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ABSTRACT

Artemisinin, the main antimalarial compound of *Artemisia annua* L., is currently attracting increasing interest for its antiproliferative properties, but its content is highly variable, depending on several genetic, environmental and processing conditionsAim of the present study is to analyse the artemisinin content in different plant extract, to test their *in vitro* activity on cell proliferation and then to correlate these data to the active principle concentration. For this purpose, an innovative miniaturised sample pretreatment strategy based on microextraction by packed sorbent (MEPS) was developed and coupled to an original advanced method based on liquid chromatography with diode array detection and tandem mass spectrometry (LC-DAD-MS/MS). The method was fully validated, granting consistent data. ~~it was linear~~ Good linearity was found over a suitable concentration range, i.e. 5-1000 ng/mL. Extraction yields (>85%), precision (RSD < 3.5%) and accuracy (recovery 88-93%) were all within acceptable levels of confidence. After validation, the method was successfully applied to the determination of artemisinin in *A. annua* extracts. Analyte content was widely variable (up to twenty-fold) according to the starting material and the extraction procedure, ranging between 6 and 109 µg/mL.

The cytotoxic activity of all analysed extracts was also tested on human leukemic cells by viable cell count and cell cycle analysis. Artemisinin concentrations and biological activity were carefully evaluated and the observed antiproliferative effects varied according to artemisinin content in each extract type. This highlights the need to quantitatively analyse the main active constituent of plant extracts and the obtained data have shown to be promising for the choice of the related herbal product dosage.

Keywords: *Artemisia annua;* artemisinin; sample treatment miniaturisation; microextraction by packed sorbent; liquid chromatography-mass spectrometry.

1. INTRODUCTION

Artemisia annua L. is an annual short-day plant, belonging to *Asteraceae* family. It is native of China, where was traditionally used as a remedy for fever, chill and haemorrhoids [1-3]. Recently, *A. annua*, together with its metabolite artemisinin and its semisynthetic derivatives artemether and arteether, received worldwide attention, since artemisinin-based combination therapy has been established as standard treatments of severe and drug-resistant malaria [4].

In addition to the sesquiterpene lactone artemisinin, *A. annua* produces a wide variety of other secondary metabolites, generating a very complex phytochemical profile containing terpenes, flavonoids, coumarins and other shikimate metabolites [2]. Artemisinin has been generally recognised as the main active principle of *A. annua*, effective against both malaria and a number of other diseases. In particular, it resulted to be able to contrast Lupus erythematosus-related nephritis, neuroinflammation, virus infections, schistosomiasis and trypanosomiasis [5-8]. Moreover, artemisinin and its derivatives also exert remarkable antiproliferative effects towards cell lines from a variety of tumour types, causing cell cycle arrest and cell death [9-12]. It also shows hypoglycaemic activities, useful in diabetes mellitus treatments [13]. On these bases, *A. annua* results an interesting medicinal plant to develop herbal preparations both for human and animal treatment.

Nevertheless, despite the increasing use of herbal products, their quality control is often lacking or performed through inadequate procedures. Only in a few cases, the qualitative and quantitative composition of these herbal products is properly investigated and reported, at least for what is related to the main active compounds. Taking into account also that artemisinin content is highly variable, depending on cultivars and growing conditions of *A. annua* [14,15], it results evident how crucial is to be able to normalise the results obtained from biological activities, based on the

concentration of artemisinin in plant extracts and related commercial products obtained from this plant. For this reason, in order to evaluate the content in a sound and reliable way, it is essential to have an effective analytical methodology characterized by high sensitivity, selectivity and accuracy when applied to complex samples of different nature and origin, which have been subjected to different preparation procedures [16-20].

Analytical methods based on liquid chromatography, in particular coupled to MS/MS [21-24], can be found in the scientific literature for artemisinin quantitative analysis alone or together with other compounds in *A. annua* extracts and derived products. Solvent extraction was applied to different foods (tea, biscuits, and porridge) enriched with dried leaves of *A. annua* prior to LC-MS/MS analysis [21]. Organic solvents were also used to obtain extracts by maceration of the herbal drug to study spontaneous plants [22]. An LC-MS/MS method was proposed for the analysis of artemisinin together with six analogues to be applied to *A. annua* crude plant [2323]. Finally, rapid resolution liquid chromatography coupled to triple quadrupole mass spectrometry (RRLC-QQQ) was exploited for the determination of artemisinin in *A. annua* raw material after ultrasound-assisted extraction ~~(UAE)~~ [24].

To the best of our knowledge, this work is the first one addressed to quantify artemisinin in *A. annua* extracts, also commercialised for therapeutic use, and to test their *in vitro* antiproliferative activity. An advanced analytical strategy is herein proposed, exploiting innovative and sound analytical aspects provided by miniaturised sample pretreatment and mass spectrometry. A liquid chromatographic method coupled to diode array detection and tandem mass spectrometry (LC-DAD-MS/MS) was developed and fully validated in order to accurately quantify artemisinin in plant extracts and different commercial products, after sample pretreatment by means of a fast and feasible microextraction by packed sorbent (MEPS) procedure [16]. In order to study the

antiproliferative effects of each extract, cell counting and DNA profile analysis have been performed on HL60 cell line derived from human promyelocytic leukaemia.

2. EXPERIMENTAL

2.1. Chemicals and standard solutions

Artemisinin pure powder (98% purity) and acetonitrile, methanol, formic acid (all reagents for mass spectrometry) in addition to all the solvents used for the extract preparation procedures (all analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA); artemisinin-D3 pure powder (98% purity, xx% isotopic purity), used as the internal standard (IS), was provided by Biosynth (St. Gallen, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-Q apparatus from Millipore (Milford, MA, USA). The analyte and IS stock solutions (1 mg/mL) were prepared by dissolving suitable amounts of pure powders in methanol; the corresponding standard solutions were prepared daily by dilution with a water/acetonitrile mixture (50:50) containing 0.25 % formic acid. All solutions were stored protected from light in amber glass vials certified for mass spectrometry from Waters Corporation (Milford, MA, USA). Human promyelocytic leukemic cell line HL60 was purchased from American Type Culture Collection (Manassas, VA, USA). Propidium Iodide, Trypan Blue, Foetal Bovine Serum, RPMI 1640, L-glutamine, tri-sodium citrate, RNAse, Igepal and ethanol were ultrapure grade from Sigma Aldrich.

2.2. Chromatographic instrumentation and conditions

LC-DAD-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic system with autosampler coupled to a Waters 2998 photodiode array detector and a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with an electrospray ion source (ESI), working

148 in positive ionisation mode. Data processing was performed using Waters MassLynx 4.1 software. A
149 Sigma-Aldrich 6-port Mini-Vap evaporator/concentrator equipped with nitrogen as the drying gas,
150 a Crison (Barcelona, Spain) Basic 20 pHmeter, a Velp (Usmate, Italy) RX3 vortex mixer and an Elma
151 (Singen, Germany) Sonic T310 Trans ultrasonic bath were also used.
152 Separations were obtained on a Restek Ultra AQ C18 column (50 × 2.1mm I.D., 3 µm), maintained
153 at room temperature and equipped with a Restek Ultra AQ C18 guard column. The mobile phase
154 was a mixture of 0.25 % formic acid in water (A) and 0.25 % formic acid in acetonitrile (B), flowing
155 at a constant rate of 0.2 mL/min. Mobile phase composition automated gradient program started
156 with A:B (30:70), then ramped up to A:B (90:10) over 2 min; this ratio was maintained for 3 min,
157 then ramped down to A:B (30:70) over 1 min and maintained for 2 min. The injection volume was
158 10 µL.
159
160 **2.3. DAD and MS/MS parameters**
161 For artemisinin detection, DAD was operated at 232 nm, while for MS/MS analysis, multiple reaction
162 monitoring (MRM) transitions were used, acquiring in positive ionisation mode (ESI+) and exploiting
163 two different exclusive transitions for both artemisinin and IS: the most abundant one for
164 quantitative purposes, the second one for identity confirmation. All MS parameters were optimised
165 for maximum abundance of the selected ions via direct infusion of artemisinin and IS working
166 solutions (1 µg/mL methanolic solutions) at 10 µL/min. The optimised parameters were as follows:
167 ion source voltage, 3.6 kV; cone voltage, 15.0 V, ion source temperature, 100°C; desolvation
168 temperature, 200°C; desolvation gas flow, 850 L/h (nitrogen as the desolvation gas, argon as the
169 collision gas). The precursor ions and the product ions, with dwell time, cone voltage and collision
170 energy, were optimised and are shown in Table 1.
171

2.4. Plant material and extract preparation

A. annua was cultivated in "Il Giardino di Pimpinella" Marzabotto (Bologna), harvested at the flowering stage and identified by Dr. Laura dell'Aquila and Prof. Ferruccio Poli. Voucher specimens (BOLO0602000) were retained in the Herbarium of Alma Mater Studiorum - University of Bologna (SMA).

In order to prepare the hydroalcoholic extract (Extract A), 5 g of dried and grounded plant material were extracted 3 times for 24h in 250 mL of 65 % ethanol at room temperature and under constant magnetic stirring. Supernatant was filtered and evaporated under vacuum at a temperature of 40°C to yield the crude extract.

Artemisinin enriched extract (Extract B) was prepared according to the method reported by the Chinese Pharmacopeia [25]: 5 g of dried and grounded plant material were extracted using 80 mL of petroleum ether heated under reflux at 80°C for 1 h. The solvent was filtered and dried under vacuum and the obtained dried extract was dissolved in 50 mL of hexane, then subjected to liquid-liquid partition using 16 mL of 20 % acetonitrile in water. The procedure was repeated three times, the acetonitrile phases were combined and evaporated to yield the crude extract.

In order to perform the following chemical and biological analyses, Extracts A and B were redissolved in ethanol (10 mg/mL).

2.5. Commercial extracts

In addition to the two *A. annua* Extracts A and B prepared on purpose within this study, two commercial products were analysed (Extracts C and D). Extract C was a hydroalcoholic extract (65 % EtOH) obtained after dried *A. annua* maceration and sold as a dietary supplement. Extract D consisted in a dry extract of *A. annua* marketed as a veterinary food supplement sold in form of capsules.

196

197 **2.6. Sample pretreatment: microextraction by packed sorbent (MEPS)**

198 For both the *A. annua* extracts prepared *ad hoc* for this study and the commercial hydroalcoholic
199 extract sold as dietary supplement, 100 µL of the obtained solutions were diluted with 900 µL of a
200 water/acetonitrile mixture (50:50) containing 0.25 % formic acid and the IS.
201 As regards the veterinary food supplement, the content of 3 capsules was weighed. Then, 10 mg of
202 the content were added with 10 mL of methanol containing IS, subjected to ultrasound-assisted
203 extraction and vortex. After centrifugation, the supernatant was filtered by using nylon filters,
204 brought to dryness under a gentle nitrogen flow and re-dissolved in 1 mL of a water/acetonitrile
205 mixture (50:50) containing 0.25 % formic acid.
206 For all samples, a 100-µL aliquot of the obtained extract solution was subjected to MEPS
207 pretreatment before LC analysis.
208 MEPS procedure on the extracts was carried out by using an SGE Analytical Science (Melbourne,
209 VIC, Australia) apparatus, consisting of a 100 µL syringe equipped with a removable BIN (Barrel
210 Insert and Needle) containing a C8 miniaturised sorbent. The sorbent was activated with 150 µL of
211 methanol and then equilibrated with 150 µL of ultrapure water (3 cycles of 50 µL). The loading
212 solution was represented by a 100-µL aliquot of extract, 45 µL of ultrapure water and 5 µL of IS
213 standard solution; the loading mixture was drawn into the syringe and discharged back 10 times.
214 The sorbent was washed once with 100 µL of water and 100 µL of a water/methanol mixture (95:5)
215 and then eluted by drawing and discharging 500 µL of methanol (5 cycles of 100 µL each). The eluate
216 was dried under a gentle nitrogen stream, re-dissolved in 100 µL of a water/acetonitrile mixture
217 (50:50) containing 0.25 % formic acid and injected into the LC-DAD-MS/MS system.

218

219 **2.7. LC-DAD-MS/MS method validation**

The LC-DAD-MS/MS method was validated according to European Medicines Agency (EMA), Food and Drug Administration (FDA) and International Conference on Harmonization (ICH) guidelines [26-28]. The tested parameters were linearity (including limits of detection and limit of quantitation), extraction yield, precision and accuracy.

Artemisinin standard solutions at seven different concentrations, containing the IS at a constant concentration, were injected into the LC-DAD-MS/MS system. The analysis was carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding concentrations (expressed as ng/mL) and the calibration curves were obtained by means of the least-square method.

LOQ and LOD were calculated as the analyte concentrations which give rise to peaks whose areas are 10 and 3 times the baseline noise, respectively.

MEPS extraction yields were evaluated by subjecting artemisinin standard solutions at known concentrations to the previously described procedure. The obtained analyte peak areas were compared with those obtained by injecting standard solutions at the same theoretical concentrations in order to calculate extraction yield values.

Precision assays were carried out on both artemisinin standard solutions and analysed samples. Artemisinin standard solutions at three different concentrations (corresponding to a low, an intermediate and a high value of the linearity range) containing the IS at a constant concentration were analysed six times in the same day to obtain *intraday* precision and six times over six different days to obtain *interday* precision, expressed as percentage relative standard deviation (RSD %). Moreover, the samples were subjected to MEPS and analysed six times in the same day to obtain *intraday* precision data and six times over six different days to test *interday* precision.

Recovery assays were carried out in order to evaluate method accuracy: standard solutions containing known amounts of artemisinin (corresponding to a low, an intermediate and a high value

244 of the calibration curves) and a fixed amount of IS were added to samples which had been already
245 analysed. The obtained spiked samples were thus analysed and the recovery of the spiked analyte
246 was calculated.
247
248 **2.8. Cell culture and treatment**
249 HL60 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 % heat-
250 inactivated foetal bovine serum, in a humidified atmosphere containing 5 % CO_2. Cells were plated
251 at $1x10^5$ cells/mL in a plastic well and after 24 h *A. annua* extracts were added to the medium at 100
252 µg/mL concentration. In control cells, ethanol was added to the culture medium. The viable cells
253 were counted by using a Bürker haemocytometer in the presence of Trypan Blue after 24 h of
254 treatment.
255
256 **2.9. Cell cycle analysis**
257 Cell cycle distribution was performed on HL60 cells, control cells and cells treated with *A. annua*
258 extracts for 24 h: $1x10^6$ cells were pelleted and resuspended in tri-sodium citrate 0.1 %, RNAse 10
259 mg/L, Igepal 0.01 %, propidium iodide 50 mg/L. After 30 min at 37 °C in the dark, the isolated nuclei
260 were analysed by using a Biorad Bryte HS flowcytometer (Hercules, CA, USA) equipped with a Xe/Hg
261 lamp and a filter set to obtain an excitation at 488 nm. Propidium iodide fluorescence was collected
262 on a linear scale at 600 nm and DNA distribution was analysed by Verity ModiFit software (Topsham,
263 ME, USA).
264
265
266 **3. RESULTS AND DISCUSSION**
267 **3.1. LC-DAD-MS/MS method development**

For this study, several stationary phases for reversed phase chromatography were tested for adequate retention times, peak shape and signal-to-noise ratio. C8 and C18 sorbents were considered and tested and since C18 sorbent provided the best performance among the screened columns, this rugged, reversed-phase column with a well-balanced retention profile was selected and used for all subsequent analysis.

Together with the sorbents, different mobile phase compositions were studies: volatile acidic additives at different concentrations, namely formic acid, acetic acid and trifluoroacetic acid ranging from 0.01 % to 0.5 %, both with acetonitrile or methanol as the organic modifiers. The use of acetonitrile and H_2O flowing at 0.2 mL/min under an optimised composition gradient provided the best compromise between retention times (of both analyte and IS) and peak shape. 0.25 % formic acid provided the best analyte ionisability and thus the highest peak area, as further increases in formic acid concentrations did not provide better results.

Spectrophotometric detection by means of DAD detector was performed at 232 nm, the best option for the analyte.

MS and MS/MS spectra of the artemisinin and IS were first acquired in full-scan mode (50–600 m/z), then optimised mass spectrometry conditions were obtained by direct syringe infusion into the ESI source of 1 µg/mL solutions. Optimised m/z transitions, together with cone voltage and collision energy, for both artemisinin and the IS are reported in Table 1.

In Figure 1, a full scan mass spectrum of the analyte standard solution is presented, together with its chemical structure.

3.2. MEPS procedure development

MEPS is a recent sample pre-treatment procedure that can be envisioned as a miniaturised version of conventional solid phase extraction (SPE). In fact, a MEPS procedure has the same use cases and

292 includes the same steps as an SPE procedure, but all of them take place within a much smaller
293 amount of sorbent (a few milligrams as opposed to 30-1000 mg or more), which is contained in the
294 BIN of a modified liquid chromatography syringe. MEPS offers several advantages over SPE: For
295 example, it needs much smaller amounts of sample and of solvents (up to 200 µL vs. 1-5 mL);
296 automation is straightforward and requires inexpensive equipment (basically, an automated syringe
297 handler). Procedure development is based on the same principles, so the steps are quite similar and
298 adaptation of SPE procedures to MEPS application is relatively simple. Although a MEPS BIN is more
299 expensive than a single SPE cartridge, it can be regenerated and re-used multiple times (up to 200,
300 according to the manufacturer's specifics), leading to expenses per analysis which are almost
301 identical, but with significant environmental benefits due to the sharply reduced amounts of waste
302 (in terms of both equipment and solvents). As can be expected, MEPS is particularly advantageous
303 in case of small or very expensive samples.
304 MEPS procedure was developed and optimised in terms of sorbent type, volumes and nature of the
305 washing and elution solvents, number of cycles and speed of drawing and discharging. A careful
306 sorbent selection was important to achieve satisfying purification and extraction yields: the
307 performance of different commercially available types of sorbents such as C2, C8, C18 and M1 (M1,
308 mixed mode: C8 + strong cation exchange, SCX) was tested for extraction efficiency. C2 and C8
309 sorbents both led to acceptable extraction yields in comparison with C18 and M1, however C8
310 sorbent provided also better results in terms of clean-up (Figure 2a). All the main parameters
311 involved in the MEPS procedure steps (loading, washing and elution) were carefully optimised.
312 Artemisinin absorption onto the sorbent was satisfactory after 10 draw/discharge cycles (Figure 2b)
313 of the loading solution at the speed of 2 µL/s. Different solvents and mixtures were tested for the
314 washing and elution steps and good purification was obtained by washing the sorbent from matrix
315 components with 100 µL of ultrapure water followed by 100 µL of a water/methanol 95:5 mixture

316 at a speed of 10 µL/s. Finally, elution with 5 cycles of 100 µL of pure methanol each (Figure 2c), at a
317 speed of 2 µL/s, was established as the best combination in order to maximize elution of both
318 artemisinin and IS.
319 The eluate was dried under a gentle nitrogen stream, redissolved with 100 µL of a water/acetonitrile
320 mixture (50:50) containing 0.25 % formic acid and injected into the LC-DAD-MS/MS system. After
321 each extraction cycle, the sorbent was washed and re-conditioned by applying a cleaning step with
322 200 µL of methanol followed by 200 µL of water.
323 Figure 3 shows the MRM chromatograms of Extract B spiked with a 15 µg/mL standard solution of
324 IS and subjected to MEPS pretreatment. The artemisinin concentration found in the injected
325 solution was 11.33 µg/mL. As one can see, the chromatogram is devoid of any interference and the
326 baseline signal is remarkably stable. This means that the MEPS procedure has significantly enhanced
327 selectivity. Although no direct sensitivity increase is achieved with the described MEPS procedure
328 (the sample is not pre-concentrated), the increased selectivity also increases the analyte signal to
329 noise ratio. This leads to a more accurate detection of low-concentration samples, and thus to an
330 effectively higher sensitivity in comparison to untreated samples.
331
332 **3.3. LC-DAD-MS/MS method validation**
333 The LC-DAD-MS/MS method coupled to MEPS was validated in terms of linearity, precision,
334 extraction yield and accuracy, according to the main international regulatory guidelines [26-28].
335 Good linearity (r^2 > 0.9995) was obtained over to the 5 ng/mL to 100 µg/mL artemisinin
336 concentration range (regression line equation: $y = a\,x + b$; SD_{slope} = xx; $SD_{intercept}$ = xx). LOQ and LOD
337 values were 5 ng/mL and 1.5 ng/mL, respectively. Extraction yield and precision assays were carried
338 out subjecting standard solutions containing artemisinin and IS to the MEPS procedure: three
339 different levels represented the whole linearity range (5 ng/mL, 100 ng/mL and 50 µg/mL), with the

IS at a constant concentration (15 µg/mL). RSD values for intraday precision were always <2.3%, while interday precision assays led to RSD always <3.5%. Extraction yield was always higher than 85% (>86% for the IS). Accuracy, evaluated as recovery values, was also tested at three concentration levels, and ranged from 88 to 93%. The complete results are reported in Table 2.

3.4. Analysis of *A. annua* extracts

The validated method was applied to the analysis of two *A. annua* extracts prepared *ad hoc* for the purposes of the present work (i.e. a hydroalcoholic extract, Extract A, and an artemisinin-enriched extract following the Chinese Pharmacopeia, Extract B) and two commercial ones sold as food supplements (i.e. hydroalcoholic extract, Extract C; dry extract, Extract D). When applied to the respective extraction procedures, the LOQ values obtained during method validation were: 5 µg/g of fluid extract for Extracts A and B; 0.05 µg/g for extract C; 0.5 µg/g for extract D.

Quantitative results regarding the analysed samples are shown in Table 3; each reported value is the mean obtained from three independent analyses. Artemisinin concentrations in Extract B were significantly higher than those recorded in Extract A and comparable to those observed in Extract C. In Extract D, artemisinin content can be considered coherent with a concentrated dry extract, as reported on the label.

As comparison, the artemisinin levels found in extracts in previously published papers range from 60 µg/mL [29] to 200-500 µg/mL [30] to 6-17 mg/mL [31]. Artemisinin levels in the original plant material can also vary widely according to the source and the author, from 10-22 pg/mg of dry weight (D.W.) [32] to 1-10 µg/mg D.W. [33], and from 100-200 ng/mg D.W. in Chinese plants to 10-15 µg/mg D.W. in selected Chinese and Vietnamese hybrids [34].

The different extracts of *A. annua* were also examined for cytotoxic potential on HL60 leukemic cells and the cell samples were incubated with all extracts. Although all treatments were performed with

364 the same compound concentration (100 µg/mL), cell counts demonstrated the effectiveness of each
365 extract on cell growth was very variable (Table 4). Among different extracts, Extract B exhibited the
366 highest inhibition of cell proliferation, followed by Extract C, Extract A and finally Extract D, the latter
367 being the least active. Interestingly, the trend of obtained results correlates with the artemisinin
368 content of the tested formulation, as reported in Table 3.
369 The decrease of the viable cell number indicates the onset of cytotoxicity and in order to assess
370 whether the antiproliferative effect of *A. annua* extracts was accompanied by interference with cell
371 cycle progression, DNA profiles of cultured cells were examined by flowcytometry. The results
372 reported in Figure 4 show again the treatments caused variable effects also on cell cycle distribution:
373 a marked sub-G1 peak is visible in flow cytometry histogram of the cells treated with the richest
374 artemisinin extract, indicating the induction of apoptosis and more generally a cytotoxic effect.
375 Nevertheless, a significant decrease in the cell percentage in S phase is evident for samples treated
376 with Extract A and Extract C (13.83 % and 19.05 % *vs* 39.60 % of the control cells, respectively),
377 confirming the reduction of cell proliferation described above. Only minimal effects on cell cycle
378 profile is associated to treatment with Extract D, which contains the lowest artemisinin
379 concentration.
380
381
382 **4. CONCLUSION**
383 An original methodology based on LC-DAD-MS/MS coupled to MEPS pretreatment has been
384 developed and validated for the determination of artemisinin in different extracts obtained from *A.*
385 *annua*, including commercial ones. The developed method was validated in terms of linearity,
386 precision, extraction yield and accuracy, with good results for both artemisinin and IS, thus
387 demonstrating the full suitability of this analytical strategy and the reliability of the obtained data.

Moreover, MEPS pretreatment procedure has demonstrated to be an advanced yet feasible miniaturised approach for sample purification, very promising to be applied to a wide range of complex samples, in order to enhance method selectivity and sensitivity. After validation, the method was successfully applied to the analysis of two extracts obtained from *A. annua* raw plant material and of two commercial ones. Examining the obtained results, it can be observed how artemisinin levels in raw plant material extracts were significantly higher in that obtained by means of the method reported in the Chinese Pharmacopeia; these latter were also similar to those observed in the analysed commercial hydroalcoholic extract. As regards the extract D, artemisinin content was coherent with a concentrated dry extract, as reported on the label. The quantitative results obtained from LC-DAD-MS/MS analysis were exploited to evaluate the *in vitro* activity of *A. annua*-based extracts considered in this work, towards a deeper knowledge of the mechanisms of action and the molecular targets involved in their antiproliferative activity.

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Thanks are due to Prof. Shikai Yan from Guangdong University (China) for kindly providing and translating from the Chinese Pharmacopeia the extraction protocol used in this study for Extract B.

FIGURE LEGEND

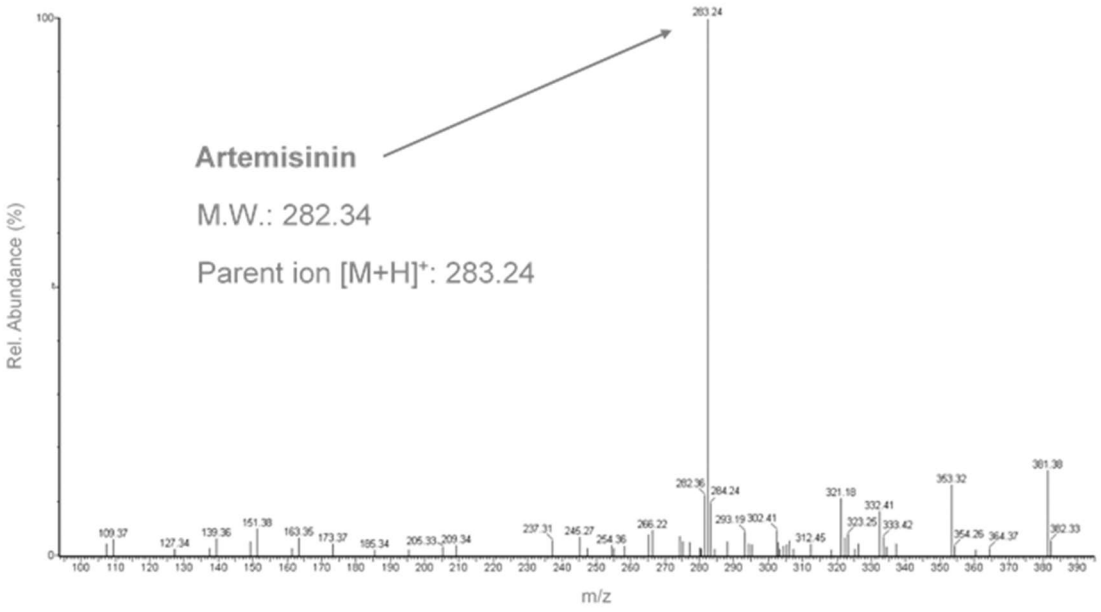

Figure 1 – MS spectrum of artemisinin.

Figure 2 – MEPS procedure development: effect on extraction yield of (a) sorbent type, (b) loading cycles, (c) elution cycles.

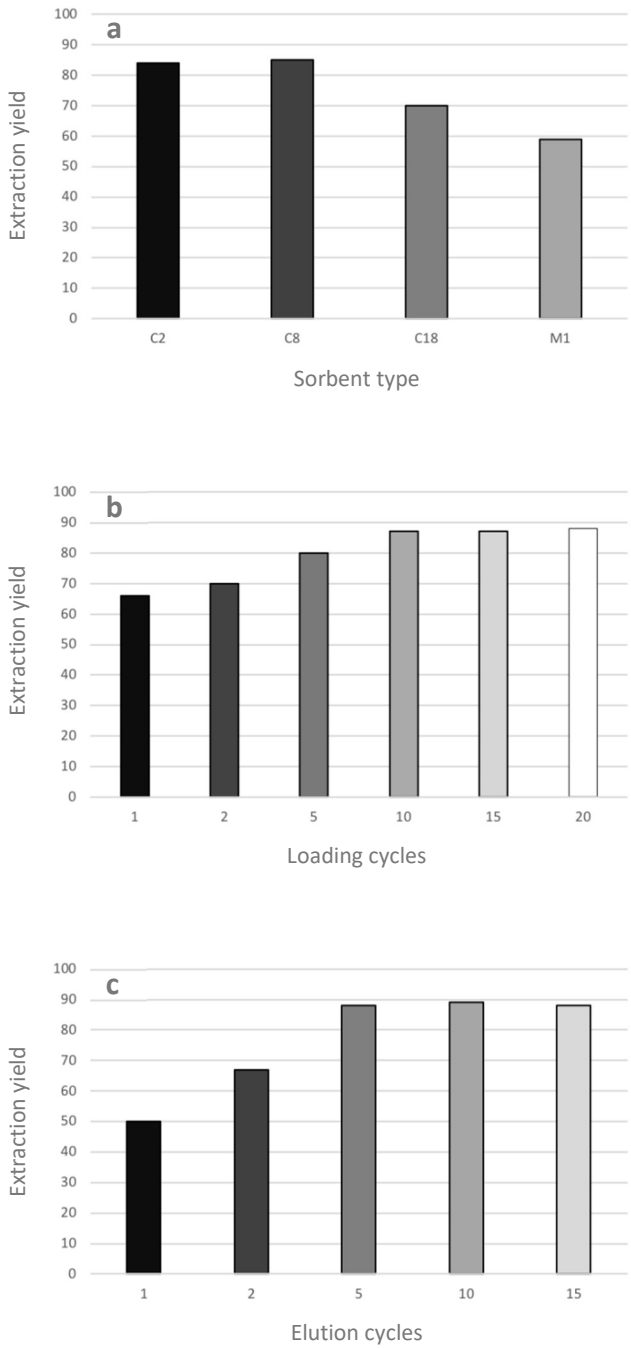

Figure 3 – Typical MRM chromatograms of Extract B spiked with a 75 ng/mL standard solution of the IS and subjected to MEPS pretreatment. Trace A: chromatogram of the 283 → 209 MRM transition corresponding to artemisinin; artemisinin peak at retention time (t_R) = 6.7 min. Trace B: chromatogram of the 286 → 212 MRM transition corresponding to the IS; IS peak at retention time (t_R) = 6.7 min.

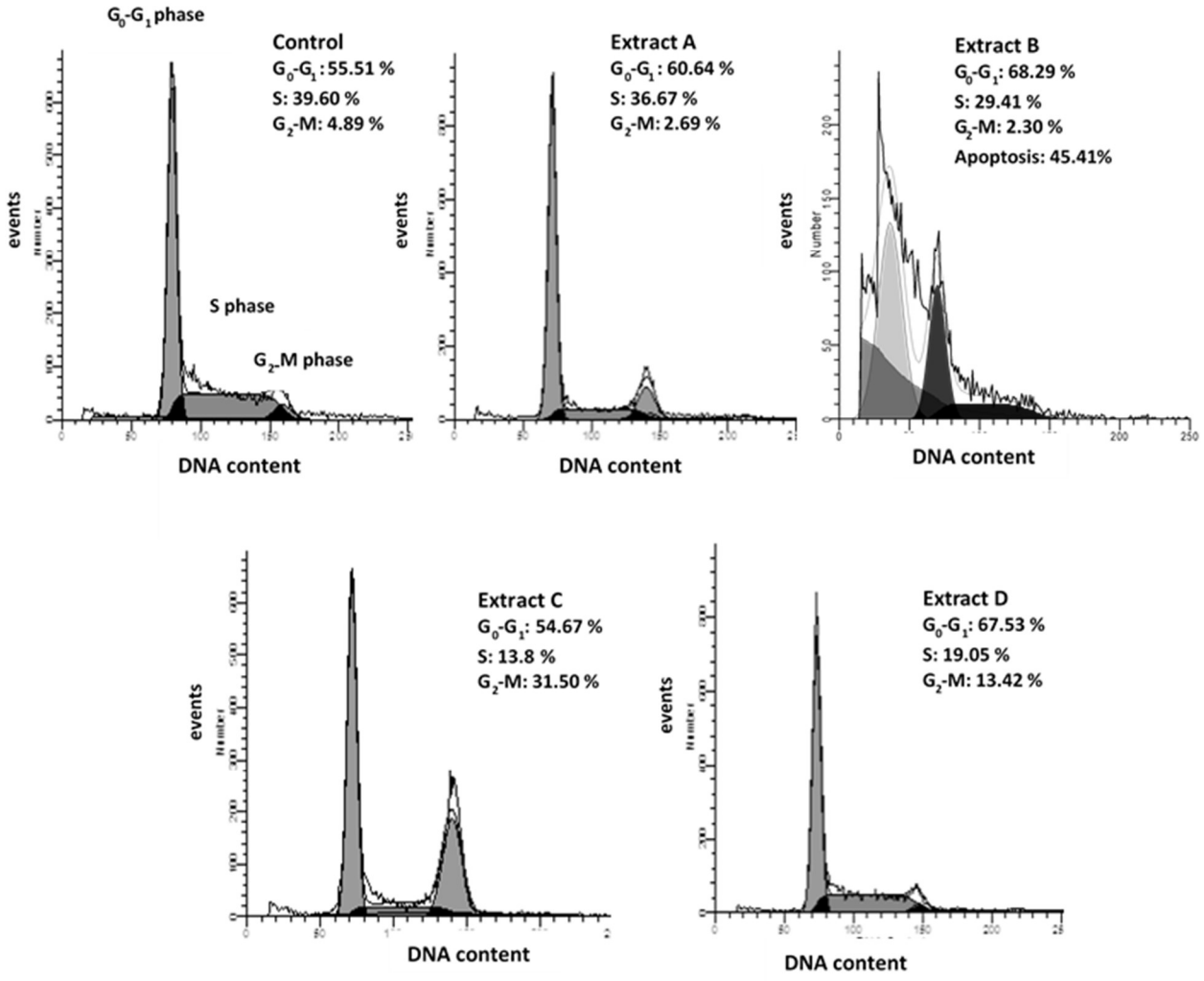

Figure 4 – Effects of *Artemisia Annua* extracts on the growth of HL-60 cells. Cells were treated with 100 µg/mL of each extract. After 24 h of incubation, viable cells were counted. Panels report the cytofluorimeter outputs obtained in one typical experiment repeated twice with similar results.

TABLE LEGEND

Table 1 – Multiple Reaction Monitoring (MRM) optimised parameters (triple quadrupole, ESI-).

Compound	Molecular weight (g/mol)	Parent ion $[M+H]^+$ (m/z)	Daughter ions (m/z)	Cone voltage (V)	Collision energy (eV)	Dwell time (ms)
Artemisinin	282.34	283.24	209.45	15	14	300
			265.36 [a]	15	12	300
IS	285.35	286.31	212.38	15	13	300
			268.34 [a]	15	11	300

[a] *confirmatory product ion*

Table 2. LC-DAD-MS/MS extraction yield and precision data

Compound	Concentration (ng/mL)	Extraction yield (%)	Intraday precision (RSD%)[a]	Interday precision (RSD%)[a]
Artemisinin	5	89	2.0	3.0
	100	88	2.0	3.3
	1000	86	2.1	3.4
IS	50	87	2.2	3.2

[a] *n = 6*

Table 3 – LC-DAD-MS/MS quantitative data from extract analysed samples.

Sample	Artemisinin content ± SD (μg/mL) [a]	IS extraction yield (%)	Accuracy (% recovery range)
Extract A	21.40 ± 1.52	89	88-90
Extract B	109.40 ± 2.63	87	91-92
Extract C	94.79 ± 2.14	88	91-93
Extract D	5.90 [b] ± 0.84	89	88-91

[a] *n = 6*
[b] *μg/mg*

Table 4 – *In vitro* activity of extracts (100 μg/mL) on leukemic cell proliferation.

Table 4. In vitro activity of extracts (100 μg/mL) on leukemic cell proliferation

Sample	10^3 cells/mL ± SD [a]
Control cells	4850 ± 50
HL60 cells + Extract A	313 ± 18
HL60 cells + Extract B	671 ± 9
HL60 cells + Extract C	154 ± 12
HL60 cells + Extract D	447 ± 62

[a] *n = 3*

503 **REFERENCES**

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[1] T. Efferth. From ancient herb to modern drug: *Artemisia annua* and artemisinin for cancer therapy. Semin. Cancer. Biol. 46 (2017) 65-83.

[2] G.D. Brown. The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). Molecules 15 (2010) 7603-98.

[3] Y. Tu. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. Nat. Med. 17 (2011) 1217-1220.

[4] M.T. Ansari, Z.S. Saify, N. Sultana, I. Ahmad, S. Saeed-Ul-Hassan, I. Tariq, M. Khanum. Malaria and artemisinin derivatives: an updated review. Mini Rev Med Chem. 13 (2013) 1879-1902.

[5] X. Mu, C. Wang. Artemisinins - A promising new treatment for systemic Lupus Erythematosus: a descriptive review. Curr. Rheumatol. Rep. 20 (2018) 55.Z.

[6] S.Y. Chen, C. Lu, L.M. Dong, J.W. Lv, Q.H. Tuo, L. Qin, S.W. Cheng, L.L. Bu, N. Lin, X.X. Zhu, D.F. Liao, X.M. Liu. Resolving neuroinflammation, the therapeutic potential of the anti-malaria drug family of artemisinin. Pharmacol. Res. 136 (2018) 172-180.

[7] T. Efferth, M.R. Romero, D.G. Wolf, T. Stamminger, J.J. Marin, M. Marschall. The antiviral activities of artemisinin and artesunate. Clin. Infect. Dis. 47 (2008) 804-11.

[8] R. Liu, H.F. Dong, Y. Guo, Q.P. Zhao Jiang MS. Efficacy of praziquantel and artemisinin derivatives for the treatment and prevention of human schistosomiasis: a systematic review and meta-analysis. Parasit. Vectors 4 (2011) 201.

[9] F. W. Michaelsen, M.E.M. Saeed, J.Schwarzkopf, T.Efferth. Activity of *Artemisia annua* and artemisinin derivatives, in prostate carcinoma. Phytomedicine 22 (2015) 1223-1231.

[10] T. Efferth. From ancient herb to modern drug: *Artemisia annua* and artemisinin for cancer therapy. Seminars in Cancer Biology 46(2017) 65-83.

[11] E.Ooko,M.E.M. Saeed, O.Kadioglu, S.Sarvi, M.Colak, K.Elmasaoudi, R.Janah, H.J.Greten, T. Efferth. Artemisinin derivatives induce iron-dependent cell death (ferroptosis) in tumor cells. Phytomedicine 22(2015)1045-1054.

[12] M.L. Abba, N. Patil, J.H. Leupold, M.E.M. Saeed, T. Efferth, H. Allgayer. Prevention of carcinogenesis and metastasis by Artemisinin-type drugs. Cancer Lett. 429 (2018) 11-18.

[13] M.U. Eteng, A.O. Abolaji, P.E. Ebong, E.A. Brisibe, A. Dar, N. Kabir, et al. Biochemical and haematological evaluation of repeated dose exposure of male Wistar rats to an ethanolic extract of *Artemisia annua*. Phytother. Res. 27 (2013) 602-609.

[14] T.E., Wallaart, N. Pras, A.C. Beekman, W.J. Quax. Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. Planta Med. 66 (2000) 57-62.

[15] N. Delabays, X. Simonnetand, M.D.A.D. Gaudin. The genetics of artemisinin content in *Artemisia annua* L. and the breeding of high yielding cultivars. Curr. Med. Chem. 8 (2001) 1795-1801.

[16] L. Mercolini, M. Protti, M.A. Saracino, M. Mandrone, F. Antognoni, F. Poli. Analytical profiling of bioactive phenolic compounds in argan (*Argania spinosa*) leaves by combined microextraction by packed sorbent (MEPS) and LC-DAD-MS/MS, Phytochem Anal. 27 (2016) 41-49.

[17] F. Bonvicini, F. Antognoni, M. Mandrone, M. Protti, L. Mercolini, M. Lianza, G. A. Gentilomi, F. Poli. Phytochemical analysis and antibacterial activity towards methicillin-resistant Staphylococcus aureus of leaf extracts from Argania spinosa (L.) Skeels, Plant Biosyst. 151 (2017) 649-656.

[18] M. Protti, I. Gualandi, R. Mandrioli, S. Zappoli, D. Tonelli, L. Mercolini. Analytical profiling of selected antioxidants and total antioxidant capacity of goji (*Lycium* spp.) berries. J. Pharm. Biomed. Anal. 143 (2017) 252-260.

[19] M. Protti, F. Valle, F. Poli, M.A. Raggi, L. Mercolini. Bioactive molecules as authenticity markers of Italian Chinotto (*Citrus* × *myrtifolia*) fruits and beverages. J. Pharm. Biomed. Anal. 104 (2015) 75-80.

[20] L. Mercolini, R. Mandrioli, A. Ferranti, V. Sorella, M. Protti, F. Epifano, M. Curini, M.A. Raggi. Quantitative evaluation of auraptene and umbelliferone, chemopreventive coumarins in citrus fruits, by HPLC-UV-FL-MS. J. Agric. Food Chem. 61 (2013) 1694-1701.

[21] A. Carrà, R. Bagnati, R. Fanelli, M. Bonati. Fast and reliable artemisinin determination from different *Artemisia annua* leaves based alimentary products by high performance liquid chromatography-tandem mass spectrometry. Food Chem. 142 (2014) 114-120.

[22] B. Ivanescu, L. Vlase, A. Corciova, M.I. Lazar. Artemisinin evaluation in Romanian *Artemisia annua* wild plants using a new HPLC/MS method. Nat. Prod. Res. 25 (2011) 716-722.

[23] J. Suberu, L. Song, S. Slade, N. Sullivan, G. Barker, A.A. Lapkin. A rapid method for the determination of artemisinin and its biosynthetic precursors in *Artemisia annua* L. crude extracts. J. Pharm. Biomed Anal. 84 (2013).

[24] X. Wang, X.E. Zhao, B. Yang, H. Dong, D. Liu, L. Huang. A combination of ultrasonic-assisted extraction with RRLC-QQQ method for the determination of artemisinin in the Chinese herb *Artemisia annua* L. Phytochem. Anal. 22 (2011) 280-284.

[25] Chinese Pharmacopoeia Commission, Pharmacopoeia of the People's Republic of China 2015 (English version), volume I, Publisher: China Medical Science Press, Beijing, 2015; p. 52.

[26] European Medicines Agency, Guideline on Bioanalytical Method Validation (2011). https://www.ema.europa.eu/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf (Accessed December 2018)

[27] Food and Drug Administration, Bioanalytical method validation - Guidance for industry (2018) https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf (Accessed December 2018)

[28] International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, Validation of Analytical Procedures: Text and methodology Q2(R1) https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf (Accessed December 2018)

[29] H. Bai, C. Wang, J. Chen, J. Peng, Q. Cao. A novel sensitive electrochemical sensor based on in-situ polymerized molecularly imprinted membranes at graphene modified electrode for artemisinin determination. Biosens. Bioelectr. 64 (2015) 352-358.

[30] T.E. Soktoeva, G.L. Ryzhova, K.A. Dychko, V.V. Khasanov, S.V. Zhigzhitzhapova, L.D. Radnaeva. Artemisinin content in *Artemisia annua* L. extracts obtained by different methods. Russ. J. Bioorg. Chem. 39 (2013) 761-764.

[31] R. Sri Indah, A. Susilowati, Y. Widyastuti, A. Yunus. Artemisinin content In *Artemisia Annua* L. treated by *Gloriosa superba* seeds' water extract. Asian J. Pharm. Clin. Res. 11 (2018) 147-149.

[32] F. Qiu, S. Wu, X. Lu, C. Zhang, J. Li, M. Gong, M. Wang, Quality evaluation of the artemisinin-producing plant *Artemisia annua* L. based on simultaneous quantification of artemisinin and six synergistic components and hierarchical cluster analysis. Ind. Crops Prod. 118 (2018) 131-141.

[33] X.-C. Tang, Y. Xiao, P. Li, L.-W. Qi. Simple, fast, and sensitive detection of artemisinin in human serum and *Artemisia annua* using microsensor array coupled with electrochemiluminescent imaging technique. Talanta 196 (2019) 124-130.

[34] J.F.S. Ferreira, J.C. Laughlin, N. Delabays, P.M. Magalha. Cultivation and genetics of *Artemisia annua* L. for increased production of the antimalarial artemisinin. Plant Genet. Resour. 3 (2005) 206-229.