of artemisinin as determined by HPLC-ELSD against the calibration curve, only slightly varied in the three plant extracts. Identical amount of artemisinin was in fact detected in the three infusions after 1 h (54 mg; 0.11% dw), 24 h (61 mg; 0.12% dw) and 48 h (64 mg; 0.12% dw).

ESI-MS/MS and spiking of the extracts with available reference standards (SensaPharm, UK) allowed to exclude the presence of artemisinin related compounds [20] such as artemisinic acid, artemisitene and deoxyartemisinin. Only dihydroartemisinin could be detected by coelution (R_t = 65.4) with the reference compound and by its ESI-MS/MS fragmentation: m/z (rel. int.), 307 [M, 284+Na]$^+$, further fragmented in the MS^2 to give a peak at 261.0 [M+Na−46]$^+$ (100%).

Chromatogram traces of the *A. annua* teas by HPLC-DAD/ELSD revealed that in addition to artemisinin and its congener, other metabolites were present in the water extracts. Comparison of HPLC compositional profiles did not disclose however any significant difference among the extracts and they were combined

Table 1
Quantification of artemisinin in *A. annua* tea.

Infusion time (h)	Amount of drug (mg)	Artemisinin	
		(mg ± SD)	(%, w/w)
1	495	0.54 ± 0 .012	0.109
24	527	0.61 ± 0.011	0.116
48	526	0.64 ± 0.010	0.121

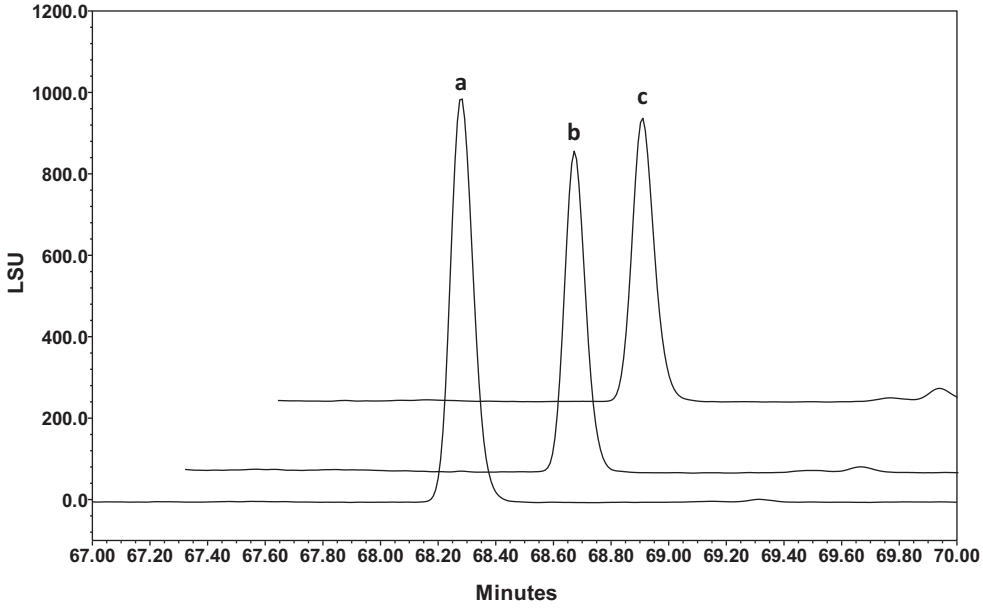


Fig. 2. HPLC-ELSD detection of artemisin in water extracts from *A. annua* after (a) 1 h, (b) 24 h and (c) 48 h infusion.

for further chemical characterization. The chromatographic trace (310 nm) is shown in Fig. 3. Inspection of the metabolic profile combining HPLC-DAD and LC/MS/MS data showed that the extracts comprise at least other two main chemical classes of constituents and the chromatogram could be divided into two main elution areas, with monoacyl chlorogenic acids eluting in the first 25–30 min, and diacylchlorogenic acids and most of the flavonoids in the following minutes of run. Compounds with absorption bands at 323.6–326.0 nm and 232.2–242.8 nm plus a diagnostic sharp shoulder at 290–300 nm could be unequivocally identified as chlorogenic acids (esters formed between (−)-quinic acid and one to four residues of some *trans*-cinnamic acids). This was also confirmed by ESI-MS/MS. A series of molecular ions $[M-H]^-$, at *m/z* 353, *m/z* 367, *m/z* 515, *m/z* 543, *m/z* 529 and *m/z* 553 were localized in the chromatogram with a general MS^2 fragmentation consistent, respectively, with caffeoylquinic acids [*m/z* 353, $M-H]^-$, [*m/z* 191, quinic acid$-H]^-$, [*m/z* 179, caffeic acid$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$; feruloylquinic acids [*m/z* 367, $M-H]^-$, [*m/z* 193, ferulic acid$-H]^-$, [*m/z* 191, quinic acid-H; $M-$feruloyl$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$; dicaffeoylquinic acids [*m/z* 515, $M-H]^-$, [*m/z* 353, $M-$caffeoyl$-H]^-$, [*m/z* 191, quinic acid$-H]^-$, [*m/z* 179, caffeic acid$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$; diferuloylquinic acids [*m/z* 543, $M-H]^-$, [*m/z* 367, $M-$feruloyl$-H]^-$, [*m/z* 349, $M-$feruloyl$-H_2O-H]^-$, [*m/z* 193, ferulic acid$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$; caffeoylferuloylquinic acids [*m/z* 529, $M-H]^-$, [*m/z* 367, $M-$caffeoyl$-H]^-$, [*m/z* 353, $M-$feruloyl$-H]^-$, [*m/z* 191, ferulic acid$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$ and di-succinoylcaffeoylquinic acids [*m/z* 553, $M-H]^-$, [*m/z* 391, M-caffeoyl-$H]^-$, [*m/z* 217, $M-2\times$succinoyl$-H]^-$, [*m/z* 179, caffeic acid$-H]^-$, [*m/z* 173, quinic acid$-H_2O-H]^-$.

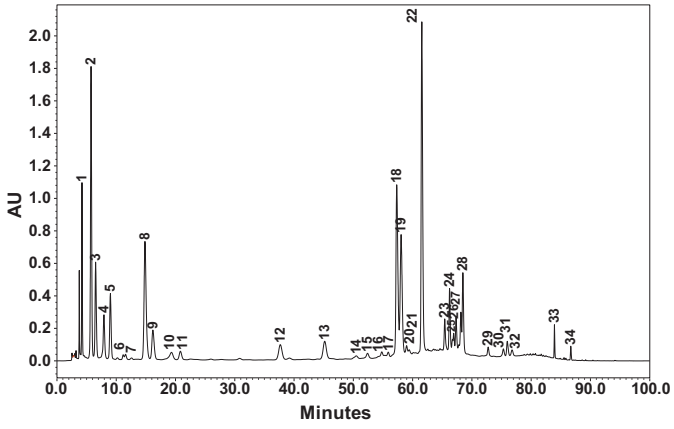


Fig. 3. HPLC-DAD chromatogram (310 nm) of *A. annua* water extract (for peak numbering see Table 3).

The simple phenolic acids, caffeic acid ([*m/z* 179 (100), $M-H]^-$, [*m/z* 135 (100), M-44 (CO_2)$-H]^-$) and quinic acid ([*m/z* 191 (100), $M-H]^-$, [*m/z* 173 (77), $M-H_2O-H]^-$, [*m/z* 127 (100), $M-CO_2-H]^-$) were also identified in the tea extract.

ESI-MS/MS fragmentation together with HPLC coelution of available reference compounds and comparison with literature data also allowed the identification of different chlorogenic acid isomers.

The monocaffeoylquinic acid isomers were identified by comparison of their MS fragmentation patterns and HPLC elution times with those of commercial chlorogenic acid (5-caffeoylquinic acid), R_t 5.76, and neo-chlorogenic acid (3-caffeoylquinic acid), R_t 4.23, and crypto-chlorogenic acid (4-caffeoylquinic acid), R_t 6.54 obtained by chemical isomerization of chlorogenic acid (see Section 2).

The three isomers, 3-, 4- and 5-*O*-feruloylquinic acids (R_t = 7.93, 11.95 and 16.01, respectively) were identified according to Clifford et al. [21] from the base peaks and the relative intensities of secondary ions formed from MS^2 fragmentation of their parent ions at *m/z* 367: respectively, [*m/z* 193.06 (100), *m/z* 173 (10)]$^-$, [*m/z* 193.07 (20), 173.06 (100)]$^-$ and, [*m/z* 191.07 (100), *m/z* 173.06 (15)]$^-$.

At least six peaks shared the same molecular weight of 515 corresponding to dicaffeoylquinic acids suggesting that the six possible isomers 1,3-, 1,4-, 1,5-, 3,4-, 3,5- and 4,5-dicaffeoyl chlorogenic acids were all present in the extract; however, only three of them could be unequivocally identified by MS fragmentation and their identity confirmed by coelution with authentic reference compounds (Phytolab, Germany; Roth, Germany) and that is the 3,4-, 3,5- and 4,5-di-*O*-caffeoylquinic acids (R_t = 57.33, 58.07 and 61.56, respectively).

Three major peaks shared the same pseudomolecular mass at *m/z* 543 and MS^2 fragmentation typical of diferuloylquinic acids.

The first eluting had the MS^2 base peak at *m/z* 349.14, while the other two showed the ion at *m/z* 367.14. In agreement with literature data [22] they were respectively assigned to the three 3,4-, 3,5- and 4,5-*O*-diferuloylquinic acids isomers (R_t = 65.43, 66.24 and 68.48 respectively).

Due to the low amount of the isomeric caffeoylferuloyl chlorogenic acids in the extract, only the 3,5-caffeoylferuloylquinic acid, R_t = 72.74 could be identified through the MS^2 fragmentation of its pseudomolecular ion at *m/z* 529 $[M-H]^-$: [*m/z* 366.80 (82), *m/z* 352.80 (100), *m/z* 191.00 (15), *m/z* 173.00 (25)]$^-$.

Among the putative succinic acid containing chlorogenic acids, mass fragmentation (see above) disclosed the presence only of one disuccinoylcaffeoylquinic acid (R_t = 75.96), which is tentatively identified as the 4-*O*-caffeoyl-3,5-di-*O*-succinoylquinic acid isomer [*m/z* 390.80 (100), *m/z* 216.90 (6), *m/z* 179.00 (4), 173.00 (3)]$^-$.

Characterization of the extracts by HPLC-DAD indicated the presence of other metabolites typically showing two maxima of absorption in the range of 257.0–270.1 nm (Band II, A-ring benzoyl system) and 333.2–349.9 nm (Band I, B-ring cynnamoyl system) with weak absorptions around nm 299*sh*, diagnostic for a flavonol or a flavone structure. Moreover, another group of compounds was characterized by one major absorption band at 283.1 nm and a weak shoulder at 318–320 nm suggesting the presence of flavanones [23].

Furthermore, inspection of the mass spectra of these components allowed to distinguish three characteristic fragmentation patterns: (a) components which showed the loss of an hexose sugar from the parent ion with a base peak corresponding to the aglycone and suggested the presence of *O*-glycosylated flavonoids; (b) components whose fragmentation was consistent with that of *C*-glycosylated flavonoids, producing the typical ions $[M-H-90]^-$ and $[M-H-120]^-$; (c) methoxylated flavonoids showing characteristic $[M-H-15]^-$ fragments.

Consistently, a series of flavonoidic constituents have also been identified in the water extract obtained from *A. annua* aerial parts. The ESI-MS/MS fragmentation of the pseudomolecular ion at *m/z* 447.00 $[M-H]^-$ produced an aglycone fragment at *m/z* 285.00 (100), $[M-H-162]^-$ consistent with the presence of a luteolin hexoside. Spiking of the extract with available reference luteolin glycosides allowed to detect the luteolin-7-*O*-glucoside (cynaroside, 256, 265*sh*, 343) at R_t = 59.00. A pseudomolecular ion at *m/z* 493.00 $[M-H]^-$ which fragmented giving a base peak at *m/z* 330.70 (100) $[M-H-162]^-$ and a less intense fragment at *m/z* 315.80 $[M-H-162-15]^-$ were detected by ESI-MS/MS analysis; only based on these spectrometric data and UV absorption (255, 267*sh*, 297*sh*, 349 nm) the peak at R_t 54.79 is tentatively identified as the patuletin-(7) or–(3)-*O*-glucoside. A polymethoxylated flavonoid with a pseudomolecular ion at *m/z* 359.11 $[M-H]^-$ and intense fragments at *m/z* 343.80 (loss of a methyl group) and *m/z* 329.08 (loss of another methyl group) showing the following UV absorbance, 258, 272*sh*, 351 nm, was identified as the jaceidin (R_t = 83.68). The presence of another polymethoxylated flavonoid was suggested by the ion at *m/z* 344 consistent with a flavone structure bearing 2x—OH and 3x—OCH_3 which was identified by combining its fragmentation pattern ([*m/z* 343.80 (100), $M-H]^-$, [*m/z* 328.80 (100), $M-H-15]^-$, [*m/z* 313.80 (10), $M-H-15]^-$) and UV spectra (256, 270, 349 nm) as the cirsilineol (R_t = 86.64). Vitexin (8-*C*-glucosyl apigenin) was identified by its mass spectrum ([*m/z* 431.00 (100), $M-H]^-$, [*m/z* 340.80 (15), $M-H-90]^-$, [*m/z* 310.80 (100), $M-H-120]^-$), by coelution (R_t = 52.41) with a reference compound (Extrasynthese, France) and by its UV spectrum (268, 302*sh*, 336 nm). Similarly, isovitexin (R_t = 56.00) could also be detected. Two compounds were found with the same pseudomolecular ion $[M-H]^-$ at *m/z* 563 whose MS/MS fragmentation gave the following ions [*m/z* 545.00, $M-H-H_2O]^-$, [*m/z* 503.00, $M-H-60]^-$, [*m/z* 473.00, $M-H-90]^-$, [*m/z* 443.00, $M-H-120]^-$, [*m/z* 383.00, $M-H-180]^-$, and [*m/z* 353.00, $M-H-209]^-$ and showed UV absorption maxima at 271 and 332 nm. Based on published data [24] were identified as the two di-*C*-hexosyl-pentosyl-flavones, 6-*C*-glucosyl-8-*C*-arabinosyl (schaftoside) and 6-*C*-arabinosyl-8-*C*-glucosyl (isoschaftoside) apigenin (R_t = 19.35, 20.91). The flavanone eriodictyol (R_t = 77.77) was identified by coelution with an authentic sample (Extrasynthese, France) and UV spectra comparison (288, 330*sh*). The pseudomolecular ion [*m/z* 609.00, $M-H]^-$, and mass fragmentation pattern [*m/z* 300.80, $M-H-162-146]^-$ suggested the presence of a rutinoside flavonoid similar to quercetin-3-rutinoside. Matching of retention time and UV spectrum allowed excluding the presence of rutin and tentatively identifying the compound as a chrysoeriol rutinoside, which has previously been reported in other species of *Artemisia* [25].

Moreover, considering the mass fragmentation of the $[M-H]^-$ ion at *m/z* 343.00 ($C_{19}H_{20}O_6$) yielding the typical *retro* Diels–Alder fragment at *m/z* 181.07 and the ion [*m/z* 166.04, $M-H-15]^-$, the presence of a tetramethoxyflavanone bearing two methoxyl groups on ring A (consistently the other two on ring B) can also be deduced.

Finally, based on comparison with literature data from other *Artemisia* sp. [26] the presence of a trimethoxycoumarin (R_t = 37.69) could also be inferred in the tea extracts (*m/z* 235.00, $[M-H]^-$ (100), *m/z* 219.00 (20), *m/z* 191.00 ($[M-H-(3x-CH_3)]^-$, 100), 143.20 ($[M-(3x-OCH_3)-H]^-$, 22); UV, 250, 296, 344 nm).

A summary of MS fragmentation data of phenolic constituents identified in *A. annua* water infusions are listed in Table 2.

The reproducibility of the HPLC analytical method for phenolic constituents in terms of retention times and peak areas, as for artemisinin (see above), was also evaluated in intra- and inter-day experiments and the calculated RSDs values were respectively within 1.0–2.0 (retention times) and 1.7–2.4% (peak areas) showing good precision of the analysis. Validation of the extraction method was also evaluated through recovery tests of the marker phenolic acids with the following results: caffeic acid, 100.45% ± 0.30 (80% addition), 101.50% ± 0.28 (100% addition) and 102.00% ± 0.20 (120% addition); chlorogenic acid, 101.40% ± 0.35 (80% addition), 102.50% ± 0.30 (100% addition) and 102.50% ± 0.30 (120% addition); 3,4-dicaffeoylquinic acid, 98.60% ± 0.25 (80% addition), 100.50% ± 0.30 (100% addition) and 101.80% ± 0.25 (120% addition) indicating a good accuracy.

Table 3 summarizes the quantitative data. As reported, quantification of the extracts obtained after 1, 24 and 48 h of infusion did not show any substantial difference in the compositional profile and yield, being the bulk of the constituents in our extracts of *A. annua* made up by chlorogenic acids namely caffeic acid derivatives.

Very interestingly, when the same extracts were reconstituted (see Section 2) in a less polar solvent, such as acetonitrile, or an apolar solvent, such as hexane, none of the phenolic metabolites discussed above were detected in the HPLC runs besides artemisinin and the amount of artemisinin detectable in the chromatograms was much less, even when the amount of hexane, the most suitable solvent to dissolve artemisinin, was tripled (Fig. 4).

4. Discussion

Since the discovery of artemisinin, several phytochemical studies have been conducted to characterize *A. annua* and also other *Artemisia* species [3,5,6]. The genus represents a high reservoir of terpenes, phenols and acetylenes, but coumarins and flavonoids have also been identified. Within the genus, *A. annua* is particularly rich in sesquiterpenoids found in the aerial parts of the plant. The most peculiar compounds are however the artemisinin related sesquiterpenes such as artemisitene, dehoxyartemisinin, arteannuin B, artemisinic and dihydroartemisinic acid, some of which are

Table 2
Summary of phenolics identified by ESI-MS/MS in *A. annua* tea.

Name	$[M-H]^-$, m/z	ESI-MS/MS (%)
Caffeic acid	179	135 (100)
Quinic acid	191	173 (77), 127 (100)
Monocaffeoylquinic acids	353	
3-Caffeoylquinic acid		191 (100), 179 (46), 173 (5), 135 (17)
5-Caffeoylquinic acid		191 (100), 179 (4), 135 (1)
4-Caffeoylquinic acid		191 (18), 179 (80), 173 (100), 135 (12)
Monoferuloylquinic acids	367	
3-Feruloylquinic acid		193 (100), 191 (4), 173 (10)
5-Feruloylquinic acid		193 (12), 191 (100), 173 (12)
4-Feruloylquinic acid		193 (20), 191 (5), 173 (100)
Dicaffeoylquinic acids	515	
3,4-Dicaffeoylquinic acid		353 (100), 191 (11), 179 (21), 173 (33)
3,5-Dicaffeoylquinic acid		353 (100), 191 (6)
4,5-Dicaffeoylquinic acid		353 (100), 191 (6), 179 (11), 173 (21),
Diferuloylquinic acids	543	
3,4-Diferuloylquinic acid		367 (50), 349 (100), 193 (20), 173 (20)
3,5-Diferuloylquinic acid		367 (100), 349 (65), 193 (30), 173 (30)
4,5-Diferuloylquinic acid		367 (100), 349 (20), 193 (10), 173 (15)
Caffeoylferuloylquinic acids	529	
3,5-Caffeoylferuloylquinic acid		367 (82), 353 (100), 191 (15), 173 (25)
Disuccinoylcaffeoylquinic acids	553	
4-Caffeoyl-3,5-disuccinoylquinic acid		391 (100), 217 (6), 179(4), 173 (3)
6-*C*-arabinosyl-8-*C*-glucosyl apigenin	563	545 (10), 503 (10), 473(45), 443 (43), 383 (82), 353 (100)
6-*C*-glucosyl-8-*C*-arabinosyl apigenin	563	545 (9), 503 (8), 473 (45), 443 (52), 383 (60), 353 (100)
Vitexin (8-*C*-glucosyl apigenin)	431	341 (15), 311 (100)
Isovitexin (6*C*-glucosyl apigenin)	431	341 (15), 311 (100)
Patuletinglycoside	493	331 (100), 315 (14)
Luteolin-7-*O*-glucoside	447	285 (100)
Chrysoeriol rutinoside	609	301 (100)
Jaceidin	359	343 (100), 329 (4)
Cirsilineol	343	329 (100), 313 (10)

Table 3
Quantification of phenolics in *A. annua* water extracts obtained after 1 h, 24 h and 48 h infusion.

Peaks[a]		R_t (min)	mg/g dw		
			1 h	24 h	48 h
1	3-Caffeoylquinic acid	4.23	1.14 ± 0.01	1.15 ± 0.01	1.21 ± 0.01
2	5-Caffeoylquinic acid	5.76	7.81 ± 0.02	9.11 ± 0.02	9.97 ± 0.03
3	4-Caffeoylquinic acid	6.54	1.24 ± 0.08	1.40 ± 0.02	1.58 ± 0.03
4	3-Feruloylquninic acid	7.93	0.70 ± 0.02	0.74 ± 0.01	0.86 ± 0.02
5	Tetramethoxyflavanone	9.01	0.89 ± 0.06	0.42 ± 0.01	0.29 ± 0.01
6/7	4-Feruloylquinic acid	11.95	0.28 ± 0.01	0.32 ± 0.01	0.29 ± 0.02
8	Caffeic acid	14.85	3.11 ± 0.02	3.12 ± 0.03	4.10 ± 0.06
9	5-Feruloylquininc acid	16.01	0.71 ± 0.02	0.78 ± 0.01	0.71 ± 0.04
10	6-*C*-arabinosyl-8-*C*-glucosyl apigenin	19.35	0.49 ± 0.01	0.48 ± 0.01	0.52 ± 0.02
11	6-*C*-glucosyl-8-*C*-arabinosyl apigenin	20.91	0.45 ± 0.01	0.45 ± 0.03	0.45 ± 0.02
12	Trimethoxycoumarin	37.69	1.31 ± 0.09	1.11 ± 0.09	1.68 ± 0.10
13	Chrysoeriol rutinoside	45.17	0.96 ± 0.06	0.91 ± 0.02	1.05 ± 0.05
15	Vitexin (8-*C*-glucosyl apigenin)	52.41	0.80 ± 0.03	0.74 ± 0.01	1.04 ± 0.02
16	Patuletinglycoside	54.79	0.69 ± 0.02	0.72 ± 0.01	0.70 ± 0.02
17	Isovitexin (6*C*-glucosyl apigenin)	56.00	1.14 ± 0.01	0.89 ± 0.07	0.85 ± 0.02
18	3,4-Dicaffeoylquinic acid	57.33	10.26 ± 0.02	8.98 ± 0.02	12.16 ± 0.06
19	3,5-Dicaffeoylquinic acid	58.07	3.15 ± 0.04	2.98 ± 0.03	3.11 ± 0.07
20	Luteolin-7-*O*-glucoside	59.00	–	–	1.88 ± 0.14
22	4,5-Dicaffeoylquinic acid	61.56	2.00 ± 0.06	1.25 ± 0.06	3.14 ± 0.06
23	3,4-Diferuloylquinic acid	65.43	0.39 ± 0.01	0.34 ± 0.01	0.60 ± 0.07
24	3,5-Diferuloylquinic acid	66.24	0.47 ± 0.02	0.44 ± 0.04	0.72 ± 0.04
28	4,5-Diferuloylquinic acid	68.48	0.58 ± 0.04	0.50 ± 0.03	0.50 ± 0.05
29	3,5-Caffeoylferuloylquinic acid	72.74	0.90 ± 0.04	0.73 ± 0.01	0.58 ± 0.06
31	4-Caffeoyl-3,5-disuccinoylquinic acid	75.96	0.78 ± 0.02	0.81 ± 0.01	0.86 ± 0.02
32	Eryodictiol	77.77	0.90 ± 0.02	0.88 ± 0.01	0.86 ± 0.02
33	Jaceidin	83.92	1.11 ± 0.02	1.15 ± 0.03	1.13 ± 0.05
34	Cirsilineol	86.68	0.06 ± 0.02	0.05 ± 0.01	0.06 ± 0.02

[a] See Fig. 3 for peak numbers.

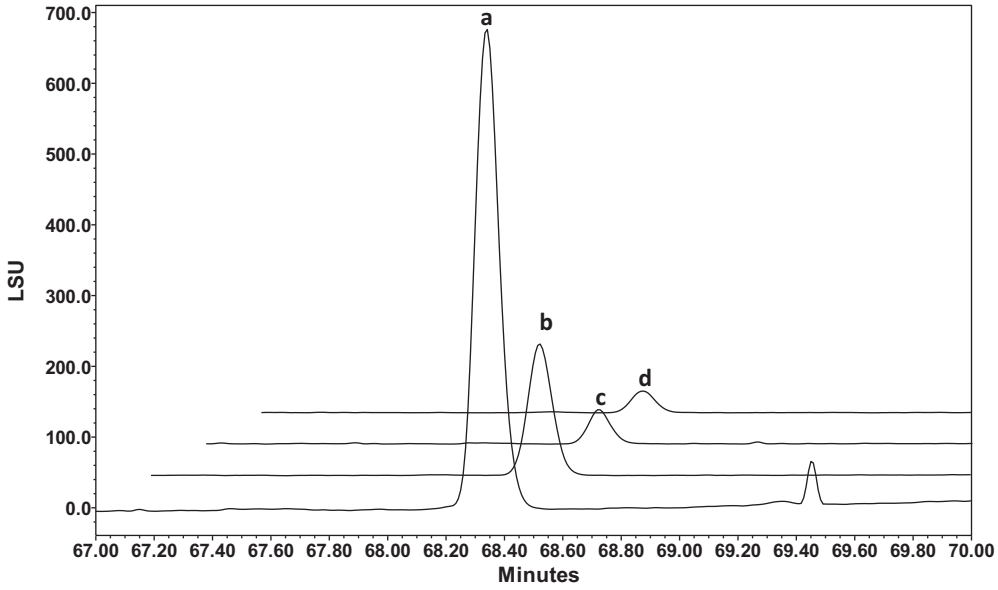


Fig. 4. Overlay of artemisinin peaks as detected by HPLC-ELSD in (a) water extract (1 ml); reconstitution of the same extract in (b) acetonitrile (1 ml); (c) hexane (1 ml) and (d) hexane (3 ml).

also involved in the biosynthesis of the active sesquiterpene lactone [27].

According to published literature [2,3,28] the use of the plant is recorded in the *Chinese Materia Medica* since antiquity. Traditional preparations of *A. annua* consisted of soaking the fresh drug in water, wringing out the plant material and then drinking the extract. In the recent years, some papers have investigated the potential use of this type of preparations bringing some evidence on their clinical efficacy [1,12,13].

Beside the determination of the content of artemisinin, to the best of our knowledge, this is the first study which gives a full characterization of the aqueous extract from *A. annua.* Data obtained and here described should then be regarded with interest to further study the pharmaceutical application of *A. annua* water extracts.

In previous investigations, artemisinin has been generally quantified after re-extraction with an organic solvent from the tea preparations and detected with HPLC-DAD [3–6,29,30] or alternatively, given the low UV absorbance of artemisinin, with HPLC-ELSD [31]. In our investigation we have determined the content of artemisinin and the full composition of the extract in water, without any further re-extraction.

It has been previously shown that extraction of artemisinin by organic solvents is influenced by a combination of factors such as solvent polarity and temperature [32,33] and, in general, medium polarity solvents have been found to have better dissolving capacity for pure artemisinin than strong polar and non polar solvents. Nevertheless, we have demonstrated (Fig. 4) that when the same water extract is reconstituted in an organic solvent, detected concentration of artemisinin is much less. Mueller et al., [1] reported that concentration of extracted artemisinin increases with the amount of drug, accounting for 24–33 mg/l when 10–20 g of dried leaf material was used. The herbal tea approach for the therapeutic use of *A. annua* has been longer discussed, due to the known low solubility in water of artemisinin [34]. Recently, in a specific study [33] the water solubility of the molecule has been determined as 49.7 ± 3.7 mg/l. As shown above, the extraction yield of the active compound obtained in our experiments is in very good agreement with this value. Consistently, we found that the amount of extracted artemisinin only slightly increase (Table 1) with the time of infusion, clearly depending on the coefficient of its solubility in water.

It is reported [8] that main metabolite of artemisinin in human plasma is represented by dihydroartemisinin, moreover it has been demonstrated that artemisinin can degrade into dihydroartemisinin in aqueous solutions in presence of metal ions through a metal-catalyzed oxidation [35]. Our results show that in water extracts dihydroartemisinin was present in very small amount compared to artemisinin confirmed the quality of our extraction procedure that is the stability of the active molecule in the used experimental conditions.

To the best of our knowledge, this is the first report *in extenso* analyzing in detail the chemical profile of *A. annua* tea. According to our chemical data on the metabolite characterization as supported by means of a combination of chromatographic, spectroscopic and spectrometric advanced techniques, extraction of the plant with water (see Section 2) allows to obtain a preparation very rich in phenolic acids, mainly represented by caffeic acid derivatives such as chlorogenic acids. In contrast, flavonoids were not very abundant in the water extracts. The identification of caffeic acid, quinic acid, chlorogenic acid and 3,5-di-*O*-caffeoyl quinic acid as constituents of *A. annua* and *A. afra* has been already reported, although obtained following drug extraction procedures different from ours or using different plant material [36,37]. The two 3,5- and 1,5-di-*O*-caffeoylquinic acids have also been isolated from the flowers of *A. vulgaris* [38]. The other chlorogenic acids described here have never been reported as constituents of *A. annua* tea preparations.

It has been suggested in several occasions [30,36] that activity of artemisinin may be enhanced by the tea infusion due to the synergistic effect with other metabolites which occur to be extracted altogether from the drug. About 40 flavonoids have been isolated from *A. annua* and they are the most favoured candidates for the potentiation of artemisinin bioactivity. Some of them confirmed their antimalarial synergistic activity when assayed in combination with artemisinin [2].

Our results on the metabolic profile of *A. annua* preparation leave open new suggestions for the efficacy of the tea infusion from the aerial parts of the plant. Our data clearly show that *A. annua* teas main phenolic constituents

such as chlorogenic acids, highly soluble in polar solvents, have a role in enhancing artemisinin solubility and extraction efficiency when water is used. Decreasing of the solvent polarity to reconstitute the water extract led in fact to extracts with no phenolics and much lower amounts of artemisinin.

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