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1 **Coffee silverskin extracts: quantification of 30 bioactive compounds by a new HPLC-**
2 **MS/MS method and evaluation of their antioxidant and antibacterial activities.**
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26 **Abstract**

27 The research of value-added applications for coffee silverskin (CSS) requires studies to
28 investigate potential bioactive compounds and biological activities in CSS extracts. In this
29 study, different ultrasound-assisted extraction (UAE) methods have been tested to extract
30 bioactive compounds from CSS. The obtained extracts, were characterized using a new HPLC-
31 MS/MS method to detect and quantify 30 bioactive compounds of 2 classes: alkaloids and
32 polyphenols (including phenolic acids, flavonoids, and secoiridoids). CSS extracts obtained
33 with ethanol/water (70:30) as extraction solvent showed the highest levels ($p \leq 0.05$) of
34 bioactive compounds (4.01 ± 0.34 % w/w). High content of caffeine was observed with levels
35 varying from 1.00% to 3.59% of dry weight of extract (dw). 18 phenolic compounds were
36 detected in CSS extracts with caffeoylquinic acids (3-CQA, 5-CQA and 3,5-diCQA) as the
37 most abundant polyphenols (3115.6 µg g^{-1} to 5444.0 µg g^{-1}). This study is also one of the first
38 to characterize in-depth flavonoids in CSS revealing the levels of different flavonoids
39 compounds such as rutin (1.63 – 8.70 µg g^{-1}), quercetin (1.53 – 2.46 µg g^{-1}), kaempferol (0.76
40 – 1.66 µg g^{-1}) and quercitrin (0.15 – 0.51 µg g^{-1}). Neuroprotective activity of silverskin extracts
41 against H_2O_2-induced damage was evaluated for the first time suggesting for methanol and
42 ethanol/water (70:30) extracts a potential role as protective agents against neurodegeneration
43 due to their ability to counteract oxidative stress and maintain cell viability. Silverskin extracts
44 were not inhibiting the growth of anyone of the bacterial species included in this study but data
45 obtained by water extract might deserve a deeper future investigation on biofilm-related
46 activities, such as quorum sensing or virulence factors' expression. From their composition and
47 their evidenced biological activities, CSS extracts could represent valuable ingredients in
48 nutraceutical formulations.

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53 **Keywords:** Coffee silverskin, coffee by-product, polyphenols, caffeine, Caffeoylquinic acids,
54 flavonoids, value-added applications**,** antioxidant activity, antimicrobial activity, antibiofilm
55 formation

56

57 **1. Introduction**

58 Coffee is one of the most consumed beverages in the world with a global consumption of 161
59 million of 60 kg coffee bags per year. As consequences, millions of tons of coffee by-products
60 such as spent coffee ground (SCG) and coffee silverskin (CSS) are produced each year.
61 Silverskin, the thin tegument covering the coffee bean, is a by-product of green coffee roasting
62 procedure. Indeed, from eight tons of coffee roasted, around 60 kg of CSS are produced (Alves,
63 Rodrigues, Nunes, Vinha, & Oliveira, 2017). Therefore, with the increasing coffee production
64 and the environmental impact of waste accumulation, CSS disposal needs to be properly
65 managed (Janissen & Huynh, 2018). That is why in recent years, a lot of research has been
66 carried out in order to find newer CSS applications. Various value adding applications have
67 been proposed for CSS such as feedstock for biofuel production and adsorbent material to
68 remove potential toxic metals in water (Hijosa-Valsero et al., 2018, Malara et al., 2018).
69 Another promising approach is the use of CSS as raw material for the recovery of functional
70 compounds of potential interest. Indeed, CSS is a good source of insoluble and soluble dietary
71 fibers (around 50% and 15% respectively) which can be used for food-products enrichment
72 (Iriondo-DeHond et al., 2019). Moreover, recent studies have evidenced that CSS is a rich
73 source of bioactive compounds such as melanoidins, caffeine and polyphenols which allow
74 potentials applications of CSS extracts as functional ingredient in cosmetic (Bessada, Alves,
75 Oliveira, & Beatriz, 2018) and nutraceutical (Bertolino et al., 2019) formulations. These
76 applications require a preliminary separation of bioactive molecules from CSS and thus, it is
77 important to develop simple and efficient extraction methods in order to obtain CSS extracts
78 with high levels of bioactive compounds. Different approaches have been proposed for CSS
79 extracts preparation such as solid–liquid extraction (Iriondo-DeHond et al., 2019),
80 hydrothermal extraction (Conde & Mussatto, 2016), pulsed electric field assisted extraction
81 (Barbosa-Pereira, Guglielmetti, & Zeppa, 2018), microwave assisted extraction and ultrasound
82 assisted extraction (UAE) (Guglielmetti, 2017). Among these different extraction techniques,
83 UAE has shown the advantage of being sustainable, efficient and economically viable (Chemat
84 et al., 2017, Wen et al., 2019). However, regarding CSS extraction, no study has been yet
85 performed in order to compare the effect of solvents during UAE on the composition and
86 bioactivities of CSS extracts. Previous analytical studies performed on CSS extracts mainly
87 focused on caffeine, total phenolic content and chlorogenic acids quantification (Conde and
88 Mussatto, 2016, Costa et al., 2018, Panusa et al., 2017). However, despite the growing interest
89 in CSS, deep characterization of the minor bioactive compounds in CSS is lacking. Indeed,

beyond chlorogenic acids, other classes of polyphenols such as flavonoids (flavonols, flavan-3-ols, and anthocyanins), stilbenes, secoiridoids or xanthones have been poorly assessed in CSS. Moreover, to our knowledge, research of minor alkaloids such as quinine doihas never been performed in CSS. The innovation of this study also lies in the analytical method developed for the analysis of CSS. Indeed, high-performance liquid chromatography (HPLC) coupled to diode array detector (DAD) has been widely used for the quantification of polyphenols in coffee by-products (Regazzoni et al., 2016). In addition, some studies have analyzed some classes of bioactive compounds in CSS extracts using more sophisticated detection systems such as tandem mass spectrometry (HPLC-MS/MS) (Fernandez-Gomez et al., 2016, Panusa et al., 2017). However, to our knowledge, no HPLC-MS/MS method is reported to analyze contemporaneously in coffee, 30 bioactive compounds of different subclasses, including alkaloids, chlorogenic acids, phenolic acids, flavonoids, xanthones and secoiridoids. In this context, the main aim of this work was the development of effective UAE methodologies with different solvents for the extraction of bioactive compounds from CSS. Obtained extracts were characterized by the developed and validated HPLC-MS/MS method. Another objective of this work was the evaluation, for the first time, of the neuroprotective activity against H2O2-induced damage together with the antimicrobial and anti-biofilm activities of the CSS extracts. Oxidative stress is an imbalance between reactive oxygen species (ROS) production and endogenous antioxidant defenses and it is involved in several chronic degenerative diseases (cardiovascular diseases, cancer and neurodegeneration). Neurodegenerative disorders are becoming a primary health problem with the rapid increase of the aging population. Thus, the identification of effective phytochemicals against neurodegeneration, like CSS polyphenols, could be of great importance. From antimicrobial point of view, the continuous emergence and spread of bacteria resistant to antibiotics has become one of the most severe threats to human health. Therefore the search for new compounds with antimicrobial activity has become urgent. Among these, there are extracts from natural sources, which have attracted great attention by the scientific community. Moreover, microorganisms preferentially grow as biofilms. They are mono- or multi-species complex and structured communities attached to an inert or living surface and embedded into a self produced extracellular matrix. They are characterized by intrinsic emergent properties representing an advantage for the microorganism over the mode of growth in a liquid suspension as single cellular entities (Flemming et al., 2016). Biofilms from pathogenic bacteria, for instance, may form onto different surfaces of the human host such as the skin, respiratory tract, urinary tract and the gastrointestinal tract mucosae. They are recognized as an

124 important factor contributing to persistence of infections due to the peculiar physiology of the
125 composing microorganisms. Moreover, the microorganisms into a biofilm are highly tolerant
126 to several antimicrobials with MIC values 100 to 1000 times higher than those measurable
127 against planktonic cells from the same species (Macia, Molinero, & Oliver, 2014). For this
128 reason, searching new molecules or strategies to combat biofilms has gained a lot of interest in
129 the last twenty years and plants/food extracts rich in bioactive compounds such as polyphenols
130 could represent an interesting alternative. The results shown in this study represent a clear
131 evidence of potential value-added applications of CSS extracts as valuable ingredients in
132 nutraceutical formulations.
133

134 **2. Materials and methods.**

135 **2.1. Reagents and standards**

136 Analytical standards of the 30 bioactive compounds were supplied by Sigma -Aldrich (Milan,
137 Italy). HPLC-grade formic acid 99–100% was purchased from J.T. Baker B.V. (Deventer,
138 Holland) while Hydrochloric acid (HCl) 37% and HPLC-grade ethanol (EtOH) and methanol
139 (MeOH) were supplied by Carlo Erba (Milan, Italy). Deionized water was obtained from a
140 Milli-Q Reagent Water System (Bedford, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-
141 2,5diphenyl-tetrazolium bromide (MTT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-
142 DA), H_2O_2, dimethyl sulfoxide (DMSO), Phosphate Buffered Saline (PBS), bovine serum
143 albumine (BSA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS),
144 penicillin/streptomycin, all trans retinoic acid (RA) and all other chemicals of the highest
145 analytical grade were purchased from Sigma Chemical (Milan, Italy).

146 **2.2. Coffee silverskin preparation**

147 Coffee silverskin was obtained from the roasting of green C. *arabica* beans of Ethiopian origin
148 (supplied by Gardelli specialty coffees, Forli, Italy). The roasting process was carried out during
149 9 min at a maximum temperature of 195°C using an Ikawa coffee roaster (IKAWA Ltd,
150 London, UK). The removed silverskin was then ground under nitrogen in a fine powder and
151 was stored at 4˚C until extraction.

152 **2.3. Coffee silverskin extraction**

153 The extraction of the bioactive compounds was based on extraction methods optimized by
154 Caprioli, Nzekoue, Giusti, Vittori, and Sagratini (2018) with slight modifications. Eight
155 extraction procedures were tested in order to determine the best extraction methods for the 30
156 bioactive compounds in CSS (Table 1S). Briefly, 10 g of CSS powder was sonicated with 50
157 mL of solvent using a FALC ultrasonic bath (FALC, Treviglio, Italy) at a frequency of 40 kHz
158 for 120 min at 20 °C. After the extraction, the sample was filtrated with a filter paper and the
159 obtained extract was collected, lyophilized and stored in darkness at a temperature of −20 °C
160 until analysis. Among the different extraction procedures tested, four different extracts were
161 chosen according to recoveries and extraction yields and finally considered for further studies:
162 MeOH extract (E1), water extract (E2), MeOH/water (50:50) extract (E3) and EtOH/water
163 (70:30) extract (E4). Before analyses, 5 mg of lyophilized extract were dissolved in 5 mL of
164 MeOH (1 mg mL−1) sonicated for 10 min. For HPLC analyses, aliquots of the solutions were
165 filtrated with 0.2 μm pore size filter and then injected in HPLC-MS/MS.
166
167 **2.4. HPLC-MS/MS analyses**

168 The analysis of the bioactive compounds has been carried out using an Agilent 1290 Infinity
169 series coupled to a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA, USA)
170 equipped with an electrospray (ESI) source operating in negative and positive ionization modes.
171 Their separation was performed using a Kinetex PFP analytical column (100 mm × 2.1 mm i.d.,
172 particle size 2.6 μm) from Phenomenex (Torrance, CA, USA). The mobile phase was a mixture
173 of water (A) and methanol (B) both with formic acid 0.1% at a flow rate of 0.2 mL min−1 in
174 gradient elution mode. The composition of the mobile phase varied as follows: 0–2 min, 20%
175 B; 2–15 min, 80% B; 15–18 min, 80% B; 18–23 min, 100% B, 23–35 min, 20% B. The volume
176 of injection was 2 μL. The temperature of the column was 30 °C while the temperature of the
177 drying gas in the ionization source was 350 °C. The nebulizer pressure was 25 psi, the gas flow
178 was 10 L min−1, and the capillary voltage was 4000 V. Detection was performed in dynamic
179 "multiple reaction monitoring" (Dynamic-MRM) mode monitoring specific precursor/product
180 ions transitions for each analyte (Table 2S).
181
182 **2.5. Spectrophotometric analyses**

183 **2.5.1. Total phenolic content (TPC) and total flavonoid content (TFC) determination**

184 The TPC was determined spectrophotometrically according to Siatka and Kašparová (2010)
185 method with some modifications. Briefly, 0.5 mL of extracts solution (1 mg mL−1 in methanol)
186 was introduced into test tubes, then 2.5 mL of Folin–Denis reagent solution and 7 mL of
187 Na2CO3 (7.5% w/w in water) solution were added. The reaction mixture allowed to stand at
188 room temperature in the dark for 2 h and absorption was measured at 765 nm. The TPC
189 quantification in the extracts was made using gallic acid calibration curve and was expressed
190 as mg of gallic acid equivalents (GAE) per g of dry weight of CSS extract.
191 The TFC of the different extract were evaluated following a method described by Chen, Chen,
192 Xiao, and Fu (2018) with slight variations. In a 15 mL test tube, 0.5 mL of extract solution (1
193 mg mL−1), 0.15 mL of NaNO2 (0.5 M), 3.2 mL of methanol (30% V/V) and 0.15 mL of
194 AlCl3·6H2O (0.3 M) were mixed. After 5 min, 1 mL of NaOH (1 M) was added. The solution
195 was mixed well and the absorbance was measured against the blank reagent at 506 nm. The
196 standard calibration curve for TFC was made using rutin standard solution (0 to 100 mg l−1)
197 under the same procedure as described above. TFC was expressed as mg of rutin equivalents
198 (RE) per g of dried extract.
199

200 **2.5.2. DPPH radical scavenging activity**

201 The ability of the extracts to scavenge the radical 2,2-diphenyl-1-picrydrazyl (DPPH) was
202 investigated spectrophotometrically according to the method proposed by Venditti et al (2017)
203 with modifications. Briefly, in a 15 mL test tube, 0.5 mL of extract solution (1 mg mL^{-1} in
204 methanol) was mixed with 4.5 mL of ethanolic solution of DPPH (0.1 mM). After 30 min of
205 incubation in the dark at room temperature, the DPPH disappearance was measured
206 spectrophotometrically measuring the absorption at 517 nm. The percentage of DPPH
207 scavenging was calculated following the formula: $\% I = [(A_{control} - A_{sample})/A_{control}] \times 100$.
208 Where $A_{control}$ and A_{sample} represent the absorbance obtained without and with antioxidants
209 respectively. The scavenging activity was expressed as the IC_{50} value (μg mL^{-1}), which is the
210 concentration of the extract necessary to cause 50% of DPPH inhibition. The IC_{50} value was
211 obtained by interpolation from the linear regression analysis. Trolox® was used as the reference
212 antioxidant (1 – 50 μg mL^{-1}).

213 **2.6. Biological analysis**

214 **2.6.1. Cell culture and treatment**

215 The SH-SY5Y human neuroblastoma cell line was obtained from Sigma-Aldrich (cat. n°
216 94030304) (St. Louis, MO, USA). Cells were grown in culture medium, composed of high
217 glucose DMEM supplemented with 10% (V/V) of FBS, 2 mM L-Glutamine, 50 U mL^{-1} of
218 penicillin, and 50 µg mL^{-1} of streptomycin and maintained at 37 °C in a humidified incubator
219 with 5% CO_2, as previously reported (Giusti et al., 2018). Cell differentiation was induced with
220 10 µM retinoic acid (RA) and 1% FBS for seven days prior treatments (Lopes, 2010). The 4
221 silverskin extracts have been solubilized in MeOH extract (E1), water extract (E2),
222 MeOH/water extract (E3) and EtOH/water extract (E4) and 10 mg mL^{-1} stocks were kept at
223 −20 °C until use. Differentiated SH-SY5Y were treated with 1, 10, 50, 100, and 200 µg mL^{-1}
224 of the 4 extracts for 24 h. The controls were prepared adding the highest volume of the
225 respective vehicle. At this concentration the vehicles did not influence cell viability (data not
226 shown). Oxidative stress was induced, as previously reported (Giusti et al., 2018) exposing cells
227 to 700 µM H_2O_2 in 1% FBS DMEM.

228 **2.6.2. MTT viability test**

229 Cell viability was evaluated by measuring MTT reduction as previously reported (Angeloni et
230 al., 2015). Briefly, at the end of each experiments, MTT was added to the medium at the
231 concentration 0.5 mg mL^{-1} and incubated for 90 min at 37 °C. After incubation, MTT solutions
232 were removed, DMSO was added, and the absorbance was recorded at λ = 595 nm using a
233 microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA,
234 USA). Data are expressed as % in respect to control cells. Control cells are considered as 100%
235 cell viability.

236

237 **2.6.3. Intracellular ROS measurement**

238 The formation of intracellular reactive oxygen species (ROS) was evaluated using the
239 fluorescent DCFH-DA probe as previously reported (Marrazzo et al., 2018). Briefly, at the end
240 of each experiments, SH-SY5Y cells were incubated with 10 μM DCFH-DA in DMEM 1%
241 FBS w/o phenol red for 30 min. After removal of DCFH-DA, cells were incubated with 400
242 μM H2O2 in DMEM 1% FBS w/o phenol red for 15 min. Then, H2O2 was removed and
243 replaced by PBS. Cells fluorescence was measured using 485 nm excitation and 535 nm
244 emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter,
245 PerkinElmer). Data are expressed as % in respect to H2O2 treated cells. Peroxide treated cells
246 are considered as 100% ROS production.

247 **2.7. Antibacterial activity**

248 **2.7.1. Susceptibility testing**

249 Maintenance, cultivation and antimicrobial susceptibility testing were carried out following
250 international guidelines used for testing antibiotics as per the European Committee for
251 Antimicrobial Susceptibility Testing - EUCAST (http://www.eucast.org/ast_of_bacteria/).
252 Reference bacterial strains used in the study were *Staphylococcus aureus* ATCC 6538,
253 *Pseudomonas aeruginosa* ATCC 9027, and *Escherichia coli* ATCC 25922. The extracts were
254 dissolved in 1:1 H_2O/DMSO at a stock concentration of 4.096 mg mL^{-1}. A DMSO concentration
255 up to 8% was not toxic to the bacterial species considered and in the experimental conditions
256 used.

257 **2.7.2. Anti-biofilm activity**

258 The effect of the coffee silverskin extracts on the ability of the selected bacterial strains to form
259 a mature biofilm was evaluated by measuring the biofilm biomass accumulated on the surface
260 of polystyrene 96-well tissue culture treated cluster microtiter plates (Corning Inc., NY, USA).
261 The biomass was quantified by the crystal violet method, with minor modifications. Briefly,
262 two different media were used in independent experiments: Tryptone Soya Broth (TSB)

263 supplemented with 1% (w/v) glucose and Muller-Hinton II broth (Oxoid,). A two-fold serial
264 dilution of the extracts was set in medium (concentration range 1.024 – 0.5 mg L^{-1}) and
265 inoculated with 100 µL of a 1:100 dilution of a starting bacterial suspension prepared at 0.5
266 McFarland units. Biomass was evaluated after 24 h of incubation by removal of supernatant
267 and gently washing with distilled water. Cells in the biofilm were stained with a 0.01% crystal
268 violet water solution, left at room temperature for 15 min, washed three times with distilled
269 water and incubated at 37°C to dryness. Two-hundred µL of 95% ethanol was then added to the
270 wells and shaken for 10 min. 125 µL of the solution of each well were transferred in a new
271 microtiter plate and the optical densities (OD) determined using ELISA microplate reader at
272 540nm wavelength. The test was performed in duplicate.

273 **2.8. Statistical analysis**

274 All the analyses were performed in triplicate (n=3) and values were expressed as mean ±
275 standard deviation. The statistical significance of the differences between the extracts was
276 determined using Student's t-test. Differences at the level $p < 0.05$ were considered significant.
277 In experiments with SH-SY5Y cell cultures, one-way ANOVA was used to compare
278 differences among groups followed by Dunnett's or Bonferroni's test (Prism 5; GraphPad
279 Software, San Diego, CA). Differences at the level $p < 0.05$ were considered significant.

280 **3. Results and discussions**

281 **3.1. Validation of the HPLC-MS/MS method.**

282 The analytical method was validated by investigating the linearity, reproducibility and the
283 sensitivity of the method for all the bioactive compounds considered in dynamic MRM mode
284 (Table 1). The linearity of the analytical method was assessed by injecting standard solutions
285 of different concentration ranges from 0.001 µg mL−1 to 5 µg mL−1 in order to build analytical
286 curves of 8 points for each analyte. The coefficients of correlation obtained ranged from 0.993
287 to 1 confirming thus the high linearity of the method (Table 1). The reproducibility of the

288 HPLC-MS/MS method was evaluated by determining the relative standard deviations (% 289 RSDs) after three replicated injections of mix standard solution (0.5 μg mL−1) on the same day 290 (intraday precision) and on three consecutive days (interday precision). The intraday precision 291 ranged from 0.4% to 7.6%, while the intraday precision was between 4.7% and 15.2% for all 292 the targeted compounds. The sensitivity of the analytical method was validated by assessing 293 the limits of detection (LODs) and the limits of quantification (LOQs) of each monitored 294 bioactive compound. After the injections of standard solutions of known concentrations, the 295 signal to noise ratios (S/N) were calculated. The LODs and LOQs were estimated as the 296 concentrations of analytes giving the S/N of 3:1 and 10:1 respectively. The LODs obtained 297 ranged from 0.0003 μg mL−1 to 0.05 μg mL−1, while the LOQs were between 0.001 μg mL−1 298 and 0.2 μg mL−1 (Table 1).

299 **Table 1**. HPLC-MS/MS method validation parameters: regression equation, linearity (R^2). limits of detection (LODs), limits of quantification
300 (LOQs) and reproducibility for the thirty monitored compounds.

Compounds	Concentration range (µg ml^{-1})	Regression Equation	R^2	LOQs[a]	LODs[b]	Reproducibility (%RSD)	
						Intraday	Interday
Shikimic acid	0.05-5	y = 7937.8x +736.11	0.994	0.1	0.03	3.4	9.9
Gallic acid	0.01-5	y = 13243x - 1329.5	0.993	0.05	0.02	2.1	6.8
Loganic Acid	0.01-5	y = 6662x + 48.058	1	0.01	0.003	5.1	9.7
5-caffeylquinic acid	0.01-5	y = 16814x - 1057.5	0.997	0.01	0.003	3.6	8.6
Swertiamarin	0.005-5	y = 7685.1x + 15.302	1	0.005	0.002	3.0	7.7
Gentiopicroside	0.05-5	y = 1706.4x - 1.6666	1	0.2	0.05	5.3	8.6
(+) Catechin	0.01-5	y = 6481.3x + 31.934	1	0.01	0.003	5.6	7.6
3-caffeylquinic acid	0.1-5	y = 4483.2x - 433.35	0.998	0.1	0.03	4.1	7.8
Sweroside	0.01-5	y = 2109.5x + 6.4729	1	0.01	0.003	2.1	6.8
Chlorogenic acid	0.005-5	y = 29047x - 610.29	0.999	0.005	0.002	2.7	6.6
Caffeine	0.005-5	y = 135892x + 1311.1	1	0.004	0.001	0.4	4.0
Cyanidin 3-glucoside	0.005-5	y = 241096x - 15411	0.997	0.004	0.001	1.0	9.3
Vanillic acid	0.05-5	y = 857.52x - 19.701	0.999	0.05	0.02	5.6	14.2
Caffeic acid	0.01-5	y = 23436x - 537.21	0.999	0.01	0.003	2.2	13.0
(-)Epicatechin	0.01-5	y = 6524.8x + 151.77	1	0.01	0.003	2.6	4.7
Syringic acid	0.005-5	y = 2125.6x - 154.2	0.995	0.005	0.002	6.2	15.2
P-coumaric acid	0.005-5	y = 28093x - 2.4157	0.999	0.005	0.002	3.1	10.3
Ferulic acid	0.01-5	y = 5853.5x - 113.69	0.999	0.01	0.003	6.0	13.5
3.5-Dicaffeoylquinic acid	0.005-5	y = 22621x - 1430.1	0.996	0.005	0.002	1.4	10.6
Quinine	0.001-5	y = 129606x - 3926.8	0.998	0.001	0.0003	3.0	13.8
Naringin	0.001-5	y = 9379.7x + 110.96	1	0.001	0.0003	5.1	7.3

Rutin	0.001-5	y = 15411x - 532.59	0.998	0.001	0.0003	3.3	8.9
Hyperoside	0.001-5	y = 31512x + 325.65	1	0.001	0.0003	2.3	9.8
Trans-Cinnamic acid	0.005-5	y = 54199x + 1479.1	0.999	0.05	0.02	4.3	1.0
Resveratrol	0.005-5	y = 11091x + 297.04	1	0.005	0.002	4.4	14.6
Amarogentin	0.001-5	y = 26116x + 212.77	0.999	0.001	0.0003	4.2	13.9
Quercitrin	0.001-5	y = 24198x + 468.93	0.999	0.001	0.0003	1.8	15.0
Kaempferol	0.001-5	y = 24224x + 500.82	0.999	0.001	0.0003	1.8	8.5
Quercetin	0.005-5	y = 19350x + 913.34	0.997	0.005	0.002	1.9	7.2
Isogentisin	0.001-5	y = 175952x + 19759	0.993	0.001	0.0003	7.6	12.7

301

302 [a] LOD (limit of detection) = 3 × signal-to-noise (S/N) ratio. [b] LOQ (limit of quantification) = 10 × signal-to-noise (S/N) ratio.

303 **3.2. Determination of bioactive compounds in CSS extracts by HPLC-MS/MS analyses.**

304 8 extraction procedures were tested to optimize the extraction of the 30 targeted compounds
305 (Table 1S). The optimized HPLC-MS/MS method was used to analyze the different extracts.
306 From these experiments, four ultrasound-assisted extraction methods with different extraction
307 solvents (MeOH, water, MeOH/water 50:50, EtOH/water 70:30) were selected considering not
308 only the extraction yields, the effectiveness of compounds extraction and recoveries but also
309 the potentials nutraceutical applications of the extracts. The extraction yields of the four extarcts
310 were respectively of 4.4%, 4.3%, 3.7% and 2.86% for MeOH, EtOH/water 70:30, MeOH/water
311 50:50 and water, expressed as w/w dry weight. According to the effectiveness of compounds
312 extraction, the four solvents have been chosen among all, also because the provided the highest
313 total bioactive compounds concentration respectively of 745.4 mg kg−1, 725.19 mg kg−1,
314 724.17 mg kg−1, and 714.26 mg kg−1, for EtOH/water 70:30, MeOH, MeOH/water 50:50 and
315 water. Interesting, the total concentration of bioactive compounds extracted through UAE with
316 water (714.26 mg kg−1) was higher with respect to non UAE (661 mg kg−1) treatment (method
317 8 of Table 1S). The mean recoveries obtained by extracting the sample with ethanol:water
318 (70:30) for the 30 bioactive compounds by spiking the sample at 0.25 and 25 mg kg−1 were
319 from 88.9 to 100.3%, with RSDs lower than 11.1% (Table 3S). The mean recoveries obtained
320 spiking the sample at 0.5 and 50 (only for caffeine and chlorogenic acids) kg L−1 were from
321 89.1 to 102.4%, with RSDs lower than 8.4% (Table 3S). Also matrix effect has been assessed
322 through matrix-matched-calibration curve and it was negligible for all compounds (Data not
323 shown).

324 The Table 2 reports the concentrations (expressed as μg g−1 of dry weight extract) of the 30
325 bioactive compounds in the 4 different extracts: E1 (MeOH extract), E2 (water extract), E3
326 (MeOH/water extract) and E4 (EtOH/water extract). 20 of the 30 monitored compounds were
327 revealed in the analyzed extracts. As an example we reported an HPLC-MS/MS chromatogram

328 of a standard mixture of the 30 bioactive compounds (Fig. 1) and a chromatogram of CSS

329 extract (Fig. 1S). The difference of levels could be due to the polarities of the different solvents

330 tested and their preferential interaction with specific classes of compounds.

331

332 **Table 1.** HPLC-MS/MS method validation parameters: regression equation, linearity (R2).

333 limits of detection (LODs), limits of quantification (LOQs) and reproducibility for the thirty

334 monitored compounds.

n.	Compounds	E1 (MeOH)	E2 (H_2O)	E3 (MeOH: H_2O)	E4 (EtOH: H_2O)
	ALKALOIDS				
1	Caffeine	10010.22 ± 389.40	19599.04 ± 1842.31	25176.74 ± 1072.53	35879.16 ± 3236.30
2	Quinine	0.23 ± 0.01	0.49 ± 0.02	0.43 ± 0.04	0.61 ± 0.03
	POLYPHENOLS				
	Phenolic acids				
3	3,5-diCQA	43.79 ± 2.04	47.82 ± 3.93	145.9 ± 4.00	201.08 ± 2.03
4	3-CQA	3390.85 ± 221.08	2748.70 ± 83.56	4014.6 ± 307.52	2725.61 ± 57.24
5	5-CQA	2009.35 ± 153.92	319.08 ± 6.70	396.51 ± 14.87	388.39 ± 0.66
6	Caffeic Acid	n.d	79.00 ± 0.13	112.65 ± 16.36	212.38 ± 27.86
7	Ferulic Acid	n.d	66.52 ± 3.09	104.64 ± 8.60	226.23 ± 6.20
8	Gallic Acid	16.59 ± 1.41	24.36 ± 1.59	31.07 ± 0.94	15.76 ± 1.21
9	Loganic acid	n.d	n.d	n.d	n.d
10	p-Coumaric Acid	2.96 ± 0.39	7.75 ± 0.87	9.91 ± 0.46	18.18 ± 1.49
11	Shikimic Acid	n.d	n.d	n.d1	n.d
12	Syringic Acid	n.d	39.00 ± 5.12	52.5 ± 5.86	77.5 ± 3.60
13	Trans-cynnamic acid	1.10 ± 0.05	2.98 ± 0.27	3.55 ± 0.18	4.20 ± 0.27
14	Vanillic Acid	n.d	138.27 ± 5.19	184.73 ± 0.31	345.13 ± 50.11
	Flavonoids				
15	Catechin	n.d	n.d	n.d	n.d
16	Cyanidin 3-glucoside	n.d	n.d	n.d	n.d
17	Delphinidin 3,5-diglucoside	n.d	n.d	n.d	n.d
18	Epicatechin	151.07 ± 0.26	n.d	n.d	n.d
19	Hyperoside	0.39 ± 0.00	0.28 ± 0.00	0.37 ± 0.02	n.d
20	Kaempferol	0.96 ± 0.08	0.76 ± 0.06	1.4 ± 0.01	1.66 ± 0.03

n.	Compounds	E1 (MeOH)	E2 (H_2O)	E3 (MeOH: H_2O)	E4 (EtOH: H_2O)
21	Naringin	n.d	0.32 ± 0.01	n.d	n.d
22	Quercetin Dihydrate	2.13 ± 0.17	2.19 ± 0.01	2.46 ± 0.04	1.53 ± 0.26
23	Quercitrin	0.38 ± 0.04	0.15 ± 0.01	0.33 ± 0.03	0.51 ± 0.00
24	Resveratrol	n.d	n.d	n.d	n.d
25	Rutin Hydrate	1.63 ± 0.05	1.74 ± 0.02	8.7 ± 0.12	3.37 ± 0.19
	Xanthone				
26	Isogentisin	0.032 ± 0.00	0.40 ± 0.01	0.5 ± 0.08	0.31 ± 0.06
	Secoiridoids				
27	Swertiamarin	n.d	n.d	n.d	n.d
28	Sweroside	n.d	n.d	n.d	n.d
29	Gentiopicroside	n.d	n.d	n.d	n.d
30	Amarogentin	n.d	n.d	n.d	n.d
	Total level of bioactive compounds	15631.69 ± 768.88	23078.85 ± 1952.89	30247.00 ± 1431.96	40101.63 ± 3387.54
	% of bioactive compounds (%w/w)	1.56 ± 0.08	2.31 ± 0.20	3.02 ± 0.14	4.01 ± 0.34
	Total level of polyphenols	5621.23 ± 379.48	3479.32 ± 110.59	5069.83 ± 359.40	4221.85 ± 151.21

335

336 n.d : not detectable. the signal was lower than LOQ; CQA: caffeoylquinic acid.

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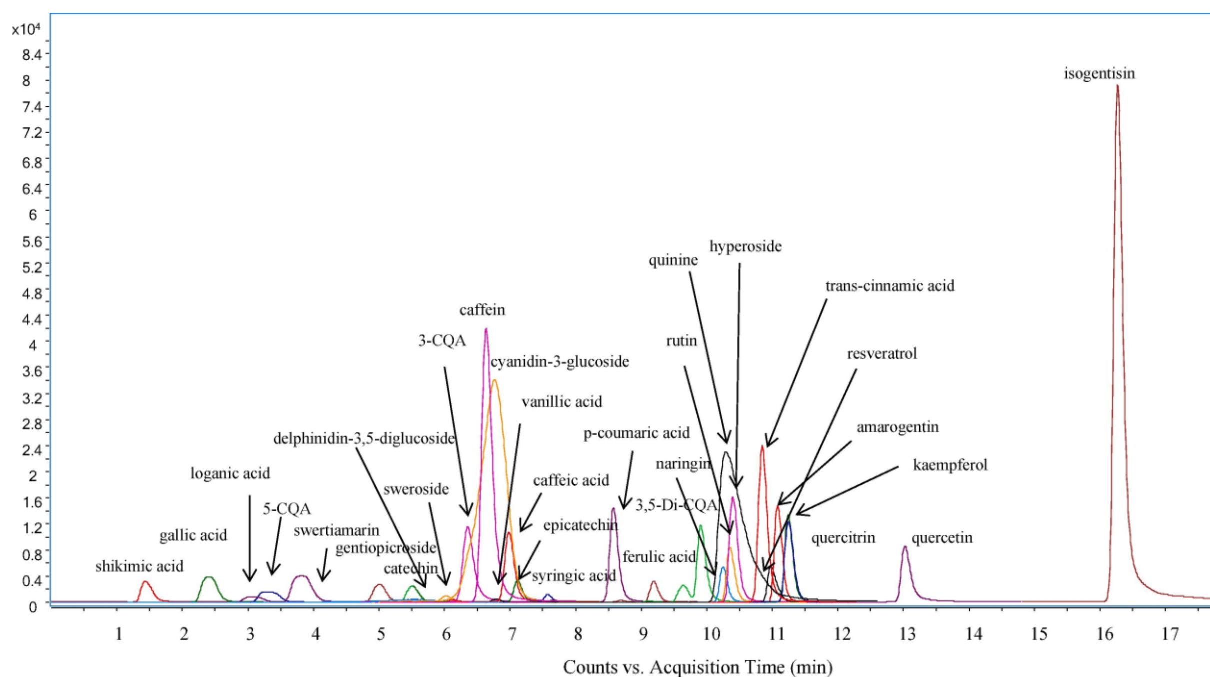


345

346 **Fig. 1.** HPLC-MS/MS chromatogram of a standard mixture of the 30 bioactive compounds

347 plotted as overlapped multiple reaction monitoring (MRM) transition of each compound.

348

349

350 **3.2.1. Alkaloids.**

351 Caffeine was the most abundant bioactive compound in all the extracts with content varying

352 from 1.00% to 3.59% of dry weight of extract (dw). These levels are quite high considering that

353 caffeine levels can range between 1.01% and 8.16% of dry coffee beans and dry coffee extracts

354 (Belay et al., 2008, Jeszka-Skowron et al., 2016). Nowadays, the market value of energy drink

355 is considerably increasing. Considering that main component in most energy drinks is caffeine

356 (Alsunni, 2015), CSS extracts could represent an ideal ingredient for the preparation of energy

357 drinks and energy bars (Bondesson, 2015) given their high caffeine content and their low

358 purchase cost. The level of caffeine extracted varied with the extraction solvent used. Indeed,

359 the highest level of caffeine was observed in E4 (3.59%) using ethanol/water (70:30) as

360 extraction solvent. These levels resulted higher than those reported by previous studies, in

361 which the highest caffeine levels were: 1.42% of dw after UAE with water (Guglielmetti, 2017);

362 2.64% of dw after subcritical water extraction (Narita & Inouye, 2012) and 2.4% using boiling
363 water for extraction (Iriondo-DeHond et al., 2019). Good levels were also observed using water
364 (E2) with caffeine contents closed to 2% of dry weight of CSS extract (Table 2). These results
365 showed that UAE can be considered as a good extraction method for the production caffeine-
366 enriched extracts from CSS. Moreover, the simplicity of UAE and the affordable cost of the
367 extraction equipment could allow the application of the proposed method to small and medium-
368 sized enterprises. Quinine was found in all the analyzed extracts with levels ranging from 0.23
369 μg g−1 (E1)–0.61 μg g−1 (E4). Quinine is an alkaloid first isolated from the bark of cinchona
370 tree and known as a potent antimalarial agent (Jones, Panda, & Hall, 2015). Its presence in
371 coffee has been reported in various studies (Tongcumpou, Usapein, & Tuntiwiwattanapun,
372 2019). However, to our knowledge, this study is the first to reveal the levels of quinine in CSS
373 extracts.

374 **3.2.2. Polyphenols.**

375 18 phenolic compounds were quantified in the 4 CSS extracts. Considering the targeted
376 analytes, the total polyphenols contents ranged from 0.35% (E2) to 0.56% (E1) of dry weight
377 of the extracts.

378 *Phenolic acids.*

379 Caffeoylquinic acids (3-CQA, 5-CQA and 3,5-diCQA) were the most abundant polyphenols in
380 the different CSS extracts. Their total concentrations ranged from 3115.6 μg g^{-1} to 5444.0 μg g^{-}
381 ^{1}, corresponding to 78.5 – 96.8% of the total level of polyphenols detected in CSS. These results
382 are in accordance with related studies on CSS phenolic composition (Regazzoni et al., 2016).
383 However, contrary to previous studies, which stated that there is no unconjugated phenolic acid
384 in CSS extract (Bresciani, Calani, Bruni, Brighenti & Del Rio, 2014), this study is one of the
385 first to identify and quantify 7 unconjugated phenolic acids in CSS. Indeed, gallic acid, vanillic
386 acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and trans-cynnamic acid were

387 detected in CSS with a total concentration between 20.65 μg g^{-1} (E1) and 899.39 μg g^{-1} (E4).
388 Just 3 unconjugated phenolic acids (gallic acid, p-coumaric acid and trans-cynnamic acid) were
389 present in the methanolic extract (E1) while all the 7 were present in the other extracts with
390 highest levels of vanillic acid (345.13 ± 50.11 μg g^{-1}), ferulic acid (226.23 ± 6.20 μg g^{-1}) and
391 caffeic acid (212.38 ± 27.86 μg g^{-1}) in E4.

392 *Flavonoids.*

393 Among the different flavonoids targeted in this study, 7 polyphenols belonging to various
394 flavonoids subclasses were detected. E1 resulted to be the extract with the highest total level of
395 monitored flavonoids (156.56 ± 0.60 μg g^{-1}). Concerning flavonols subclass, rutin (1.63 – 8.70
396 μg g^{-1}), quercetin (1.53 – 2.46 μg g^{-1}), kaempferol (0.76 – 1.66 μg g^{-1}) and quercitrin (0.15 –
397 0.51 μg g^{-1}) were found in all the CSS extracts. Hyperoside (0.28 – 0.39 μg g^{-1}) was not detected
398 in E4. Concerning the flavan-3-ols subclass, epicatechin (151.07 ± 0.26 μg g^{-1}) was found only
399 in E1 while catechin was not detected in all the extracts. Naringin, which belongs to flavanones
400 subclass was found only in E2 (0.32 ± 0.01 μg g^{-1}). The monitored anthocyanins (cyanidin and
401 delphinidin) and resveratrol were not detected in CSS.

402 Few studies have performed a deepen characterization of flavonoid polyphenols in coffee by-
403 products. Ramón-Gonçalves et al. (2019) attempted to quantify some flavonoids in spent coffee
404 grounds extracts. However, just 3 flavonoid compounds (rutin, naringin and resveratrol) were
405 detected and their analyses were performed by HPLC-DAD, which is less sensible than HPLC-
406 MS/MS. Therefore, to our knowledge, this study is the first to report the flavonoid composition
407 of CSS extracts.

408 *Xanthone*

409 Furthermore, small quantities of isogentisin (0.03 – 0.50 μg g^{-1}) were found in all the CSS
410 extracts. This compound, which is a polyphenol of the xanthone class, is an important bioactive
411 compound present in *Gentiana lutea* L. Isogentisin is reported to prevent tobacco-induced

412 endothelial diseases (Schmieder et al., 2007) and to inhibit monoamine oxidase (Mustafa et al., 413 2015). It is important to consider that this study is the first to report the presence and the levels 414 of isogentisin in coffee by-product such as CSS.

415 *Secoiridoids.*

416 We can also note that none of the targeted secoiridoids (amarogentin, swertiamarin, sweroside 417 and gentiopicroside) was found in the analyzed extracts.

418 Various studies have highlighted the beneficial biological properties of CQAs and flavonoids 419 such as antioxidant, anti-inflammatory, anti-carcinogenic and cardiovascular disease risk-420 reduction (Cvejić, Krstonošić, Bursać, & Miljić, 2017; Maaliki, Shaito, Pintus, El-Yazbi, & 421 Eid, 2019; Martini et al., 2019). Therefore, high levels in polyphenols, could suggest a possible 422 application of CSS extracts as ingredients in functional food and food supplement preparation 423 (Del Castillo, Fernandez-Gomez, Martinez-Saez, Iriondo-DeHond, Martirosyan, & Mesa, 424 2016; Gocmen, Sahan, Yildiz, Coskun, & Aroufai, 2019).

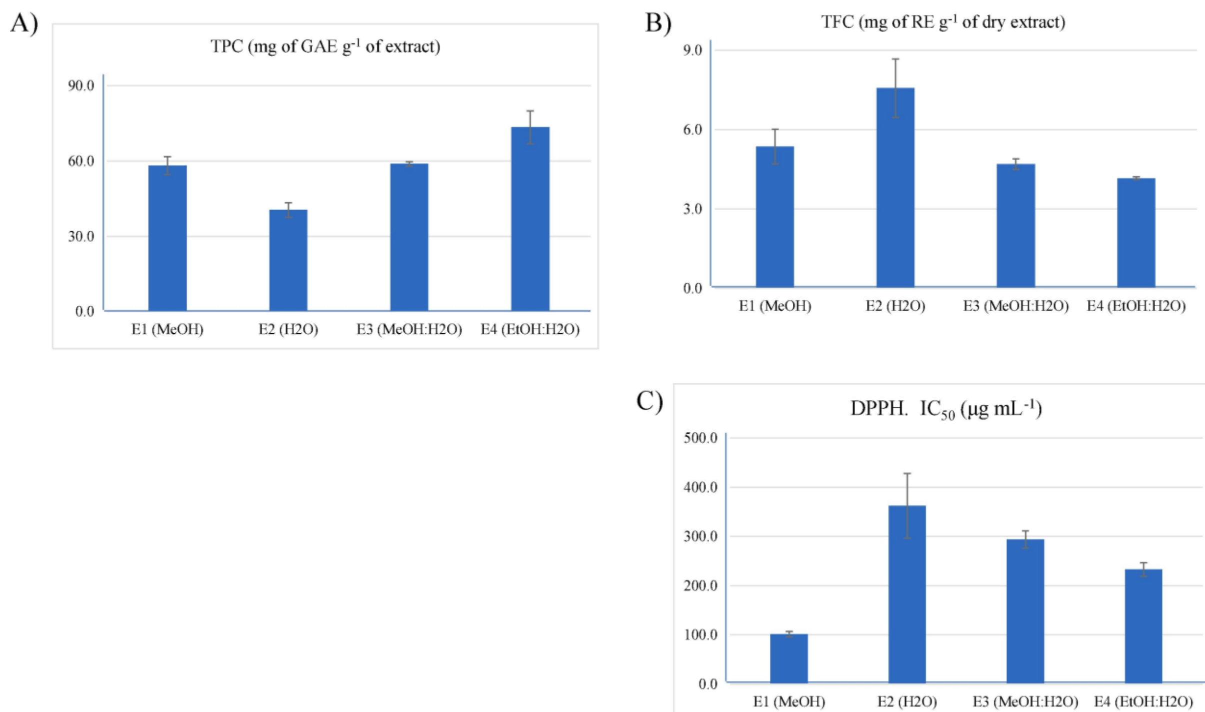
425 **3.3. Spectrophotometric analyses.**

426 **3.3.1. Total phenolic content (TPC) and total flavonoid content (TFC) determination.**

427 **Figure 2** shows the TPC and TFC of the different CSS extracts**.** TPC measured 428 spectrophotometrically provided an estimation of the total levels of polyphenols in the analyzed 429 extracts. The TFC of the studied extracts ranged from 4.2 mg of RE g^{-1} to 7.6 mg of RE g^{-1} of 430 dw of CSS extract, while the TPC varied according to the solvent of extraction with means 431 levels between 40.4 mg of GAE g^{-1} and 73.4 mg of GAE g^{-1} of dw of extract. The levels 432 obtained in the analyzed extracts were higher than the values reported in other studies using 433 conventional solid-liquid extraction methods. For instance, Panusa et al., (2017) obtained CSS 434 extracts with TPC ranging from 4.35 to 12.82 mg of GAE g^{-1} of dw after conventional 435 extraction with water at 60 °C for 30 min. EtOH: H_2O (70:30) allow to obtain the highest TPC 436 in CSS extracts (73.4 ± 6.6 mg of GAE g^{-1} dw). As reported by Costa et al. (2014), 100% water

437 resulted the worst solvent to extract phenolic compounds from CSS. In fact, the lowest TPC
438 was observed in CSS extracts prepared with water as extraction solvent (40.4 ± 2.9 mg of GAE
439 g^{-1} dw). This result is in accordance with the levels observed after HPLC quantification.
440 Nevertheless, the TPC of the aqueous extract (E2) resulted higher than those reported in
441 previous studies, in which water was used as solvent for solid-liquid CSS extraction. Indeed,
442 Conde, & Mussatto, (2016) obtained CSS extracts with TPC of 19.2 mg of GAE g^{-1} of dw and
443 TFC of 2.73 mg of RE g^{-1} of dw after hydrothermal treatment at 120°C for 20 min. These results
444 highlight therefore, the effectiveness of UAE in obtaining CSS extracts with high levels of
445 polyphenols. Narita &Inouye (2012) obtained CSS extracts with highest TPC of 130 ± 6
446 mg GAE g^{-1} of dw by using subcritical water extraction (240 °C, 3.2 MPa). These levels are
447 higher than those obtained in this study. However, supercritical extraction requires significant
448 investments in special equipment, unlike UAE, which provides high extraction efficiency while
449 remaining affordable for laboratories and small industries.
450 The difference observed between the spectrophotometric and HPLC results can be explained
451 by the higher selectivity of the HPLC analysis, which provided a quantification of specific
452 analytes. Therefore, considering both approaches is useful to understand the effect of solvent
453 on bioactive compounds extraction.

454



455

456 **Fig. 2.** Spectrophotometric analyses of coffee silver skin (CSS) extracts (n = 3). (A) Total
457 phenolic contents (TPC) of CSS extracts, expressed as mg of gallic acid equivalents per g of
458 dry weight of extract (mg of GAE g−1 of dry extract). (B) Total flavonoid contents (TFC) of
459 CSS extracts, expressed as mg of rutin equivalents per g of dry weight of extract (mg of RE/g
460 of dry extract). (C) DPPH radical scavenging activity of the different CSS extracts, expressed
461 as IC50 value (μg mL−1). DPPH: 2,2-diphenyl-1-picrydrazyl; IC50 which is the concentration
462 of the extract necessary to cause 50% of DPPH inhibition.

463

464 **3.3.2. Radical scavenging activity (RSA)**

465 The radical scavenging activity of the investigated CSS extracts was determined by DPPH
466 assays. The DPPH values varied in the different extracts, showing that the type of solvent
467 affected the antioxidant capacity of CSS extracts (**Figure 2**). The methanolic extracts (E1)
468 showed the highest RSA with an IC_{50} of 101.7 ± 5.5 µg mL^{-1}, while the lowest RSA were
469 observed in the aqueous extract (E2) with an IC_{50} of 362.1 ± 65.7 µg mL^{-1}. It could be

470 interesting to note that according to HPLC-MS/MS analyses results, E1 and E2 tended to be
471 respectively the most and the least concentrated extracts in monitored polyphenols (**Table 2**).
472 However, there was no evidenced correlation between the DPPH inhibition of the studied
473 extracts and their respective TPC. This might suggest that the antioxidant activity of CSS
474 extracts may be due to other compounds than polyphenols such as melanoidins and diterpenes
475 which are present in CSS and possess relevant antioxidant activities (Costa et al., 2014; Mesías,
476 & Delgado-Andrade, 2017). These antioxidant compounds are less soluble in water (Belandria
477 et al., 2016) and this could explain why CSS extracts obtained from water (E2) and water
478 containing solvents (E3 and E4) showed lowers DPPH inhibition than E1, which was obtained
479 from 100% methanol. Moreover, DPPH is a lipophilic radical, which could limit the
480 accessibility of hydrophilic antioxidant present in CSS extracts (Choi, & Koh, 2017).
481

482 **3.4. Neuroprotective activity of silverskin extracts against H_2O_2-induced damage**

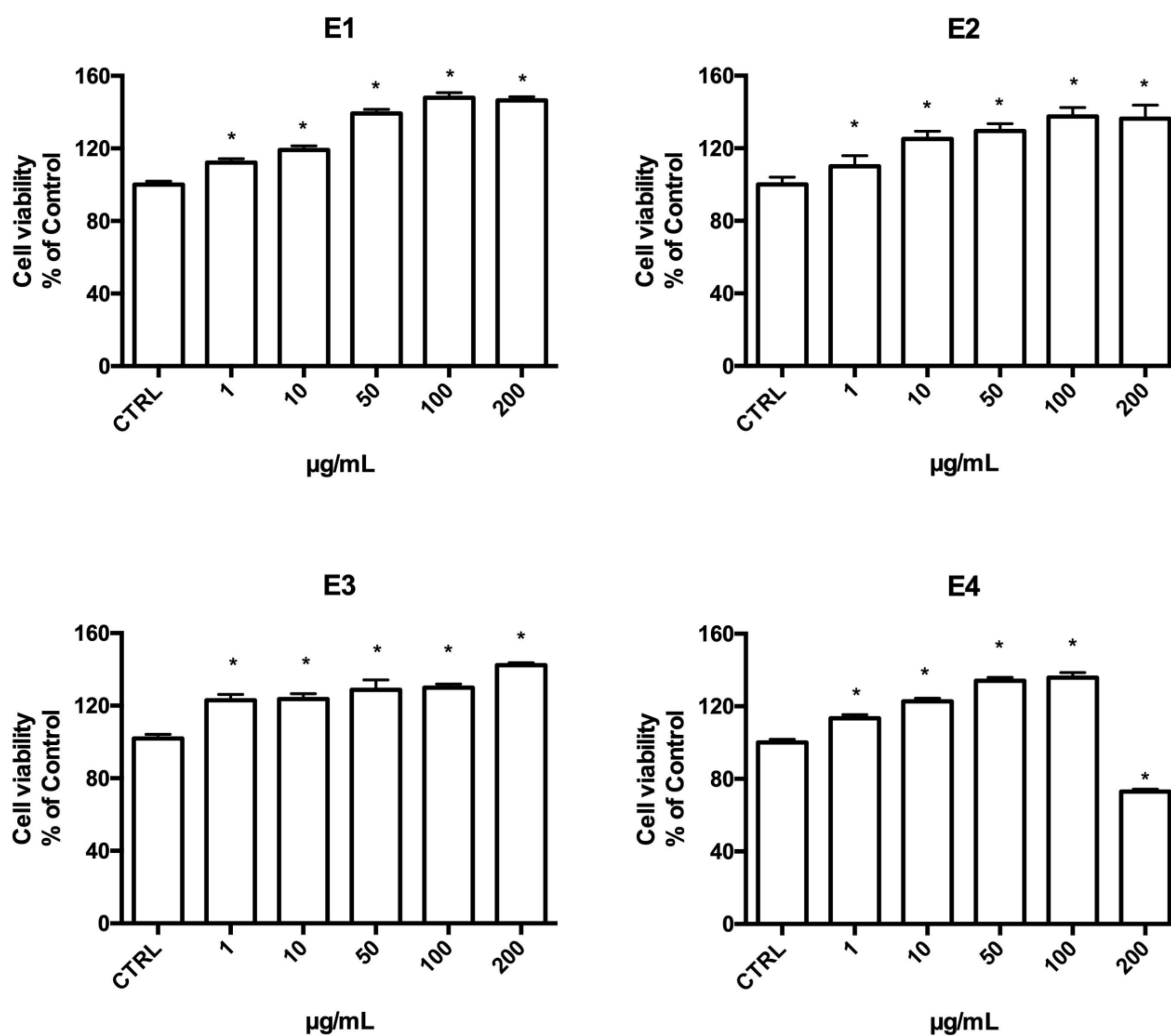
483 To investigate the potential cytotoxicity of E1, E2, E3, and E4 extracts, differentiated SH-SY5Y
484 cells were treated with different concentrations (1–200 μg mL−1) of the four extracts for 24 h
485 (Fig. 3). Until 100 μg mL−1 all the extracts did not reduce cell viability in respect to control
486 cells indicating that they are not cytotoxic. Only E4 reduced cell viability at the highest
487 concentration and for this reason 200 μg mL−1 E4 has not been considered in the next
488 experiments. Of note, E1, E2, E3, and E4 significantly increased cell viability at 1–100 μg
489 mL−1. The observed increase in cell viability could be ascribed to an enhancement of
490 mitochondrial respiration. In fact, MTT measures cell viability in terms of reductive activity as
491 enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by
492 dehydrogenases occurring in the mitochondria of living cells (Mosmann, 1983). On these bases,
493 the MTT assay is dependent on mitochondrial respiration. In our opinion, the increased
494 mitochondrial respiration could be related to the presence of caffeine. Caffeine has been shown

495 to increase mitochondrial content by increasing the expression of peroxisome proliferator-
496 activated receptor gamma coactivator 1-alpha (PGC-1α), which is a regulator of nuclear
497 respiratory factors 1 and 2 (NRF1/2) (McConell et al., 2010, Ojuka et al., 2002, Schnuck et al.,
498 2018). PGC-1α and NRF1/2 modulate the expression of mitochondrial transcription factor A
499 (TFAM), which regulates the expression of respiratory components of the electron transport
500 chain (Li, Hou, & Hao, 2017) Interestingly, there is no a direct correlation between the content
501 of caffeine in the four extracts and the increase in cell viability. This could be due to the
502 presence in the extracts of compounds other than caffeine that could modulate both caffeine
503 uptake and caffeine metabolism. In the future, it would be interesting to study the effect of pure
504 caffeine on SH-SY5Y cell line to better clarify this point. Moreover, in order to verify that the
505 increase in cell viability is not due to a de-differentiation we evaluated the expression of two
506 well-known markers of neuronal differentiation: neuronal nuclear antigen (NeuN) and
507 synaptophysin (SYP), (Borsani et al., 2020). In vitro effects of concentrated growth factors
508 (CGF) on human SH-SY5Y neuronal cells. Eur Rev Med Pharmacol Sci, 24, 304–314) (Fig.
509 2S A, B). Interestingly, all the treatments did not modify the expression level of NeuN and
510 SYP. Moreover, to further demonstrate that the observed increase in cell viability is not due to
511 an increased cell replication, we treated differentiated SH-SY5Y cells with 100 μg mL−1 of
512 each extract and after 24 h the number of cells was counted. Of note, the treatments maintain
513 the number of cells to value comparable to control cells (data not shown). To study the potential
514 protective activity of the extracts against oxidative stress, cells were treated with increasing
515 concentrations (1–200 μg mL−1) of the four extracts before the induction of oxidative stress by
516 700 μM H2O2 exposure for 1 h (Fig. 4). This peroxide concentration has been chosen as it
517 reduces cell viability by 50% with respect to control cells (Fig. 4). Moreover, similar H2O2
518 concentrations have been recently used by Piras et al. (2016) in differentiated SH-SY5Y cells.
519 At the lower concentrations, only E1 and E4 extracts showed a protective activity against

520 peroxide exposure as they significantly increased cell viability in respect to H2O2 treated cells
521 at 1–10 μg mL−1. On the contrary, E2 and E3 increased cell viability in respect to peroxide
522 exposed cells only at concentrations higher than 50 μg mL−1. To verify if the observed
523 protective effects could be related to a reduction of endogenous ROS levels, differentiated SH-
524 SY5Y cells were treated with the four extracts at 1–200 μg mL−1 prior to the addition of H2O2
525 and the level of intracellular ROS was determined using the peroxide-sensitive fluorescent
526 probe DCFH-DA (Fig. 5). In agreement with viability data, E1 and E4 were the most effective
527 in reducing ROS levels. In particular, E1 significantly reduced ROS levels at 10 and 50 μg
528 mL−1, meanwhile E4 significantly reduced ROS levels at 50 and 100 μg mL−1. E2 and E3
529 significantly decreased ROS levels only at the highest concentration. Of note, these data are in
530 agreement with the RSAs of the 4 extracts obtained by DPPH assay, indicating that E1 and E4
531 extracts exert a higher antioxidant activity both in vitro and in cells. Interestingly, there is no
532 correlation between the concentrations of E1 and E4 that significantly protected against
533 peroxide and the concentrations that reduced ROS levels suggesting that the protective
534 mechanisms could be not only ascribed to an antioxidant activity. Moreover, E1 greater
535 effectiveness in countering oxidative stress in respect to E2 and E3 seems to be related to a
536 higher level of total polyphenols and in particular to a significantly higher concentration of 5-
537 CQA and epicatechin. 5-CQA is an isomer of caffeoylquinic acid (CQA) that is the ester formed
538 between one molecule of caffeic acid and one molecule of quinic acid (Liang, Dupuis, Yada,
539 & Kitts, 2019). Among CQA isomers, 5-CQA has been shown to possess the higher antioxidant
540 activity in vitro (Liang et al., 2019). This compound has also been investigated in cell model
541 system. In particular, 5-CQA lowered ROS production and recovered depleted GSH in
542 endothelial EA.hy926 cells exposed to TNF-α. (Wang, Sarriá, Mateos, Goya, & Bravo-
543 Clemente, 2018). Similar results were observed in Caco2 cells challenged with the
544 inflammatory and oxidative stress mediators PMA + IFNγ (Liang & Kitts, 2018). Pre-treatment

545 with 5-CQA ameliorated oxidative stress by reducing intracellular ROS, increasing reduced
546 GSH, and activating Nrf2 signaling pathway. The main biological functions of epicatechin are
547 the production of protein complexes, the scavenging of free radicals, and the reduction of lipid
548 peroxidation, making it a great antioxidant (Bernatova, 2018). Rats pre-treated with epicatechin
549 showed a reduced deterioration of spatial memory induced by the Aβ25–35, related to a
550 reduction of oxidative stress and inflammation in the hippocampus (Diaz, 2019). A very recent
551 paper demonstrated the protective effect of epicatechin against neuronal cell death via oxidative
552 stress and ER stress induced by methamphetamin. In particular, epicatechin inhibited ROS
553 generation as well as MAPK activity, and CHOP and DR4 expression, during
554 methamphetamine-mediated apoptosis. Leonardo et al. (2013) showed that epicatechin protects
555 mouse cortical neurons from oxygen/glucose deprivation trough the activation of Nrf2-
556 mediated pathway and the upregulation of the antioxidant enzyme heme oxygenase 1. The
557 ability of epicatechin to modulate Nrf2 pathway and to increase HO1 activity has also been
558 demonstrated in vivo using Nrf2 and HO1 knockout mice (Shah et al., 2010). The same authors
559 demonstrated that epicatechin protects embryonic cortical neuronal cells against oxidative
560 stress induced by H2O2 and tert-butyl hydroperoxide. On the bases of these studies we
561 hypothesize that the protective effect showed by E1 could be related to its higher content of 5-
562 CQA and epicatechin in respect to the other extracts. The specific composition of E4,
563 characterized by a higher content of p-coumaric acid, ferulic acid, 3,5-diCQA, and quercitrin
564 in respect to the other extracts, could be related to its protective activity against oxidative stress.
565 All of these phenolic compounds have been demonstrated to be effective against oxidative
566 stress in different cell systems. In PC12 neuronal cells, p-coumaric acid, a hydroxy derivative
567 of cinnamic acid, strongly suppressed the accumulation of intracellular ROS and protected from
568 Aβ toxicity (Hong, Yeong, & Mira, 2012). Ferulic acid is one of the most studied phenolic acid
569 derivatives for its neuroprotective activity. In neuronal cells, ferulic acid counteracted

570 oxidative/nitrosative stress caused by Aβ exposure or other radical initiators, with different
571 mechanisms, including the activation of cell stress response (Picone et al., 2009). In SH-SY5Y
572 cells 3,5-diCQA, a caffeoylquinic acid derivatives, attenuated the neuronal death and caspase-
573 3 activation induced by H2O2 (Kim, Park, Jeon, Kwon, & Chun, 2005). In addition, 3,5-diCQA
574 restored H2O2-induced depletion of intracellular glutathione. Quercitrin is a glycosylated form
575 of quercetin and the sugar bound to the aglycone portion increases solubility in polar solvents
576 and consequently improves absorption of quercitrin in respect to quercetin (Gee, Dupont,
577 Rhodes, & Johnson, 1998) Different studies evidenced quercitrin anti-oxidative and anti-
578 inflammatory activity (Rattanajarasroj & Unchern, 2010). Quercitrin antioxidant effect has also
579 been demonstrated in ICR mice treated with carbon tetrachloride (Ma, Luo, Jiang, & Liu, 2015).
580 In particular a 4 weeks treatment with quercitrin suppressed the elevation of reactive oxygen
581 species (ROS) production and malondialdehyde (MDA) content, reduced tissue plasminogen
582 activator (t-PA) activity, enhanced the antioxidant enzyme activities and abrogated cytochrome
583 P450 2E1 (CYP2E1) induction in mouse brains. In conclusion data obtained in SH-SY5Y cells
584 showed a higher effectiveness of E1 and E4 in protecting cells against H2O2-induced damage
585 related to their specific pattern of phytochemicals. Moreover, these findings suggest a potential
586 role of E1 and E4 extracts as preventive/protective agents against neurodegeneration due to
587 their ability to counteract oxidative stress and maintain cell viability.



588

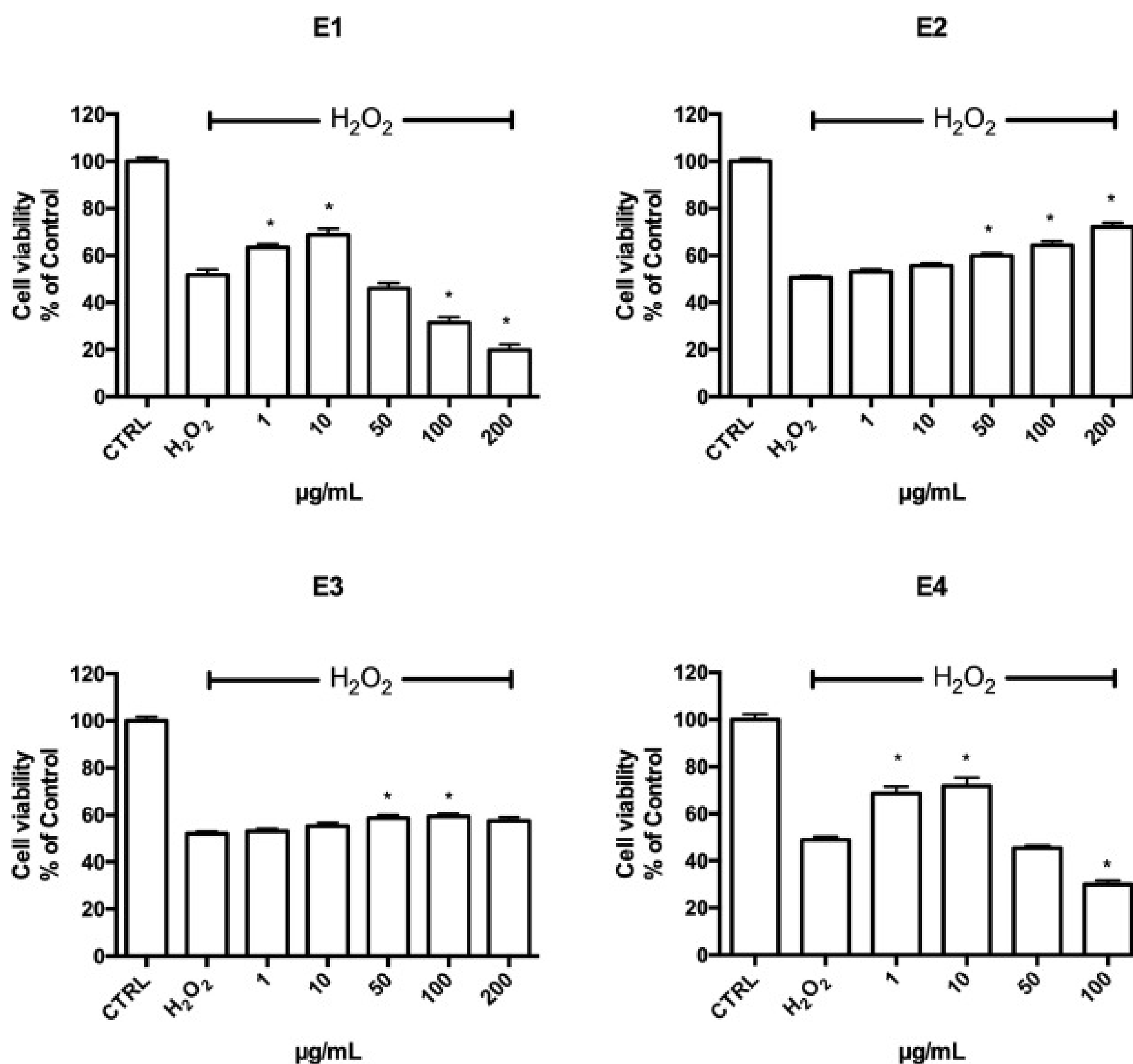
589 **Fig. 3.** Viability of differentiated SH-SY5Y treated with the extracts. Cells were treated with

590 increasing concentration of E1, E2, E3, and E4 (1–200 µg mL−1) and after 24 h cell viability

591 was evaluated by MTT assay. Each bar represents means ± SEM of at least four independent

592 experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *$p < 0.05$

593 with respect to CTRL.



594

595

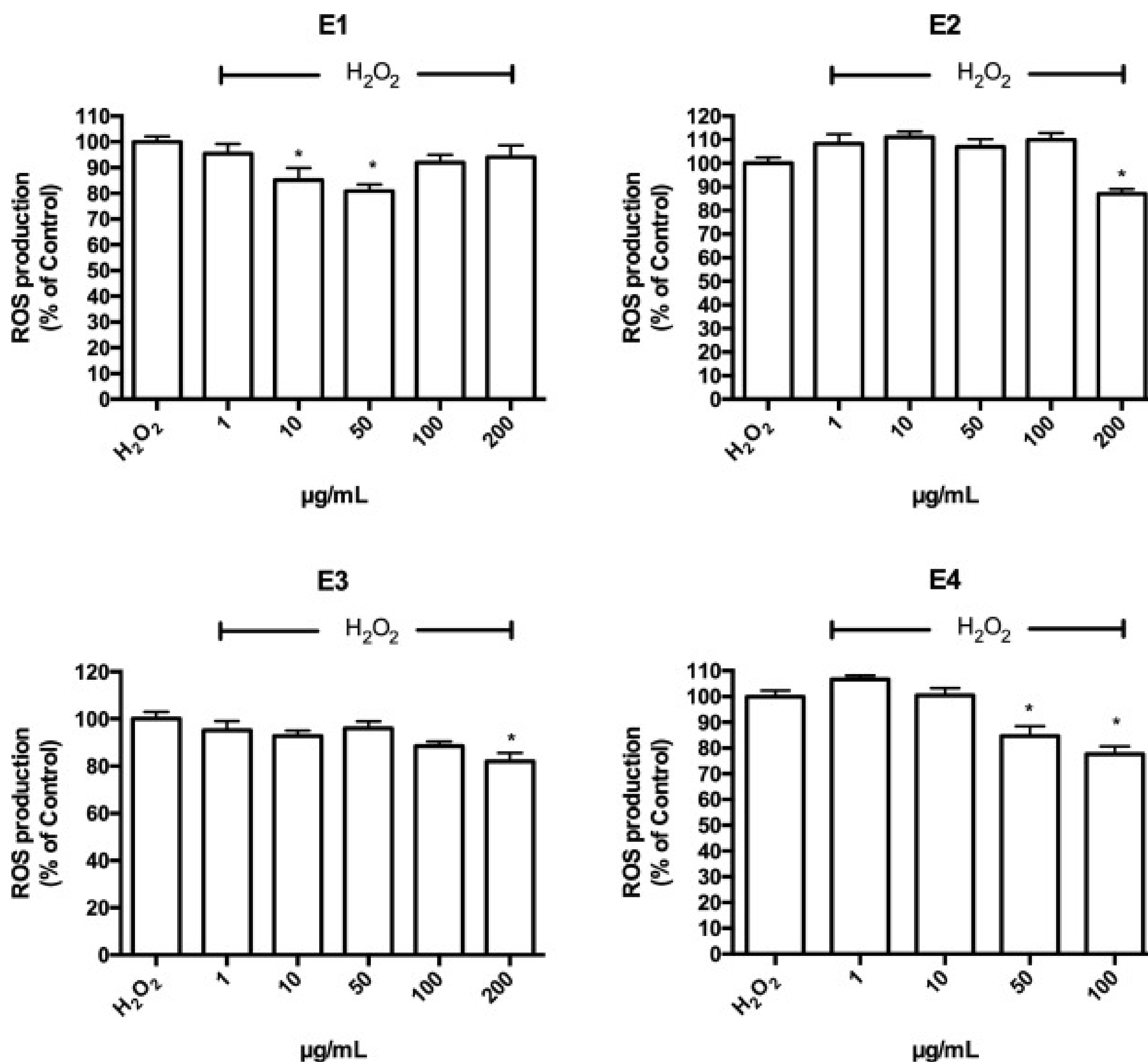
596 **Fig. 4.** Viability of differentiated SH-SY5Y treated with the extracts in the absence/presence of

597 H2O2. Cells were treated with increasing concentration of E1, E2, E3, and E4 (1–200 µg mL−1)

598 for 24 h, exposed to 700 µM H2O2 for 1 h and cell viability was evaluated by MTT assay. Each

599 bar represents means ± SEM of at least four independent experiments. Data were analyzed by

600 one-way ANOVA followed by Bonferroni's test. *$p < 0.05$ with respect to H_2O



601

602 **Fig. 5**. Antioxidant activity of the extracts against H2O2 in differentiated SH-SY5Y cells. Cells
603 were treated with E1, E2, E3, and E4 (1–200 µg mL−1) after 24 h were exposed to H2O2.
604 Intracellular ROS levels were measured with the peroxide-sensitive probe DCFH-DA. Data are
605 expressed as percentage with respect to H2O2-treated cells. Each bar represents means ± SEM
606 of at least four independent experiments. Data were analyzed by one-way ANOVA followed
607 by Bonferroni's test. *p < 0.05 with respect to H2O2.

608 **3.5. Antimicrobial activity**

609 All silverskin extracts were not inhibiting the growth of anyone of the bacterial species included
610 in this study. The minimal inhibitory concentrations (MIC) would have to be searched for at
611 concentration above 512 mg L^{-1}, values which hamper the realistic utilization of the crude

612 extracts as antimicrobials. Actually, the extracts contain many bioactive compounds with
613 proven antimicrobial activities, such as 5-CQA, caffeic acid and caffeine (Dash et al., 2008).
614 Besides their high relative amount into the extracts, their absolute amount is far below the MICs
615 reported in the literature for the single compounds. For instance, MIC of caffeine against *S.*
616 *aureus* and *E. coli* is > 200 mg L^{-1} (Dash et al., 2008). To reach these concentrations, at least 5
617 mg mL^{-1} of extract would have to be used. The inactivity at the tested concentrations
618 additionally indicated that there was not a synergism by the complex and unique mixture of
619 components, which is often an interesting feature of natural extracts. Also in this respect, the
620 silverskin extracts did not show interesting profiles of antimicrobial activity. Additionally,
621 given the importance of biofilms, a screening of the potential anti-biofilm activity of the
622 silverskin extracts has been conducted by measurement of the biomass produced by the
623 different species after 20 hours of growth in two different media. This widely used approach
624 gives an estimation of the amount of mature biofilm formed and developed onto a surface.
625 Overall the extracts did not show a strong inhibitory effect on biofilm formation and maturation.
626 For *S. aureus* and *E. coli* the trend is toward inhibition (**Table 3**). However, the highest
627 contributions to the obtained significant negative Pearson correlation coefficients (e.g. E2 and
628 E3 vs *S. aureus* and E1, E2, and E4 vs *E.coli*) was given by the higher extract concentrations
629 (> 128 mg L^{-1}). In the case of *P. aeruginosa*, instead, the trend was opposite, that is the Pearson
630 correlation coefficients were positive, indicating a general stimulatory effect of extracts on the
631 biofilm biomass accumulation. Only the water extract E2 was an exception not showing any
632 concentration dependent effect (r = -0.07). Considering the different average composition of
633 silverskin methanol extract E1 in respect to the others, a paired mean differences comparison
634 analysis was performed between E1 and E2, E1 and E3, E1 and E4 (**Figure 2S**). Results
635 indicated that in the case of *S. aureus* E2 and E4 were more effective in decreasing the biomass
636 formation, even if only E2 was doing it significantly (P = 0.006, two-sided, Wilcoxon test). *E.*

637 *coli* biofilm biomass was not differentially influenced by the E1 in respect to the extracts
638 obtained by the water based solvents, with the exception of E4 that was less effective, albeit at
639 a low level of statistical significance (P = 0.029, two-sided, Wilcoxon test). At last, *P.*
640 *aeruginosa* behaviour in the presence of different concentration of extracts was similar to that
641 shown by *S. aureus* with E4 having instead the major effect on biofilm biomass reduction (P =
642 0.006, two-sided, Wilcoxon test). Overall, data indicated that silverskin extracts obtained by
643 water-based solvents might deserve a deeper future investigation on biofilm-related activities,
644 such as quorum sensing or virulence factors' expression.

645 **Table 3.** Pearson correlation coefficients for the two variables extract concentration versus
646 biofilm biomass. Associated probability is also indicated in the corresponding column.

Extracts	*S. aureus*		*E. coli*		*P. aeruginosa*	
	Pearson's *r*	**P Value**	**Pearson's *r***	**P Value**	**Pearson's *r***	**P Value**
E1	−0.52	0.0852	−0.71	0.0144	0.70	0.0108
E2	−0.88	0.0004	−0.86	0.0007	−0.07	0.8216
E3	−0.68	0.0148	−0.24	0.4829	0.45	0.1437
E4	−0.33	0.2948	−0.76	0.0069	0.84	0.0006

647
648

649 **Conclusions.**

650 This study is one of the first to provide a deepen characterization of bioactive compounds in
651 CSS. UAE, an affordable extraction technique, allowed to obtained CSS extracts with high
652 content of caffeine, chlorogenic acids and flavonoids and the total of the 30 bioactive
653 compounds monitored in this tudy represent 1.56–4.01% w/w of CSS extracts. Data obtained
654 on SH-SY5Y cells showed a higher effectiveness of E1 and E4 extracts in protecting cells
655 against H2O2-induced damage related to their specific pattern of phytochemicals, suggesting a
656 potential role as protective agents against neurodegeneration due to their ability to counteract
657 oxidative stress and maintain cell viability. Moreover, data indicated that silverskin extracts

658 obtained by water-based solvents might deserve a deeper future investigation on biofilm-related
659 activities, such as quorum sensing or virulence factors' expression. The low cost of CSS and
660 the biological activities of the obtained extracts, which are attributed to their phytochemical
661 compositions, could suggest a possible application of CSS extracts as ingredients in food and
662 pharmaceutical formulations.

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