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# 1 **Combination of Molecular Network and Centrifugal Partition** 2 **Chromatography fractionation for targeting and identifying** 3 ***Artemisia annua* L. antioxidant compounds**

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## 13 14 **Abstract**

15 *Artemisia annua* L. is an annual weedy herb belonging to the Asteraceae family. As a  
16 traditional Chinese herb, *Artemisia annua* is a major source of artemisinin, an  
17 antimalarial drug. In addition to artemisinin, this plant contains several other  
18 molecular families presenting a wide range of biological properties. To facilitate the  
19 screening and the identification of active compounds, the present study describes  
20 their targeting by combining the dereplication information obtained by means of  
21 Molecular Networks and a crude extract fractionation by Centrifugal Partition  
22 Chromatography to obtain and test simplified fractions. This simple and fast approach  
23 was developed focused on the antioxidant activity of *Artemisia annua* with the aim of  
24 screening and identifying the antioxidant molecules for further cosmetic uses. Firstly,  
25 the aerial parts of *Artemisia annua* were extracted and their antioxidant activity was  
26 evaluated by DPPH, ABTS, CUPRAC, FRAP and iron (II) chelating assays. Extract  
27 with a positive response was subjected to UHPLC-HRMS with autoMS/MS  
28 experiments in order to build a Molecular Network using the GNPS (Global Natural  
29 Products Social Molecular Networking) platform. Secondly, the crude extract was  
30 fractionated using CPC with an adapted Arizona solvent system. The fractions  
31 obtained were evaluated for antioxidant activity to focus on active compounds, which  
32 were located on the Molecular Network and identified thanks to their MS/MS spectra.  
33 Using this approach, the major phenolic compound contributing to the antioxidant  
34 activity of *Artemisia annua* extract was identified.

## 35 **Keywords**

36 *Artemisia annua* L.; Molecular Network; Centrifugal Partition Chromatography;  
37 Antioxidant activity.

## 38 39 40 **1. Introduction**

41 *Artemisia annua* is one of the 500 species of the *Artemisia* genus [1] belonging to the  
42 Asteracea family. It is an annual weedy herb traditionally used for the treatment of  
43 malaria throughout Asia and Africa in the form of tea or pressed juice [2]. *Artemisia*  
44 *annua* is the major source of artemisinin, an endoperoxide sesquiterpene lactone [3]

45 responsible for antiplasmodial activity [4] used as the active ingredient in anti-malarial  
46 drugs. In addition to artemisinin, *Artemisia annua* has abundant nutrient profiles such  
47 as amino acids, vitamins and mineral elements [5]. Over the years, more than 600  
48 secondary metabolites have been identified in the whole plant [6] including several  
49 sesquiterpenoids, triterpenoids, monoterpenoids, steroids, flavonoids, coumarins,  
50 alkaloids and benzenoids [7]. This wide variety of compounds and extract richness  
51 gives *Artemisia annua* a number of biological properties that have attracted the  
52 interest of researchers e.g. hepatoprotective [8], antifungal [9], antitumoral [10], and  
53 antioxidant [11] activities. In order to promote *Artemisia annua* in the cosmetic field  
54 and to develop new cosmetic ingredients, the antioxidant activity was targeted in this  
55 study. Antioxidant activity is important to protect the biological system, since reactive  
56 oxygen species (ROS) play an important role in oxidation, which may oxidize  
57 proteins, lipids, or DNA in tissues, leading to degenerative/chronic diseases including  
58 cancer, diabetes or cardiovascular disease [12]. The main role of antioxidant  
59 compounds is to delay or inhibit the oxidation of lipids or other molecules by  
60 preventing the initiation or propagation of oxidizing chain reactions [13]. Antioxidant  
61 compounds are of great interest in cosmetic applications as they prevent skin  
62 damage and early aging. A number of studies describe simple, cost effective, easily  
63 interpreted, colorimetric techniques adapted for the estimation of antioxidant power.  
64 The most commonly used methods include DPPH (2,2-diphenyl-1-picrylhydrazyl),  
65 ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)), FRAP (ferric reducing  
66 antioxidant power), cupric reducing antioxidant capacity (CUPRAC) assays, and total  
67 reducing capacity [14,15]. Such assays rely on either single electron transfer (SET)  
68 or hydrogen atom transfer (HAT) reaction kinetics and display either reduction  
69 capacity (FRAP, CUPRAC) or direct free radical inhibition (DPPH, ABTS) [16]. The  
70 results obtained with *in vitro* tests cannot directly be transferred to an *in vivo*  
71 antioxidant effect for cosmetic applications to prevent skin damage and early aging,  
72 but these assays are still first-line tests before a deeper study of plant extract activity  
73 and the development of a cosmetic product. Several studies have demonstrated the  
74 antioxidant capability of *Artemisia annua* [11,17,18], which could be due to the  
75 presence of phenolic compounds [10, 18]. However, the active molecules were not  
76 identified. In cosmetic products, entire extracts being generally used directly in the  
77 formulas, we sought to develop an approach to target the molecules that contribute  
78 the most to the extract activity without purifying and identifying all the extracted  
79 compounds. Therefore we combined: i) the dereplication information obtained by  
80 means of Molecular Networks (MN) that compare the MS/MS spectra of the analyzed  
81 compounds and group them in clusters according to the similarities of their  
82 fragmentation pathway [19-21]. MN are used to visualize the structural relationship  
83 between compounds belonging to a molecular family, facilitating the identification of  
84 unknown metabolites; ii) the crude extract fractionation by Centrifugal Partition  
85 Chromatography (CPC), a liquid-liquid preparative chromatography technique which  
86 presents numerous benefits over preparative LC due to the absence of solid  
87 stationary phase (e.g. irreversible adsorption, solute deactivation, solid waste and the  
88 ability to recover all the extract without loss) [22], making possible to obtain different  
89 simplified fractions from a complex crude extract. Using this combination of  
90 techniques, a simple and fast approach to produce and test simplified fractions, was  
91 developed, thus facilitating the screening and identification of the bioactive  
92 metabolites.

93

## 2. Material and methods

### 2.1 Chemicals

The following reagents were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France): 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium persulfate (99.99%), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-*p,p'*-disulfonic acid monosodium salt hydrate (97%), copper (II) chloride (99.99%), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) ( $\geq 98\%$ ), sodium acetate ( $\geq 99\%$ ), 2,2'-azobis(2-methylpropionamide) dihydrochloride granular (97%), trolox ( $\geq 98\%$ ), ferric chloride (97%), neocuproine ( $\geq 98\%$ ), glacial acetic acid, formic acid, ammonium acetate and ferric chloride hexahydrate. 2,2'-azino-bis-(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS) (98%) reagent was purchased from ThermoFisher-AlfaAesar (Kandel, Germany). All the solvents used for the plant extraction, CPC fractionation and UHPLC-HRMS analysis were HPLC grade: ethanol, isopropanol, *n*-heptane, methanol and ethyl acetate were purchased from VWR (Fontenay-sous-Bois, France); HPLC PLUS gradient grade acetonitrile used for UHPLC-HRMS analysis was from SDS Carlo Erba (Val de Reuil, France). Ultrapure water was produced with a PurelabFlex system from Veolia (Wissous, France). Artemisinin (98%) was purchased from Extrasynthese (Genay, France). The stock solution of artemisinin was prepared by dissolving pure powder in ethanol at 1 mg/mL.

### 2.2 Plant material and sample preparation

A sample of *Artemisia annua* was obtained from stems and leaves collected in 2017 at the Comité de Développement Horticole de la Région Centre-Val de Loire (CDHRC, Saint Cyr en Val, France). 5 g of crushed dried areal parts were extracted in 50 mL of ethanol during 1 h using ultrasound assisted extraction. The supernatant was recovered and centrifuged 10 min at 10000 g before evaporation of the solvent under nitrogen flow to obtain the dry crude extract. Extraction yield defined as the mass of dry crude extract on the mass of dry raw material was 2.3%.

### 2.3 UHPLC-UV-MS/HRMS and FIA-HRMS analysis

Chromatographic analyses were performed using an ultimate 3000 RSLC system composed of an autosampler, a binary pump, a thermostated column compartment and a DAD detector (200-800 nm) (Dionex, Germering, Germany). The column was a (150 x 2.1 mm; 1.7  $\mu$ m) UHPLC Fortis-C18 (AIT, Houilles, France). The mobile phase was composed of water (A) and acetonitrile/isopropanol (50/50) (B) both acidified with 0.1% formic acid at a flow rate of 300  $\mu$ L/min. The mobile phase gradient was: 0-0.1 min, 10% B; 0.1-9 min, 100% B; 9-11.5 min, 100% B; 11.5-12 min, 10% B; 12-15 min, 10% B. The compound separation was carried out at 60°C. The extract was dissolved in ethanol at a concentration of 10 mg/mL, and 0.2  $\mu$ L were injected.

The MS and MS/MS experiments were carried out on a maXis UHR-Q-TOF mass spectrometer (Bruker, Bremen, Germany) using the data dependent acquisition mode (DDA) with an electrospray ion source (ESI) working in positive ionization mode. Nitrogen was used as drying gas at a flow rate of 9 L/min heated at 200°C and as nebulizing gas at a pressure of 2 bar. The mass spectra were recorded in the *m/z* range 50-1650 at 1.6 Hz for MS and MS/MS. Capillary voltage was set at 4.5 kV. Three precursor ions were selected per cycle and fragmented at two collision energies, averaged into one MS/MS spectrum, set at 20 and 45 eV for *m/z* 140; 35 and 78 eV at *m/z* 1000; collision energies were proportional for other *m/z*. All the MS

141 data were processed using DataAnalysis 4.4 (Bruker). The molecular formulae were  
142 generated using elemental composition (C, H, O) without any restriction, Na  $\leq$  1 and  
143 N  $\leq$  5 with a mass accuracy  $\leq$  3 ppm.

144 CPC fractions were quickly analyzed (less than 1 min) by Flow Injection Analysis  
145 (FIA) in the mass spectrometer. 15  $\mu$ L of the fraction were diluted in 250  $\mu$ L of  
146 methanol and 0.2  $\mu$ L were injected. The solvent was a mixture of acidified water  
147 (0.1% formic acid) and acetonitrile (35/65) at 200  $\mu$ L/min.

148 The AutoMS(n) algorithm of DataAnalysis 4.4 software was applied to generate the  
149 peak-list with the following parameters: retention time window: 0.3 min, intensity  
150 threshold TIC (AllMSn): 50, maximum number of compounds: 7500. The generated  
151 peak-list was exported into a mgf file including deconvoluted ions and the 10 most  
152 abundant non deconvoluted ones with an intensity superior to 100. This format  
153 expresses the MS/MS spectra into a peak-list containing precursor ions (tr,  $m/z$ ) and  
154 fragment ions ( $m/z$ , intensity, charge).

## 155 **2.4 Molecular Network design**

156 The Molecular Network was designed using the Global Natural Products Social  
157 Molecular Networking (GNPS) platform [<http://gnps.ucsd.edu>]. All the MS/MS spectra  
158 obtained were exported to the GNPS platform in the form of a peaklist (mgf file)  
159 containing all the needed information about the analysis. Then, MS/MS spectra were  
160 compared pairwise to search for spectral similarities *i.e.* the same fragment ions  
161 and/or neutral losses. The optimum parameters were: tolerance 0.02 Da for parent  
162 mass and fragments; cosine score  $\geq$  0.65; matched peaks  $\geq$  3; network topK 10;  
163 maximum connected component size 100; minimum cluster size 1, No run  
164 MSCluster. The results were downloaded and exported to be visualized on  
165 Cytoscape 3.6.1 software [<https://cytoscape.org>].

166 The identification of compounds was supported by GNPS's spectral libraries. The  
167 MS/MS spectra of *Artemisia annua* compounds were compared to MS/MS spectra of  
168 compounds contained in the GNPS library platform, using the following parameters:  
169 library search min matched peaks: 4; score threshold: 0.7; maximum analog search  
170 mass difference: 100.

## 171 **2.5 Fractionation by Centrifugal Partition Chromatography** 172 **(CPC)**

173 CPC experiments were performed with a semi-preparative 50 mL FCPC (Rousselet  
174 Robatel Kromaton, Annonay, France) associated to a preparative LC-8A pump  
175 (Shimadzu, Japan). The sample was introduced in the column using a 6-port high  
176 pressure Rheodyne injection valve (AIT, Houilles, France) equipped with a 10 mL  
177 loop.

178 The biphasic solvent system Arizona M (heptane / ethyl acetate / methanol / water  
179 with a 5/6/5/6 volumetric ratio) was prepared by mixing the different solvents in a  
180 separating funnel. The two phases were separated after funnel agitation and  
181 equilibration. The aqueous phase was selected as the mobile phase and the organic  
182 one as the stationary phase (descending mode).

183 The stationary phase was firstly introduced into the column (9 mL/min, 500 rpm), then  
184 the mobile phase was pumped through the stationary phase (3 mL/min, 2000 rpm)  
185 until an equilibrium stage was reached ( $V_{\text{mobile phase}} = 20$  mL, 60% of retention).

186 116 mg of the *Artemisia annua* ethanolic extract were solubilized in 4 mL of a mixture  
187 stationary phase/mobile phase (50/50) at a concentration of 29 mg/mL and were  
188 injected after the equilibration step. The elution (3 mL/min, 2000 rpm) and extrusion  
189 (9 mL/min, 500 rpm) steps were performed to recover all the compounds from the  
190 column.

## 191 **2.6 *In-vitro* antioxidant capability**

192 Antioxidant capability was evaluated using DPPH, ABTS, FRAP, CUPRAC and Iron  
193 (II) chelating assays. It was determined by measuring the absorbance of reagent in  
194 the presence of the different samples (solvent for blank, trolox for positive control,  
195 crude extract or fractions) because the oxidation/reduction and complexation  
196 reactions are highlighted by a color change of the solution.

197 Evaluation of biological activity was performed in 96-well plates; each sample was  
198 deposited at 3 cascading concentrations. Ethanol was used to dilute the samples and  
199 for the negative control. Trolox was the positive control and was evaluated at 6  
200 cascading concentrations ranging from 0.005 mg/mL to 0.5 mg/mL. Stock solutions of  
201 crude extract and CPC fractions were at 10 mg/mL, thus after dilution, the  
202 concentrations of 0.1, 0.25 and 0.5 mg/mL were evaluated. All the assays were done  
203 in triplicate (n=3).

### 204 **DPPH radical scavenging activity assay**

205 DPPH assays were performed by readjusting the method described by Lee *et al.* [23].  
206 Briefly, 10  $\mu$ L of sample were mixed with 190  $\mu$ L of DPPH reagent at 60  $\mu$ M in  
207 ethanol and incubated 30 min in the dark at room temperature. The absorbance was  
208 recorded using a microplate reader (MULTISCAN GO, ThermoFisher, USA) at  
209 516 nm.

### 210 **ABTS radical cation decolorization assay**

211 ABTS assays were carried out by readjusting the method described by Tagliazucchi  
212 *et al.* [24]. In brief, the mixture of 7 mM of ABTS solution and 2.45 mM potassium  
213 persulfate solution at equal volume were incubated in the dark for 16 h under  
214 agitation at room temperature. Next, the mixture was diluted with ethanol/water  
215 (25/75) in the following volume proportion (1:12.5). Then, 190  $\mu$ L of the diluted  
216 mixture were mixed with 10  $\mu$ L of sample and the absorbance was recorded after 30  
217 min of incubation in the dark at room temperature using a microplate reader at 734  
218 nm.

### 219 **FRAP (Ferric ion Reducing Antioxidant Power) assay**

220 A modified FRAP protocol was used according to the method outlined by Benzie and  
221 Strain [25]. Concisely, the FRAP reagent is composed of FeCl<sub>3</sub> (20 mM) and TPTZ  
222 (10 mM, solubilized in HCl 40 mM) with acetate buffer (300 mM) in a 1:1:10 (v/v/v)  
223 ratio. Thereafter, 10  $\mu$ L of sample were mixed with 190  $\mu$ L of FRAP solution and  
224 incubated 30 min in the dark at room temperature. Finally, the absorbance was  
225 measured using a microplate reader at 590 nm.

### 226 **CUPRAC (Cupric ion reducing antioxidant activity) assay**

227 CUPRAC assays were carried out according to the method described by Apak *et al.*  
228 [26] with slight modifications. In short, 10  $\mu$ L of sample were mixed with 190  $\mu$ L of  
229 CUPRAC reagent prepared by combining 10 mM Cu(II) with 7.5 mM Neocuproine

230 and 1 M acetate ammonium buffer in a 1:1:1 (v/v/v) ratio. Finally, the mixture was  
231 incubated 30 min in the dark at room temperature and the absorbance was measured  
232 at 450 nm using a microplate reader.

### 233 **Iron (II) chelating assay**

234 The ability of *Artemisia annua* compounds to chelate Fe<sup>2+</sup> was evaluated using a  
235 method described by Denis *et al.* [27] with some modifications. The reagent was  
236 prepared by combining 1 mM FeCl<sub>3</sub> and 0.3 mM ferrozine at a 1:1 (v/v) ratio. Next,  
237 10 µL of sample were mixed with 190 µL of reagent and incubated 30 min in the dark  
238 at room temperature. Finally, the absorbance was measured using a microplate  
239 reader at 562 nm.

### 240 **Statistical Analysis**

241 Absorbance results (n=3) were average and data were presented as mean ±  
242 standard deviation. MetaboAnalyst 4.0 software was used to perform statistical  
243 analyses. The samples were from the same population. Finally, the multi-comparison  
244 was performed using the one-way analysis of variance (ANOVA) followed by *F*-  
245 protected least significant difference (Tukey's HSD) *post hoc* comparisons.

### 246 **Results and discussion**

247 The objective of this work was to target and identify the main antioxidant markers  
248 from *Artemisia annua* extract. The Base Peak Chromatogram (BPC) obtained with  
249 MS detection is presented in Figure 1. It offers a first molecular fingerprint of the  
250 sample and highlights its complexity. This analysis allows the detection of hundreds  
251 of chromatographic peaks with a wide range of polarities, from 10 up to 100% of  
252 organic solvent. Positive ionization mode with an ESI source was chosen because it  
253 allowed the ionization of more compounds than the negative one and a good  
254 fragmentation of the molecular ions giving suitable MS/MS spectra. It would be  
255 possible to determine the antioxidant compounds by identifying each peak of the  
256 chromatogram in Figure 1, knowing that each peak is composed of an MS and an  
257 MS/MS spectrum for the precise identification. However, to dispense with the time-  
258 consuming interpretation of numerous spectra, the Molecular Network approach was  
259 used to sort and group molecules according to spectral similarities.

### 260 **2.7 Molecular Network**

261 Molecular Networks constitutes an accessible and adaptable means to visualize and  
262 target natural products, enabling biological research and biotechnological  
263 applications in a wide range of fields [28]. Most of highlighted strategies aimed  
264 efficient isolation of natural products exhibiting significant levels of bioactivity and/or  
265 novel and chemo-diverse chemical entities mainly for drug discovery purposes [29]. A  
266 review focused on illustrating the new thinking on the isolation of natural products by  
267 drawing a parallel between the different philosophies underlying the use of Molecular  
268 Networks in the targeting of natural compounds, as developed by a growing number  
269 of research groups [30].

270 Generation of the Molecular Network was based on mass spectrometry analysis of  
271 the crude extract. MS/MS spectra of the compounds were compared pairwise to find  
272 similarities in fragmentation pathways, *i.e.* the same fragment ions or similar neutral  
273 losses. To obtain suitable MS/MS spectra with a convenient number of fragment ions,  
274 several collision energies were tested for the MS/MS experiments to find the optimal

275 ones that fragmented the rigid structures while producing good spectra for molecules  
276 that fragment more easily. The molecules presenting similar fragmentation patterns  
277 were grouped in the same cluster while the molecules with different MS/MS spectra  
278 were not associated [31-33]. Once the clusters had been built, the identification of  
279 some compounds by the injection of standard molecules or by using public spectral  
280 libraries such as GNPS or by reference to the known literature is sufficient to  
281 determine the molecular family of the associated compounds that facilitates the  
282 identification of unknown compounds. In addition, the emergence of the annotation  
283 tools enable, today, to search for *in silico* generated structures in natural product  
284 databases, which is a huge step forward compared to the traditional dereplication  
285 process based on molecular formulas and/or exact masses or NMR chemical shifts  
286 searches [30].

287 The Molecular Network of *Artemisia annua* crude extract visualized using  
288 Cytoscape 3.6.1 software is presented in Figure 2. This MN contains 2 major clusters  
289 and 8 small clusters which can be linkened to molecular families or molecular groups.  
290 Each green node represents a compound, inside which the molecular mass of the  
291 parent ion *i.e.* molecular ion, fragment ion, or adduct mass of the compound is  
292 indicated, linked by a line designating the similarity between the compounds. It is  
293 therefore possible to find several nodes that express the same compound, because if  
294 a molecule is ionized under different forms (adducts, multi-charged ions) or  
295 fragmented during the ionization process, these different ions may give MS/MS  
296 spectra with many similarities, and then the nodes representing them will be linked.  
297 Considering selected parameters it is also possible to find different nodes indicating  
298 the same *m/z*, they represent different isobaric compounds eluted at different  
299 retention times.

300 The main clusters were associated to molecular families by annotating the standards  
301 (*e.g.* artemisinin) and some compounds representative of the different molecular  
302 families already described in the literature as present in aerial parts of *Artemisia*  
303 *annua* ethanolic extract (*e.g.* coumarin, circiliol, casticin...) [7] on the MN. The  
304 structures of annotated compounds presented in Table 1 were also recognized and  
305 confirmed by GNPS spectral libraries (*e.g.* Massbank: FIO00415 Artemisinin). These  
306 results highlight the presence of 3 major molecular families in *Artemisia annua*  
307 extract: terpenes, coumarins and flavonoids. Small clusters containing only a few  
308 nodes were not identified. In accordance with the well-known richness of *Artemisia*  
309 *annua*, terpenes appear to be the largest family with numerous associated nodes.  
310 Coumarins were also well represented in this ethanolic extract with a fairly large  
311 cluster, while flavonoids were less numerous.

312 The MN allowed the rapid identification of the major molecular families and their  
313 molecules contained in the plant extract by clustering compounds according to their  
314 structures without interpreting all the MS spectra and without annotating the entire  
315 network but only those compounds already described in the literature. Before further  
316 detailing the results, in view of the molecular families identified, it can be  
317 hypothesized that the antioxidant molecules should belong more to flavonoids and/or  
318 even coumarins [34,35] than to terpenes. But, in order to target only the antioxidant  
319 compounds among all these molecules, the extract was simplified into several  
320 fractions using CPC.

## 321 2.8 Centrifugal Partition Chromatography



322 The choice of a good biphasic system is important for the separation of compounds  
323 in CPC because it represents the mobile and stationary phases. Since the antioxidant  
324 molecules were not determined, the objective of the CPC method was to split the  
325 complex crude extract of *Artemisia annua* without losing any molecules and obtain  
326 simplified fractions to focus on the active ones. To do this, several biphasic systems  
327 were tested to find the optimal one that allowed a good solubility of the extract and  
328 different partition of the compounds between the two phases leading to their  
329 separation in different fractions.

330 Six different solvent systems from the ARIZONA table (heptane / ethyl acetate /  
331 methanol / water) were tested: D (1/6/1/6), H (1/3/1/3), M (5/6/5/6), P (6/5/6/5), R  
332 (2/1/2/1) and U (4/1/4/1). These systems cover a wide range of polarities related to  
333 the identified molecular families, thus increasing the chances of targeting the  
334 optimum biphasic system. The D and H systems resulted in very long settling times;  
335 the U system has a good stabilization time but the majority of compounds remained  
336 in the organic phase. Finally, the M and P systems with short settling times of about  
337 10 s gave a satisfactory partition of the compounds between the two phases. Organic  
338 and aqueous phases of both systems were analyzed by HPLC and the M system was  
339 selected as it offered a better compound partitioning. The K<sub>d</sub> values of the main  
340 compounds were greater than 1 to avoid eluting in the dead volume and sufficiently  
341 different from each other to be separated in different fractions. The compounds with  
342 an intermediate K<sub>d</sub> exited the column during the elution step, while some non-polar  
343 compounds with high K<sub>d</sub> were recovered in the organic phase during the extrusion  
344 step.

345 After having fractionated the complex crude extract of *Artemisia annua* into 13  
346 fractions with different molecular distributions, the antioxidant activity of the simplified  
347 fractions was evaluated using antioxidant assays to target the active fractions.

## 348 **2.9 *In-vitro* antioxidant capability**

349 A complete evaluation of the antioxidant activity of *Artemisia annua* extract and all  
350 CPC fractions was performed using five different types of assays. The five assays  
351 were based on different mechanisms *i.e* hydrogen atom transfer (DPPH, ABTS),  
352 single electron transfer (FRAP, CUPRAC) or iron (II) chelating assays. Figure 3  
353 presents the results of antioxidant assays; absorbance was plotted as a function of  
354 the concentration of the sample.

355 The antioxidant capabilities of all the CPC fractions of *Artemisia annua* were  
356 compared with the positive control and the crude extract to target the active fractions.

357 The 5 graphs in Figure 3 show the same order of activity indicating that the 5 tests  
358 rank the fractions in the same order. Crude extract and Fraction 2 invariably showed  
359 the best results, with the highest antioxidant activity. Fraction 1 showed a slight  
360 activity but much lower than that of Fraction 2, while no activity was observed for the  
361 other fractions. DPPH and iron (II) chelating assays gave better results with  
362 absorbance values at 0.5 mg/mL for Fraction 2 close to that of trolox. Activities  
363 determined with ABTS, FRAP and CUPRAC assays were weaker, with values far  
364 from trolox. Table 2 presents the percentage of activity calculated for the highest  
365 tested concentration: 0.5 mg/mL for the crude extract and the 13 fractions. ANOVA  
366 analysis was performed on these results and showed that for all the assays, crude  
367 extract, Fraction 2 and Fraction 1 were significantly different from other fractions,  
368 confirming the tendency observed in Figure 3. Differences of activity level between

369 assays based on hydrogen atom transfer mechanism such as DPPH and ABTS have  
370 already been observed [15,16]. It can probably be explain because DPPH is a free  
371 radical that accepts electrons or hydrogen radicals from donor compounds [36] while  
372 ABTS assay determines the scavenging ability of antioxidant activity by reacting with  
373 a strong antioxidant agent (potassium persulfate) in the presence of ABTS salt so it is  
374 based on the inhibition of the formation of ABTS by one-electron oxidants [37,38].  
375 The reduction capacity of the *Artemisia annua* extract and of the fractions was low  
376 compared to that of positive standard (trolox) indicating that compounds mainly act  
377 by hydrogen atom transfer rather than single electron transfer. High values obtained  
378 for crude extract and Fraction 2 for Iron chelation assay suggest the ability of  
379 compound to easily form complex that could be due to specific structure such as  
380 flavonoids [39].

381 The combination of these results indicated that the antioxidant compounds of  
382 *Artemisia annua* extract were mainly eluted in CPC Fraction 2. This method  
383 highlighted the most active fraction among the 13 CPC ones. In order to identify the  
384 antioxidant compounds of *Artemisia annua*, the fractions were analyzed by mass  
385 spectrometry

386

## 387 2.10 FIA-HRMS Analysis

388 To avoid long post fractionation analysis time, CPC fractions were quickly analyzed  
389 using FIA-HRMS. Main ions obtained on the mass spectrum could be reported on the  
390 crude extract Molecular Network to identify molecules contained into the fraction.  
391 Fraction 2 analysis in Figure 4 showed one main ion at  $m/z$  361.0915 and its sodium  
392 adduct at  $m/z$  383.0728. Although the original purpose of the CPC was not to purify  
393 molecules, it turned out that this fraction seemed to contain one major compound.  
394 UHPLC-DAD analysis (Figure 4B) of Fraction 2 gave the same result detecting one  
395 main peak at 6.3 min. The molecular formula for the ion of interest  $m/z$  361.0915 was  
396 calculated using SmartFormula algorithm from DataAnalysis software and gave two  
397 propositions:  $C_{18}H_{17}O_8$  (0.1 ppm) and  $C_{19}H_{13}N_4O_4$  (3 ppm). The second formula was  
398 discarded because its isotopic pattern is too different from the experimental one.

399 The molecular formula  $C_{18}H_{16}O_8$  was then submitted to public databases for structure  
400 search PubChem, Chemspider, ChEBI, DNP. Between 161 and 179 compounds  
401 were proposed as potential structures corresponding to this formula. The ion of  
402 interest was extracted from the mass spectrum and located on the Molecular Network  
403 to facilitate compound identification and structure selection among all the  
404 propositions. The active compound was found in the flavonoid cluster as shown in  
405 Figure 4 D. Its MS/MS spectrum showed fragment ions characteristic to methylated  
406 flavonoids ( $m/z$  346.0674  $C_{17}H_{14}O_8$  due to  $\cdot CH_3$  loss,  $m/z$  328.0577  $C_{17}H_{12}O_7$  due to  
407  $\cdot CH_3$  and  $H_2O$  loss,  $m/z$  303.0497  $C_{15}H_{11}O_7$  due to  $\cdot CH_3$  and  $C_2H_2O$  loss,  $m/z$   
408 285.0391  $C_{15}H_9O_6$  due to  $H_2O$  loss from  $m/z$  303.0497,  $m/z$  257.0443  $C_{14}H_9O_5$  due to  
409 CO loss from  $m/z$  285.0391,  $m/z$  161.0233  $C_9H_5O_3$  and 137.0231  $C_7H_5O_3$  obtained by  
410 Retro Diels Alder cleavage). This MS/MS spectrum was also submitted to the GNPS  
411 libraries and the 2-(3,4-dihydroxyphenyl)-5-hydroxy-3,6,7-trimethoxy-4H-1-  
412 benzopyran-4-one (CCMSLIB00000848806) also named chrysosplenol D was found  
413 with the CAS Registry Number 14965-20-9 in SciFinder. The confidence level of  
414 identification is assigned as 2 according to the Metabolomics Standards Initiative of  
415 the Metabolomics Society [40]. Moreover Wang *et al.* recently studying major

416 flavonoid from *Artemisia Annua* reported the MS/MS spectrum of chrysosplenol D  
417 which is consistent with our results [41].

418 The ion of interest was rapidly obtained by FIA-HRMS analysis of the active fraction.  
419 After its annotation on the Molecular Network, the molecular family of the compound  
420 was determined and its identification facilitated. This molecule has already been  
421 described in the literature as being present in *Artemisia annua* [7,41], but it was never  
422 correlated to the antioxidant capability of this plant. This compound was also  
423 detected in the low active Fraction 3 but at a lower concentration than in Fraction 2

424 Focusing on this cluster revealed that four additional methylated flavonoid molecules  
425 were present in the extract and could have antioxidant activity given their similar  
426 structure. Thus these compounds were also targeted in the CPC fractions. The  
427 molecule with  $m/z$  345.0897 which could correspond to penduletin was contained in  
428 Fraction 1 which is moderately active. This molecule possess only one free hydroxyl  
429 group on the B ring that could explain its lower antioxidant activity. The other  
430 compounds in the group *i.e.* Casticin  $m/z$  375.1074, Retusin  $m/z$  359.1125 and  
431 Artemitin  $m/z$  389.1231 were found in other fractions that did not present antioxidant  
432 activity. This can be explained either by their presence in low concentrations in the  
433 fractions or by the lack of activity of these structures despite their similarity to the  
434 active compound, they have only OMe group on B ring so they possess less H to be  
435 transferred.

436

### 437 **3. Conclusion**

438 A combination of Molecular Network with a CPC fractionation approach was  
439 developed to target and identify the active compounds of crude extract. This method  
440 was applied to *Artemisia annua* crude ethanolic extract in order to highlight and  
441 identify the molecules responsible for its antioxidant capability.

442 The Molecular Network designed by means of MS/MS spectra of *Artemisia annua*  
443 showed terpenes, flavonoids and coumarins as the major families of the ethanolic  
444 crude extract. The complex extract was thereafter split and simplified into CPC  
445 fractions with different molecular distributions. Then, antioxidant properties of CPC  
446 fractions were evaluated using DPPH, ABTS, FRAP, CUPRAC and iron (II) chelating  
447 assays, and only one of the fractions showed high antioxidant capability at the same  
448 level as the total extract. FIA-HRMS analysis of the active fraction highlighted one  
449 main compound of interest, which was identified using the Molecular Network and  
450 GNPS spectral library. Finally, thanks to the combination of these two approaches,  
451 the flavonoid compound chrysosplenol D with the molecular formula  $C_{13}H_{16}O_8$  was  
452 targeted, among the hundreds of compounds of the extract, and identified as the  
453 major molecule contributing for the antioxidant activity of *Artemisia annua* extract.  
454 The developed methodology could be extended to target new bioactive compounds  
455 with different kind of activity.

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461 **References**

- 462 [1] Bora, K. S., & Sharma, A. (2011). The genus *artemisia* : A comprehensive review.  
463 *Pharmaceutical Biology*, 49(1), 101-109.  
464 <https://doi.org/10.3109/13880209.2010.497815>
- 465 [2] Ćavar, S., Maksimović, M., Vidic, D., & Parić, A. (2012). Chemical composition  
466 and antioxidant and antimicrobial activity of essential oil of *Artemisia annua* L. from  
467 Bosnia. *Industrial Crops and Products*, 37(1), 479-485.  
468 <https://doi.org/10.1016/j.indcrop.2011.07.024>
- 469 [3] Zalkow, L. H., Harris, R. N., Van Derveer, D., & Bertrand, J. A. (1977).  
470 Isocomene : A novel sesquiterpene from *Isocoma Wrightii*. X-Ray crystal structure of  
471 the corresponding diol. *Journal of the Chemical Society, Chemical Communications*,  
472 (13), 456. <https://doi.org/10.1039/c39770000456>
- 473 [4] Who. (1994). The role of artemisinin and its derivatives in the current treatment of  
474 malaria (1994-1995) (Geneva : World health organization), (Unpublished document,  
475 who/mal/94. 1067).
- 476 [5] Brisibe, E. A., Umoren, U. E., Brisibe, F., Magalhães, P. M., Ferreira, J. F. S.,  
477 Luthria, D., Prior, R. L. (2009). Nutritional characterisation and antioxidant capacity of  
478 different tissues of *Artemisia annua* L. *Food Chemistry*, 115(4), 1240-1246.  
479 <https://doi.org/10.1016/j.foodchem.2009.01.033>
- 480 [6] Van der Kooy, F., & Sullivan, S. E. (2013). The complexity of medicinal plants :  
481 The traditional *Artemisia annua* formulation, current status and future perspectives.  
482 *Journal of Ethnopharmacology*, 150(1), 1-13.  
483 <https://doi.org/10.1016/j.jep.2013.08.021>
- 484 [7] Tu, Y. (2017). From *Artemisia annua* L. to Artemisinins : The discovery and  
485 development of artemisinins and antimalarial agents.  
486 <http://public.eblib.com/choice/publicfullrecord.aspx?p=4908207>
- 487 [8] El-Askary, H., Handoussa, H., Badria, F., El-Khatib, A. H., Alsayari, A., Linscheid,  
488 M. W., & Abdel Motaal, A. (2019). Characterization of hepatoprotective metabolites  
489 from *artemisia annua* and *cleome droserifolia* using hplc/pda/esi/ms–ms. *Revista*  
490 *Brasileira de Farmacognosia*, 29(2), 213-220.  
491 <https://doi.org/10.1016/j.bjp.2018.10.001>
- 492 [9] Li, K.-M., Dong, X., Ma, Y.-N., Wu, Z.-H., Yan, Y.-M., & Cheng, Y.-X. (2019).  
493 Antifungal coumarins and lignans from *Artemisia annua*. *Fitoterapia*, 134, 323-328.  
494 <https://doi.org/10.1016/j.fitote.2019.02.022>
- 495 [10] Lang, S. J., Schmiech, M., Hafner, S., Paetz, C., Steinborn, C., Huber, R.,  
496 Simmet, T. (2019). Antitumor activity of an *Artemisia annua* herbal preparation and  
497 identification of active ingredients. *Phytomedicine*, 62, 152962.  
498 <https://doi.org/10.1016/j.phymed.2019.152962>
- 499 [11] Wan, X. L., Niu, Y., Zheng, X. C., Huang, Q., Su, W. P., Zhang, J. F., Wang, T.  
500 (2016). Antioxidant capacities of *Artemisia annua* L. leaves and enzymatically treated  
501 *Artemisia annua* L. in vitro and in broilers. *Animal Feed Science and Technology*,  
502 221, 27-34. <https://doi.org/10.1016/j.anifeedsci.2016.08.017>

- 503 [12] Lee, J., Koo, N., & Min, D. B. (2004). Reactive oxygen species, aging, and  
504 antioxidative nutraceuticals. *Comprehensive Reviews in Food Science and Food*  
505 *Safety*, 3(1), 21-33. <https://doi.org/10.1111/j.1541-4337.2004.tb00058.x>
- 506 [13] Zheng, W., & Wang, S. Y. (2001). Antioxidant activity and phenolic compounds  
507 in selected herbs. *Journal of Agricultural and Food Chemistry*, 49(11), 5165-5170.  
508 <https://doi.org/10.1021/jf010697n>
- 509 [14] Chen, Z., Bertin, R., & Froidi, G. (2013). EC50 estimation of antioxidant activity in  
510 DPPH assay using several statistical programs. *Food Chemistry*, 138(1), 414-420.  
511 <https://doi.org/10.1016/j.foodchem.2012.11.001>
- 512 [15] Kandi Sridhar, Albert Linton Charles. (2019). In vitro antioxidant activity of Kyoho  
513 grape extracts in DPPH and ABTS assays: Estimation methods for EC50 using  
514 advanced statistical programs. *Food Chemistry* 275 (2019) 41–4942
- 515 [16] Wootton-Beard P.C., Moran A., Ryan L. (2011). Stability of the total antioxidant  
516 capacity and total polyphenol content of 23 commercially available vegetable juices  
517 before and after in vitro digestion measured by FRAP, DPPH, ABTS and Folin–  
518 Ciocalteu methods. *Food Research International* 44 (2011) 217–224
- 519 [17] Gouveia, S. C., & Castilho, P. C. (2013a). *Artemisia annua* L. : Essential oil and  
520 acetone extract composition and antioxidant capacity. *Industrial Crops and Products*,  
521 45, 170-181. <https://doi.org/10.1016/j.indcrop.2012.12.022>
- 522 [18] Iqbal, S., Younas, U., Chan, K. W., Zia-Ul-Haq, M., & Ismail, M. (2012). Chemical  
523 composition of artemisia annua l. Leaves and antioxidant potential of extracts as a  
524 function of extraction solvents. *Molecules*, 17(5), 6020–6032.  
525 <https://doi.org/10.3390/molecules17056020>
- 526 [19] Smith, C. A., Maille, G. O., Want, E. J., Qin, C., Trauger, S. A., Brandon, T. R.,  
527 Siuzdak, G. (2005). Metlin : A metabolite mass spectral database. *Therapeutic Drug*  
528 *Monitoring*, 27(6), 747-751. <https://doi.org/10.1097/01.ftd.0000179845.53213.39>
- 529 [20] Lang, G., Mayhudin, N. A., Mitova, M. I., Sun, L., van der Sar, S., Blunt, J. W.,  
530 Munro, M. H. G. (2008). Evolving trends in the dereplication of natural product  
531 extracts : New methodology for rapid, small-scale investigation of natural product  
532 extracts. *Journal of Natural Products*, 71(9), 1595-1599.  
533 <https://doi.org/10.1021/np8002222>
- 534 [21] Little, J. L., Williams, A. J., Pshenichnov, A., & Tkachenko, V. (2012).  
535 Identification of “known unknowns” utilizing accurate mass data and chemspider.  
536 *Journal of The American Society for Mass Spectrometry*, 23(1), 179-185.  
537 <https://doi.org/10.1007/s13361-011-0265-y>
- 538 [22] Marchal, L., Legrand, J., & Foucault, A. (2003). Centrifugal partition  
539 chromatography : A survey of its history, and our recent advances in the field. *The*  
540 *Chemical Record*, 3(3), 133-143. <https://doi.org/10.1002/tcr.10057>
- 541 [23] Lee SK, Mbwambo ZH, Chung H, Luyengi L, Gamez EJ, Mehta RG, Kinghorn  
542 AD, Pezzuto JM. Evaluation of the antioxidant potential of natural products. *Comb*  
543 *Chem High Throughput Screen*. 1998 Apr;1(1) 35-46. PMID: 10499128.

- 544 [24] Tagliazucchi, D., Verzelloni, E., Bertolini, D., & Conte, A. (2010). In vitro bio-  
545 accessibility and antioxidant activity of grape polyphenols. *Food Chemistry*, 120(2),  
546 599-606. <https://doi.org/10.1016/j.foodchem.2009.10.030>
- 547 [25] Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (Frap)  
548 as a measure of "antioxidant power": The frap assay. *Analytical Biochemistry*,  
549 239(1), 70-76. <https://doi.org/10.1006/abio.1996.0292>
- 550 [26] Apak, R., Güçlü, K., Özyürek, M., & Karademir, S. E. (2004). Novel total  
551 antioxidant capacity index for dietary polyphenols and vitamins c and e, using their  
552 cupric ion reducing capability in the presence of neocuproine: Cuprac method.  
553 *Journal of Agricultural and Food Chemistry*, 52(26), 7970-7981.  
554 <https://doi.org/10.1021/jf048741x>
- 555 [27] Dinis, T. C. P., Madeira, V. M. C., & Almeida, L. M. (1994). Action of phenolic  
556 derivatives (Acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of  
557 membrane lipid peroxidation and as peroxy radical scavengers. *Archives of*  
558 *Biochemistry and Biophysics*, 315(1), 161-169.  
559 <https://doi.org/10.1006/abbi.1994.1485>
- 560 [28] Allard, S., Allard, P., Morel, I., & Gicquel, T. (2019). Application of a molecular  
561 networking approach for clinical and forensic toxicology exemplified in three cases  
562 involving 3-MeO-PCP, doxylamine, and chlormequat. *Drug Testing and Analysis*,  
563 11(5), 669-677. <https://doi.org/10.1002/dta.2550>
- 564 [29] Wolfender, J.-L., Litaudon, M., Touboul, D., & Queiroz, E. F. (2019). Innovative  
565 omics-based approaches for prioritisation and targeted isolation of natural products –  
566 new strategies for drug discovery. *Natural Product Reports*, 36(6), 855-868.  
567 <https://doi.org/10.1039/C9NP00004F>
- 568 [30] Fox Ramos, A. E., Evanno, L., Poupon, E., Champy, P., Beniddir, M. A. (2019).  
569 Natural products targeting strategies involving molecular networking: different  
570 manners, one goal. *Natural Product Reports*, 36(7), 960-980.  
571 <https://doi.org/10.1039/C9NP00006B>
- 572 [31] Frank, A. M., Bandeira, N., Shen, Z., Tanner, S., Briggs, S. P., Smith, R. D., &  
573 Pevzner, P. A. (2008). Clustering millions of tandem mass spectra. *Journal of*  
574 *Proteome Research*, 7(1), 113-122. <https://doi.org/10.1021/pr070361e>
- 575 [32] Watrous, J., Roach, P., Alexandrov, T., Heath, B. S., Yang, J. Y., Kersten, R.  
576 D., Dorrestein, P. C. (2012). Mass spectral molecular networking of living microbial  
577 colonies. *Proceedings of the National Academy of Sciences*, 109(26), E1743-E1752.  
578 <https://doi.org/10.1073/pnas.1203689109>
- 579 [33] Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y.,  
580 Bandeira, N. (2016). Sharing and community curation of mass spectrometry data with  
581 Global Natural Products Social Molecular Networking. *Nature Biotechnology*, 34(8),  
582 828-837. <https://doi.org/10.1038/nbt.3597>
- 583 [34] Borges Bubols, G., da Rocha Vianna, D., Medina-Rejon, A., von Poser, G.,  
584 Maria Lamuela-Raventos, R., Lucia Eifler-Lima, V., & Cristina Garcia, S. (2013). The  
585 antioxidant activity of coumarins and flavonoids. *Mini Reviews in Medicinal*  
586 *Chemistry*, 13(3), 318-334. <https://doi.org/10.2174/138955713804999775>

- 587 [35] Ferreira, J. F. S., Luthria, D. L., Sasaki, T., & Heyerick, A. (2010). Flavonoids  
588 from artemisia annua l. As antioxidants and their potential synergism with artemisinin  
589 against malaria and cancer. *Molecules*, 15(5), 3135–3170.  
590 <https://doi.org/10.3390/molecules15053135>
- 591 [36] Chen, Z., Bertin, R., & Frolidi, G. (2013). EC50 estimation of antioxidant activity in  
592 DPPH assay using several statistical programs. *Food Chemistry*, 138, 414–420.
- 593 [37] Shalaby A., Sanaa M. M. Shanab. (2013). Comparison of DPPH and ABTS  
594 assays for determining antioxidant potential of water and methanol extracts of  
595 *Spirulina platensis*. *Indian journal of geo-marine sciences* 42(5): 556-564
- 596 [38] Tirzitis, G., & Bartosz, G. (2010). Determination of antiradical and antioxidant  
597 activity: Basic principles and new insights. *Acta Biochimica Polonica*, 57(2).  
598 [https://doi.org/10.18388/abp.2010\\_2386](https://doi.org/10.18388/abp.2010_2386)
- 599 [39] Mira, L., Tereza Fernandez, M., Santos, M., Rocha, R., Helena Florêncio, M., &  
600 Jennings, K. R. (2002). Interactions of flavonoids with iron and copper ions: A  
601 mechanism for their antioxidant activity. *Free Radical Research*, 36(11), 1199-1208.  
602 <https://doi.org/10.1080/1071576021000016463>
- 603 [40] Schymanski, E. L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H. P., &  
604 Hollender, J. (2014). Identifying small molecules via high resolution mass  
605 spectrometry: Communicating confidence. *Environmental Science & Technology*,  
606 48(4), 2097-2098. <https://doi.org/10.1021/es5002105>
- 607 [41] Wang, S., Cai, T., Liu, H., Yang, A., & Xing, J. (2019). Liquid chromatography  
608 tandem mass spectrometry assay for the simultaneous determination of three major  
609 flavonoids and their glucuronidated metabolites in rats after oral administration of  
610 *Artemisia annua* L. extract at a therapeutic ultra-low dose. *Journal of Separation  
611 Science*, 42(21), 3330–3339. <https://doi.org/10.1002/jssc.201900668>

612 Table 1: Annotated compounds on the Molecular Network of *Artemisia annua*  
 613 ethanolic extract

Measured <i>m/z</i> [M+H] <sup>+</sup>	Molecular formula [M]	Calculated <i>m/z</i> [M+H] <sup>+</sup>	Error (ppm)	Compound assignment	References	Cosine score
283.1540	C <sub>15</sub> H <sub>22</sub> O <sub>5</sub>	283.1540	0.1	Artemisinin	Standard	0.73
147.0439	C <sub>9</sub> H <sub>6</sub> O <sub>2</sub>	147.0441	1.2	Coumarin	[7]	-
331.0813	C <sub>17</sub> H <sub>14</sub> O <sub>7</sub>	331.0812	0.4	Circiliol	[7]	-
249.1485	C <sub>15</sub> H <sub>20</sub> O <sub>3</sub>	249.1485	0.0	Arteannuin acid	[7]	-
375.1074	C <sub>19</sub> H <sub>18</sub> O <sub>8</sub>	375.1074	0.1	Casticin	[7]	0.80
315.0865	C <sub>17</sub> H <sub>14</sub> O <sub>6</sub>	315.0863	0.5	Cisimaritin	[7]	-
235.1699	C <sub>15</sub> H <sub>22</sub> O <sub>2</sub>	235.1693	2.7	Artemisinic acid	[7]	0.77
389.1236	C <sub>20</sub> H <sub>20</sub> O <sub>8</sub>	389.1231	0.5	Artemetin	[7]	-
359.1126	C <sub>19</sub> H <sub>18</sub> O <sub>7</sub>	359.1125	0.3	Retusin	[7]	-
361.0915	C <sub>18</sub> H <sub>16</sub> O <sub>8</sub>	361.0915	0.1	chrysosplenol D	[7]	0.89

614

615



616 Table 2: Activity of the crude extract and the 13 CPC fractions at 0.5 mg/mL evaluated on  
 617 DPPH, ABTS, FRAP, CUPRAC and Iron chelation assays

	DPPH % radical inhibition	ABTS % radical inhibition	FRAP % reduction capacity	CUPRAC % reduction capacity	Iron Chelation % chelation capacity
<i>Artemisia annua</i> extract	64.13±0.8*	29.9±0.6*	22.2±0.7*	12.35±0.2*	100.7±0.8**
Fraction 1	24.8±0.5*	15.6±0.3*	12.6±0.5*	5.0±0.3*	74.7±0.6*
Fraction 2	76.3±0.8*	20.5±0.4*	25.9±0.7*	10.6±0.3*	100.7±0.8**
Fraction 3	19.3±0.3*	6.5±0.1	7.8±0.3	2.9±0.1	53.3±0.6
Fraction 4	5.5±0.1	4.2±0.4	7.2±0.1	3.2±0.1	55.0±0.3
Fraction 5	5.3±0.3	3.0±0.6	7.5±0.1	4.4±0.2	55.1±0.5
Fraction 6	5.3±0.1	3.1±0.4	6.5±0.1	2.4±0.1	47.5±0.2*
Fraction 7	1.7±0.1	3.3±0.2	5.0±0.1	1.6±0.05	35.9±0.2*
Fraction 8	4.2±0.2	1.4±0.3	4.6±0.2	1.6±0.05	52.2±0.8
Fraction 9	1.6±0.1	1.6±0.3	4.4±0.1	1.6±0.05	51.4±0.8
Fraction 10	2.3±0.1	0.2±0.05	4.6±0.1	1.6±0.1	52.52±0.4
Fraction 11	4.6±0.1	3.9±0.1	5.4±0.2	2.0±0.05	58.92±0.8
Fraction 12	5.5±0.2	3.0±0.1	5.2±0.1	1.7±0.05	57.3±0.6
Fraction 13	3.8±0.3	1.8±0.1	4.9±0.1	1.6±0.05	55.2±0.7

618

619 \* denotes significantly different (ANOVA  $p$ -value < 0.05)

620 \*\* denotes significantly different from the other but not significantly different between them

621

622 Figure Captions

623 Figure 1: Base Peak Chromatogram (BPC) of *Artemisia annua* ethanolic extract  
624 obtained using UHPLC-HRMS in positive ionization mode (ESI+). The separation  
625 was performed at 60°C on a Fortis C18 column (150 x 2.1 mm; 1.7 µm) with gradient  
626 elution of water and acetonitrile/isopropanol (50/50) both acidified with 0.1% of formic  
627 acid.

628 Figure 2: Molecular Network of *Artemisia annua* crude extract obtained using GNPS  
629 platform and visualized with Cytoscape 3.6.1 software.

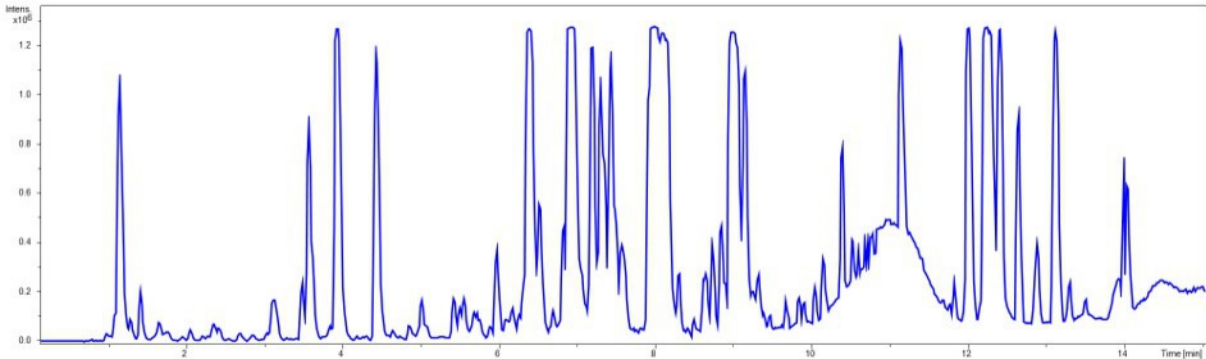
630 Figure 3: Antioxidant activity results of *Artemisia annua* extract with its CPC fractions.

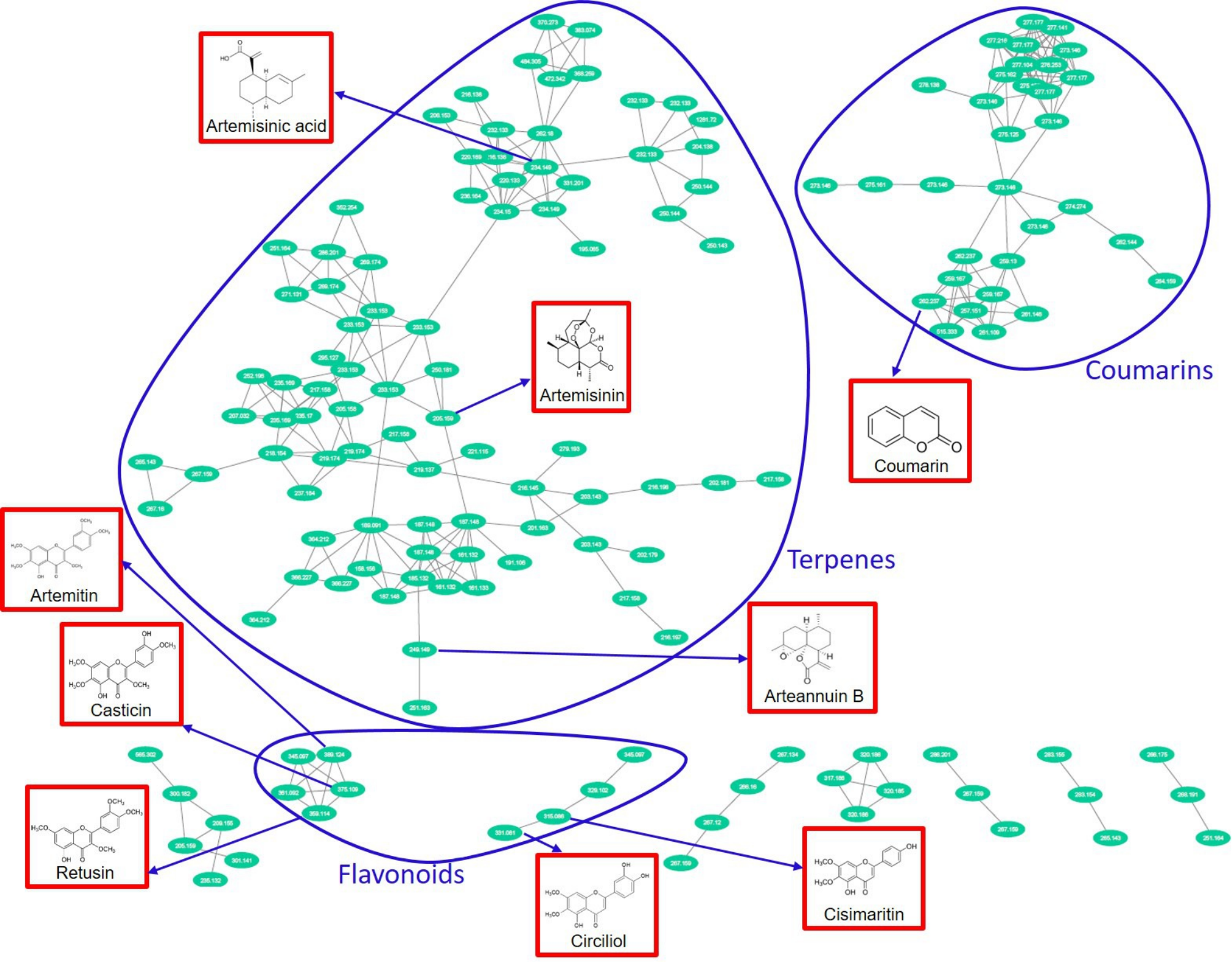
631 Figure 4: (A) Focusing in the Molecular Network on the ion of interest contained in  
632 the active CPC Fraction 2. (B) The UV chromatogram of fraction 2 at  $\lambda = 280$  nm, the  
633 separation was performed on a Fortis C18 column (150 x 2.1 mm; 1.7 µm) at 60°C  
634 using water and acetonitrile/isopropanol (50/50) both acidified with 0.1% formic acid,  
635 the flow was 300 µL/min. (C) FIA-HRMS mass spectrum of active CPC Fraction 2 of  
636 *Artemisia annua* crude extract. (D) Proposed structure for the main compound of  
637 Fraction 2.

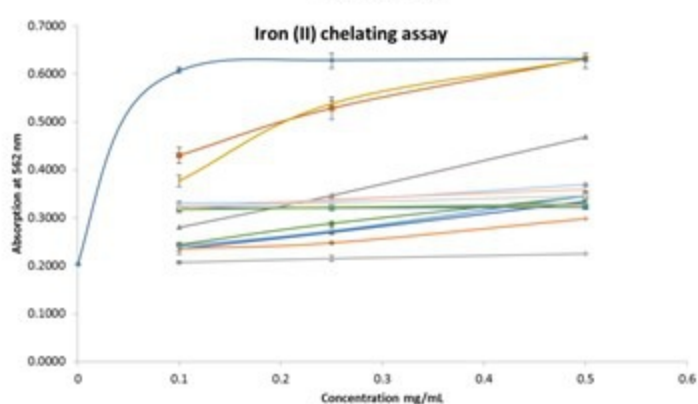
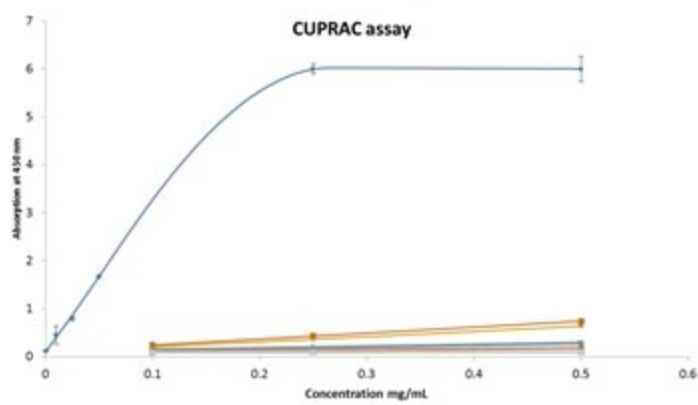
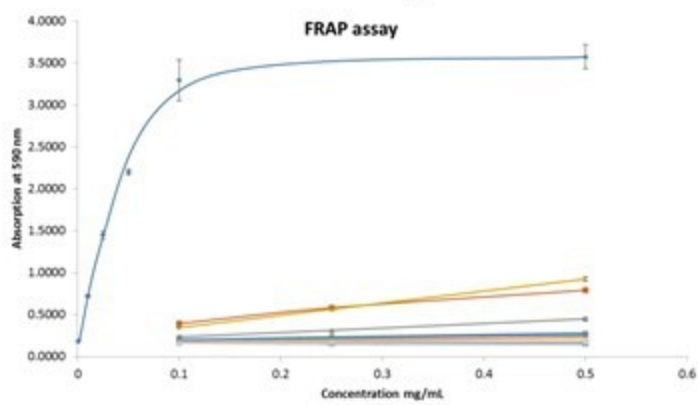
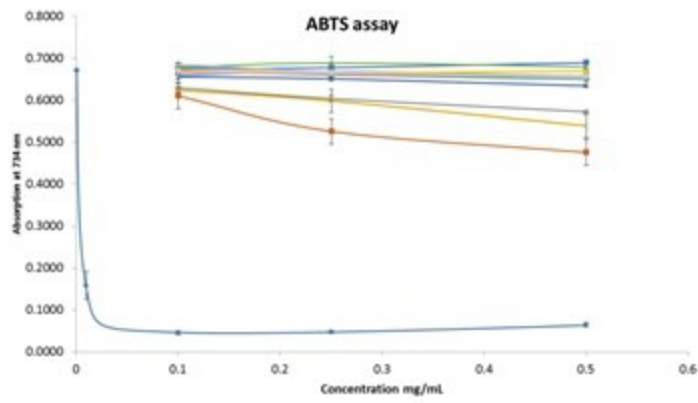
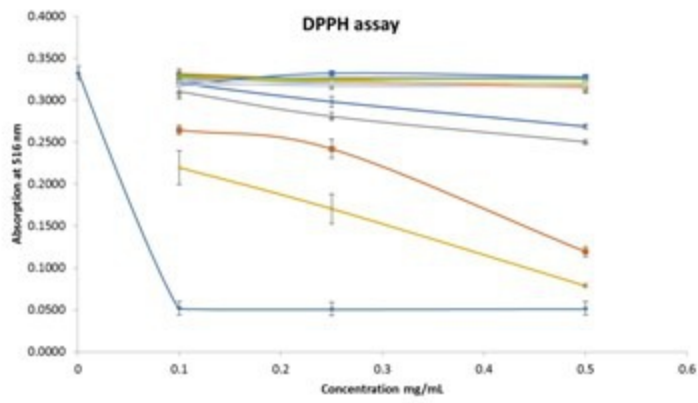
638

639

640







- Trolox
- Artemisia annua extract
- Fraction 1
- Fraction 2
- Fraction 3
- Fraction 4
- Fraction 5
- Fraction 6
- Fraction 7
- Fraction 8
- Fraction 9
- Fraction 10
- Fraction 11
- Fraction 12
- Fraction 13

