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Analysis of Artemisia annua extracts and related products by high performance liquid chromatography-tandem mass spectrometry coupled to sample treatment miniaturisation

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29 **Analysis of *Artemisia annua* extracts and related products by high performance liquid**
30 **chromatography-tandem mass spectrometry coupled to sample treatment miniaturisation**

31

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53 **ABSTRACT**

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

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

74

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

77 1. INTRODUCTION

78

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

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

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

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

106 Analytical methods based on liquid chromatography, in particular coupled to MS/MS [21-24], can
107 be found in the scientific literature for artemisinin quantitative analysis alone or together with other
108 compounds in *A. annua* extracts and derived products. Solvent extraction was applied to different
109 foods (tea, biscuits, and porridge) enriched with dried leaves of *A. annua* prior to LC-MS/MS analysis
110 [21]. Organic solvents were also used to obtain extracts by maceration of the herbal drug to study
111 spontaneous plants [22]. An LC-MS/MS method was proposed for the analysis of artemisinin
112 together with six analogues to be applied to *A. annua* crude plant [23]. Finally, rapid resolution
113 liquid chromatography coupled to triple quadrupole mass spectrometry (RRLC-QQQ) was exploited
114 for the determination of artemisinin in *A. annua* raw material after ultrasound-assisted extraction
115 (UAE) [24].

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

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

126

127

128 **2. EXPERIMENTAL**

129 **2.1. Chemicals and standard solutions**

130 Artemisinin pure powder (98% purity) and acetonitrile, methanol, formic acid (all reagents for mass
131 spectrometry) in addition to all the solvents used for the extract preparation procedures (all
132 analytical grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA); artemisinin-D3 pure
133 powder (98% purity, xx% isotopic purity), used as the internal standard (IS), was provided by
134 Biosynth (St. Gallen, Switzerland). Ultrapure water (18.2 MΩ cm) was obtained by means of a Milli-
135 Q apparatus from Millipore (Milford, MA, USA). The analyte and IS stock solutions (1 mg/mL) were
136 prepared by dissolving suitable amounts of pure powders in methanol; the corresponding standard
137 solutions were prepared daily by dilution with a water/acetonitrile mixture (50:50) containing 0.25
138 % formic acid. All solutions were stored protected from light in amber glass vials certified for mass
139 spectrometry from Waters Corporation (Milford, MA, USA). Human promyelocytic leukemic cell line
140 HL60 was purchased from American Type Culture Collection (Manassas, VA, USA). Propidium Iodide,
141 Trypan Blue, Foetal Bovine Serum, RPMI 1640, L-glutamine, tri-sodium citrate, RNase, Igepal and
142 ethanol were ultrapure grade from Sigma Aldrich.

143

144 **2.2. Chromatographic instrumentation and conditions**

145 LC-DAD-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic system with
146 autosampler coupled to a Waters 2998 photodiode array detector and a Waters Micromass Quattro
147 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

172 **2.4. Plant material and extract preparation**

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

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

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

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

189

190 **2.5. Commercial extracts**

191 In addition to the two *A. annua* Extracts A and B prepared on purpose within this study, two
192 commercial products were analysed (Extracts C and D). Extract C was a hydroalcoholic extract (65 %
193 EtOH) obtained after dried *A. annua* maceration and sold as a dietary supplement. Extract D
194 consisted in a dry extract of *A. annua* marketed as a veterinary food supplement sold in form of
195 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**

220 The LC-DAD-MS/MS method was validated according to European Medicines Agency (EMA), Food
221 and Drug Administration (FDA) and International Conference on Harmonization (ICH) guidelines [26-
222 28]. The tested parameters were linearity (including limits of detection and limit of quantitation),
223 extraction yield, precision and accuracy.

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

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

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

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

240 Moreover, the samples were subjected to MEPS and analysed six times in the same day to obtain
241 *intraday* precision data and six times over six different days to test *interday* precision.

242 Recovery assays were carried out in order to evaluate method accuracy: standard solutions
243 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 % CO₂. Cells were plated
251 at 1x10⁵ 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: 1x10⁶ 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**

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

273 Together with the sorbents, different mobile phase compositions were studied: volatile acidic
274 additives at different concentrations, namely formic acid, acetic acid and trifluoroacetic acid ranging
275 from 0.01 % to 0.5 %, both with acetonitrile or methanol as the organic modifiers. The use of
276 acetonitrile and H₂O flowing at 0.2 mL/min under an optimised composition gradient provided the
277 best compromise between retention times (of both analyte and IS) and peak shape. 0.25 % formic
278 acid provided the best analyte ionisability and thus the highest peak area, as further increases in
279 formic acid concentrations did not provide better results.

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

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

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

288

289 **3.2. MEPS procedure development**

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

includes the same steps as an SPE procedure, but all of them take place within a much smaller amount of sorbent (a few milligrams as opposed to 30-1000 mg or more), which is contained in the BIN of a modified liquid chromatography syringe. MEPS offers several advantages over SPE: For example, it needs much smaller amounts of sample and of solvents (up to 200 μ L vs. 1-5 mL); automation is straightforward and requires inexpensive equipment (basically, an automated syringe handler). Procedure development is based on the same principles, so the steps are quite similar and adaptation of SPE procedures to MEPS application is relatively simple. Although a MEPS BIN is more expensive than a single SPE cartridge, it can be regenerated and re-used multiple times (up to 200, according to the manufacturer's specifics), leading to expenses per analysis which are almost identical, but with significant environmental benefits due to the sharply reduced amounts of waste (in terms of both equipment and solvents). As can be expected, MEPS is particularly advantageous in case of small or very expensive samples.

MEPS procedure was developed and optimised in terms of sorbent type, volumes and nature of the washing and elution solvents, number of cycles and speed of drawing and discharging. A careful sorbent selection was important to achieve satisfying purification and extraction yields: the performance of different commercially available types of sorbents such as C2, C8, C18 and M1 (M1, mixed mode: C8 + strong cation exchange, SCX) was tested for extraction efficiency. C2 and C8 sorbents both led to acceptable extraction yields in comparison with C18 and M1, however C8 sorbent provided also better results in terms of clean-up (Figure 2a). All the main parameters involved in the MEPS procedure steps (loading, washing and elution) were carefully optimised. Artemisinin absorption onto the sorbent was satisfactory after 10 draw/discharge cycles (Figure 2b) of the loading solution at the speed of 2 μ L/s. Different solvents and mixtures were tested for the washing and elution steps and good purification was obtained by washing the sorbent from matrix components with 100 μ L of ultrapure water followed by 100 μ L of a water/methanol 95:5 mixture

316 at a speed of 10 $\mu\text{L/s}$. Finally, elution with 5 cycles of 100 μL of pure methanol each (Figure 2c), at a
317 speed of 2 $\mu\text{L/s}$, 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 $\mu\text{g/mL}$ standard solution of
324 IS and subjected to MEPS pretreatment. The artemisinin concentration found in the injected
325 solution was 11.33 $\mu\text{g/mL}$. 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 $\mu\text{g/mL}$ artemisinin
336 concentration range (regression line equation: $y = a x + b$; $SD_{\text{slope}} = \text{xx}$; $SD_{\text{intercept}} = \text{xx}$). 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 $\mu\text{g/mL}$), 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

the same compound concentration (100 µg/mL), cell counts demonstrated the effectiveness of each extract on cell growth was very variable (Table 4). Among different extracts, Extract B exhibited the highest inhibition of cell proliferation, followed by Extract C, Extract A and finally Extract D, the latter being the least active. Interestingly, the trend of obtained results correlates with the artemisinin content of the tested formulation, as reported in Table 3.

The decrease of the viable cell number indicates the onset of cytotoxicity and in order to assess whether the antiproliferative effect of *A. annua* extracts was accompanied by interference with cell cycle progression, DNA profiles of cultured cells were examined by flowcytometry. The results reported in Figure 4 show again the treatments caused variable effects also on cell cycle distribution: a marked sub-G1 peak is visible in flow cytometry histogram of the cells treated with the richest artemisinin extract, indicating the induction of apoptosis and more generally a cytotoxic effect. Nevertheless, a significant decrease in the cell percentage in S phase is evident for samples treated with Extract A and Extract C (13.83 % and 19.05 % vs 39.60 % of the control cells, respectively), confirming the reduction of cell proliferation described above. Only minimal effects on cell cycle profile is associated to treatment with Extract D, which contains the lowest artemisinin concentration.

380

381

382 4. CONCLUSION

An original methodology based on LC-DAD-MS/MS coupled to MEPS pretreatment has been developed and validated for the determination of artemisinin in different extracts obtained from *A. annua*, including commercial ones. The developed method was validated in terms of linearity, precision, extraction yield and accuracy, with good results for both artemisinin and IS, thus demonstrating the full suitability of this analytical strategy and the reliability of the obtained data.

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

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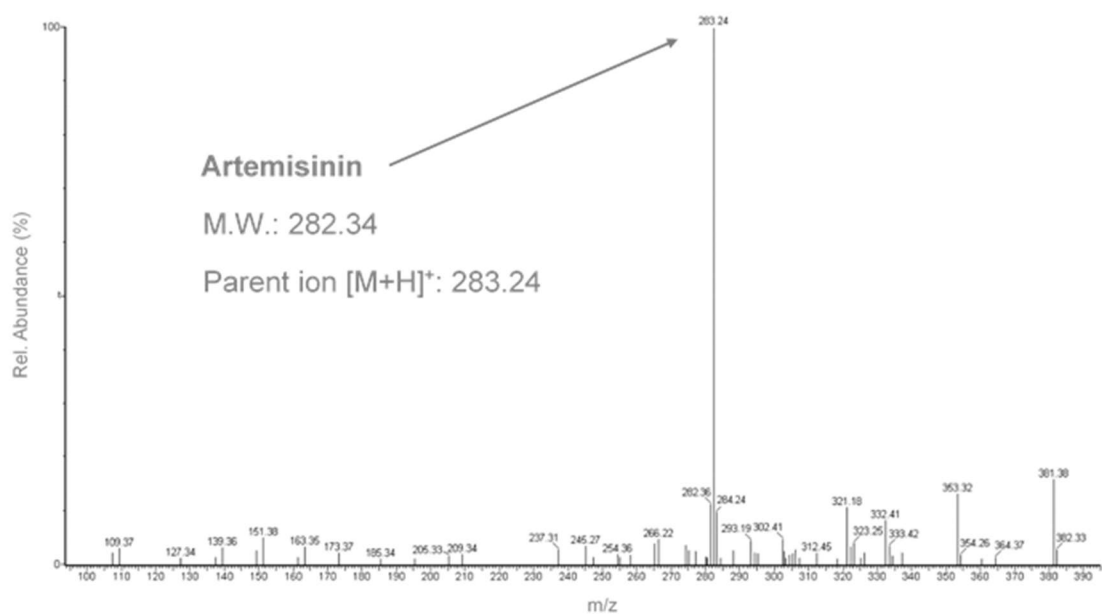
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405 Thanks are due to Prof. Shikai Yan from Guangdong University (China) for kindly providing and
406 translating from the Chinese Pharmacopeia the extraction protocol used in this study for Extract B.

407 **FIGURE LEGEND**

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410 **Figure 1** – MS spectrum of artemisinin.

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

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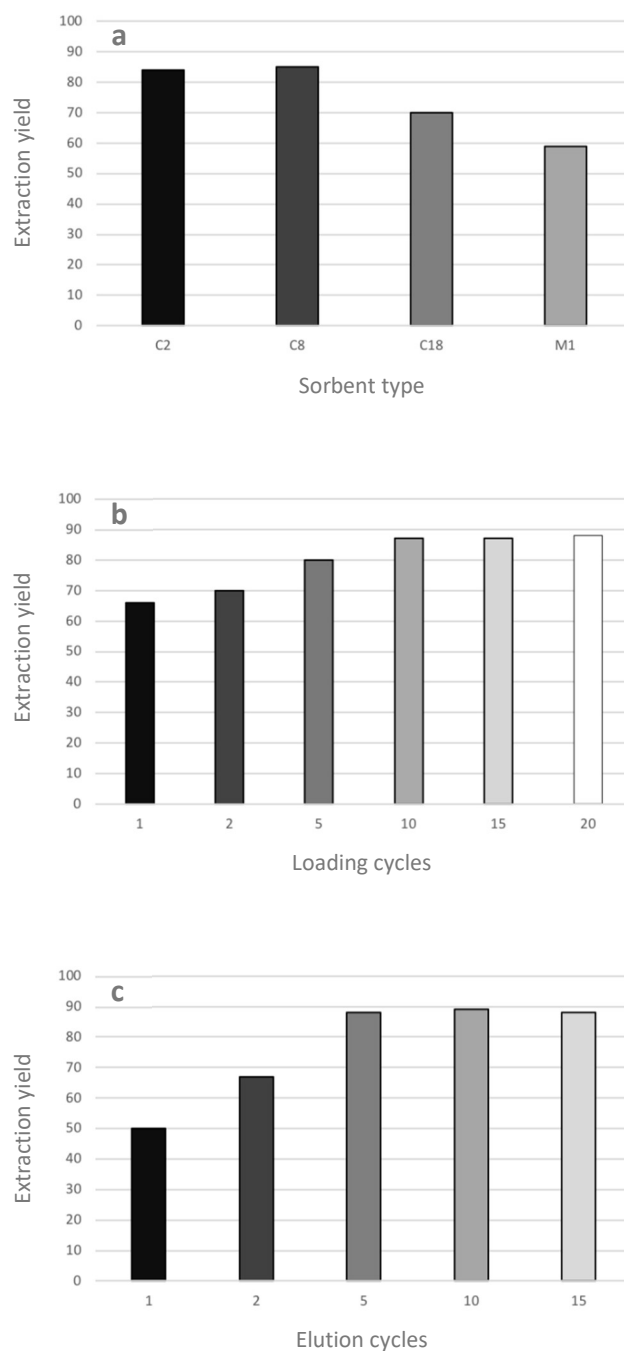


Figure 3 – Typical MRM chromatograms of Extract B spiked with a 75 ng/mL standard solution of the IS and subjected to MEPS pretreatment. Trace A: chromatogram of the 283 → 209 MRM transition corresponding to artemisinin; artemisinin peak at retention time (t_R) = 6.7 min. Trace B: chromatogram of the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

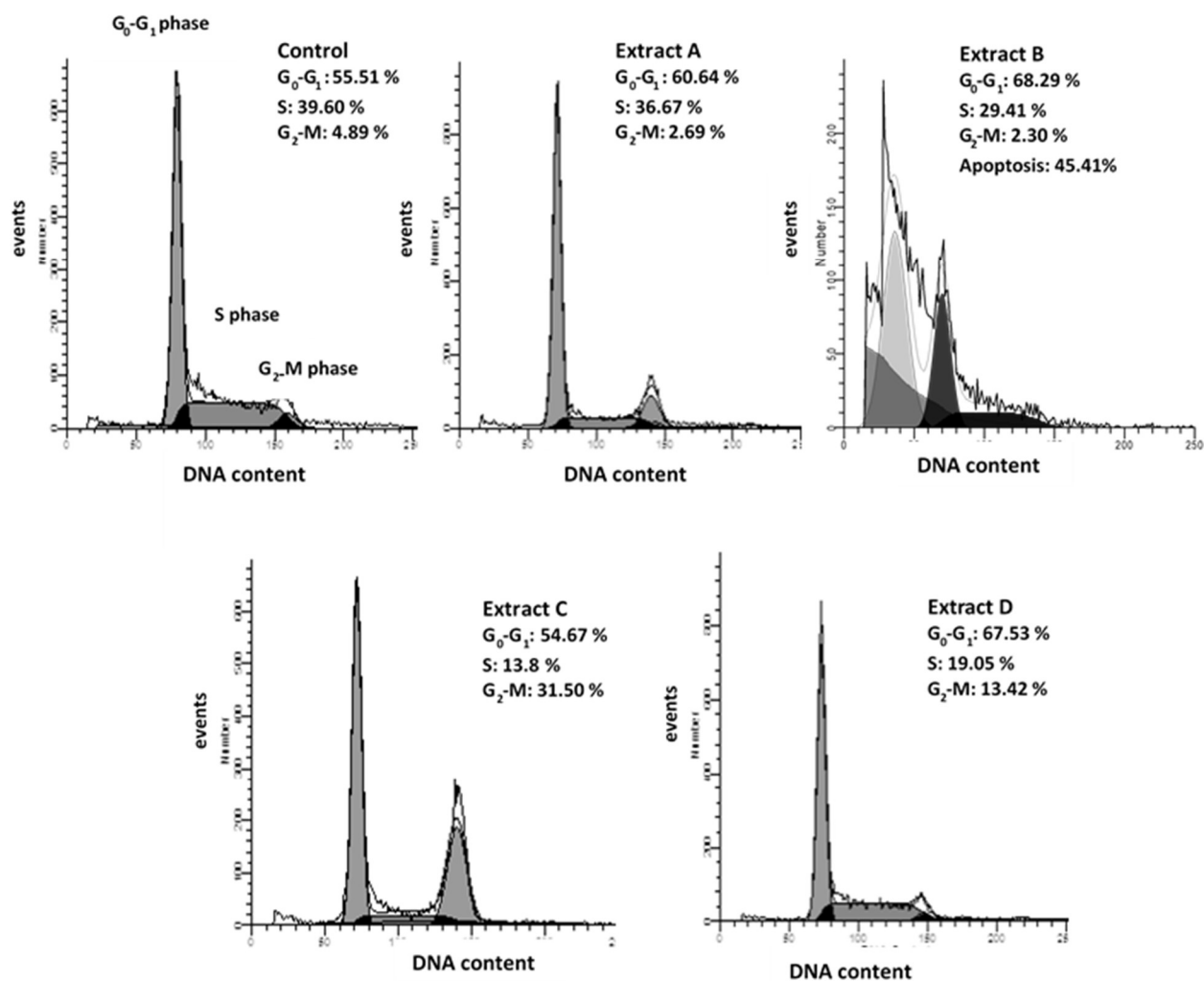


Figure 4 – Effects of *Artemisia Annua* extracts on the growth of HL-60 cells. Cells were treated with 100 $\mu\text{g/mL}$ of each extract. After 24 h of incubation, viable cells were counted. Panels report the cytofluorimeter outputs obtained in one typical experiment repeated twice with similar results.

TABLE LEGEND

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

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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

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^a confirmatory product ion

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

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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

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471 ^a *n* = 6
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473 **Table 3** – LC-DAD-MS/MS quantitative data from extract analysed samples.

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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

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 491 ^a *n* = 6
 492 ^b µg/mg
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494 **Table 4** – *In vitro* activity of extracts (100 µg/mL) on leukemic cell proliferation.

495

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

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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

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 500 ^a *n* = 3
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