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

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**Abstract**

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

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

## 1. Introduction

Coffee is one of the most consumed beverages in the world with a global consumption of 161 million of 60 kg coffee bags per year. As consequences, millions of tons of coffee by-products such as spent coffee ground (SCG) and coffee silverskin (CSS) are produced each year. Silverskin, the thin tegument covering the coffee bean, is a by-product of green coffee roasting procedure. Indeed, from eight tons of coffee roasted, around 60 kg of CSS are produced (Alves, Rodrigues, Nunes, Vinha & Oliveira, 2017). Therefore, with the increasing coffee production and the environmental impact of waste accumulation, CSS disposal needs to be properly managed (Janissen, & Huynh, 2018). That is why in recent years, a lot of research has been carried out in order to find newer CSS applications. Various value adding applications have been proposed for CSS such as feedstock for biofuel production and adsorbent material to remove potential toxic metals in water (Hijosa-Valsero, Garita-Cambronero, Paniagua-García & Díez-Antolínez, 2018; Malara et al., 2018). Another promising approach is the use of CSS as raw material for the recovery of functional compounds of potential interest. Indeed, CSS is a good source of insoluble and soluble dietary fibers (around 50% and 15% respectively) which can be used for food-products enrichment (Iriondo-DeHond et al., 2019). Moreover, recent studies have evidenced that CSS is a rich source of bioactive compounds such as melanoidins, caffeine and polyphenols which allow potentials applications of CSS extracts as functional ingredient in cosmetic (Bessada, Alves, & Oliveira, 2018) and nutraceutical (Bertolino et al., 2019) formulations. These applications require a preliminary separation of bioactive molecules from CSS and thus, it is important to develop simple and efficient extraction methods in order to obtain CSS extracts with high levels of bioactive compounds. Different approaches have been proposed for CSS extracts preparation such as solid–liquid extraction (Iriondo-DeHond et al., 2019), hydrothermal extraction (Conde & Mussatto, 2016), pulsed electric field assisted extraction (Barbosa-

Pereira, Guglielmetti, & Zeppa, 2018), microwave assisted extraction and ultrasound assisted extraction (UAE) (Guglielmetti, D'ignoti, Ghirardello, Belviso, & Zeppa, 2017). Among these different extraction techniques, UAE has shown the advantage of being sustainable, efficient and economically viable (Chemat et al., 2017; Wen, Zhang, Rai, Sun & Tiwari, 2019). However, regarding CSS extraction, no study has been yet performed in order to compare the effect of solvents during UAE on the composition and bioactivities of CSS extracts. Previous analytical studies performed on CSS extracts mainly focused on caffeine, total phenolic content and chlorogenic acids quantification (Costa et al., 2018; Conde, & Mussatto, 2016; Panusa, Petrucci, Lavecchia, & Zuorro, 2017). However, despite the growing interest 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 $H_2O_2$-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 important factor contributing to persistence of infections due to the peculiar physiology of the composing microorganisms. Moreover, the microorganisms into a biofilm are highly tolerant to several antimicrobials with MIC values 100 to 1000 times higher than those measurable against planktonic cells from the same species (Macia et al, 2014). For this reason, searching new molecules or strategies to combat biofilms has gained a lot of interest in the last twenty years and plants/food extracts rich in bioactive compounds such as

polyphenols could represent an interesting alternative. The results shown in this study represent a clear evidence of potential value-added applications of CSS extracts as valuable ingredients in nutraceutical formulations.

## 2. Materials and methods.

### 2.1. Reagents and standards

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

### 2.2. Coffee silverskin preparation

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

### 2.3. Coffee silverskin extraction

The extraction of the bioactive compounds was based on extraction methods optimized by Caprioli, Nzekoue, Giusti, Vittori & Sagratini (2018) with slight modifications. Eight extraction procedures were tested in order to determine the best extraction methods for the 30

bioactive compounds in CSS (**Table 1S**). Briefly, 10 g of CSS powder was sonicated with 50 mL of solvent using a FALC ultrasonic bath (FALC, Treviglio, Italy) at a frequency of 40 kHz for 120 minutes at 20°C. After the extraction, the sample was filtrated with a filter paper and the obtained extract was collected, lyophilized and stored in darkness at a temperature of -20°C until analysis. Among the different extraction procedures tested, four different extracts were chosen according to recoveries and extraction yields and finally considered for further studies: MeOH extract (E1), water extract (E2), MeOH/water (50:50) extract (E3) and EtOH/water (70:30) extract (E4). Before analyses, 5 mg of lyophilized extract were dissolved in 5 mL of MeOH (1 mg $mL^{-1}$) sonicated for 10 min. For HPLC analyses, aliquots of the solutions were filtrated with 0.2 μm pore size filter and then injected in HPLC-MS/MS.

**2.4. HPLC-MS/MS analyses**

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

### 2.5. Spectrophotometric analyses

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

The TPC was determined spectrophotometrically according to Siatka & Kašparová (2010) method with some modifications. Briefly, 0.5 mL of extracts solution (1 mg $mL^{-1}$ in methanol) was introduced into test tubes, then 2.5 mL of Folin–Denis reagent solution and 7mL of $Na_2CO_3$ (7.5% w/w in water) solution were added. The reaction mixture allowed to stand at room temperature in the dark for 2 h and absorption was measured at 765 nm. The TPC quantification in the extracts was made using gallic acid calibration curve and was expressed as mg of gallic acid equivalents (GAE) per g of dry weight of CSS extract.

The TFC of the different extract were evaluated following a method described by Chen, Chen, Xiao & Fu (2018) with slight variations. In a 15 mL test tube, 0.5 mL of extract solution (1 mg $mL^{-1}$), 0.15 mL of $NaNO_2$ (0.5 M), 3.2 mL of methanol (30% V/V) and 0.15 mL of $AlCl_3.6H_2O$ (0.3 M) were mixed. After 5 min, 1 mL of NaOH (1 M) was added. The solution was mixed well and the absorbance was measured against the blank reagent at 506 nm. The standard calibration curve for TFC was made using rutin standard solution (0 to 100 mg $l^{-1}$) under the same procedure as described above. TFC was expressed as mg of rutin equivalents (RE) per g of dried extract.

#### 2.5.2. DPPH radical scavenging activity

The ability of the extracts to scavenge the radical 2,2-diphenyl-1-picrydrazyl (DPPH) was investigated spectrophotometrically according to the method proposed by Venditti et al (2017) with modifications. Briefly, in a 15 mL test tube, 0.5 mL of extract solution (1 mg $mL^{-1}$ in methanol) was mixed with 4.5 mL of ethanolic solution of DPPH (0.1 mM). After 30 min of incubation in the dark at room temperature, the DPPH disappearance was measured spectrophotometrically measuring the absorption at 517 nm. The percentage of DPPH scavenging was calculated following the formula: $\% I = [(A_{control} - A_{sample})/A_{control}] \times 100$.

Where $A_{control}$ and $A_{sample}$ represent the absorbance obtained without and with antioxidants respectively. The scavenging activity was expressed as the $IC_{50}$ value (μg $mL^{-1}$), which is the concentration of the extract necessary to cause 50% of DPPH inhibition. The $IC_{50}$ value was obtained by interpolation from the linear regression analysis. Trolox® was used as the reference antioxidant (1 – 50 μg $mL^{-1}$).

**2.6. Biological analysis**

**2.6.1. Cell culture and treatment**

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

**2.6.2. MTT viability test**

Cell viability was evaluated by measuring MTT reduction as previously reported (Angeloni, Malaguti, Rizzo, Barbalace, Fabbri, & Hrelia, 2015). Briefly, at the end of each experiments, MTT was added to the medium at the concentration 0.5 mg $mL^{-1}$ and incubated for 90 min at 37°C. After incubation, MTT solutions were removed, DMSO was added, and the absorbance

was recorded at λ = 595 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA). Data are expressed as % in respect to control cells. Control cells are considered as 100% cell viability.

### 2.6.3. Intracellular ROS measurement

The formation of intracellular reactive oxygen species (ROS) was evaluated using the fluorescent DCFH-DA probe as previously reported (Marrazzo, Angeloni, Freschi, Lorenzini, Prata, Maraldi, & Hrelia, 2018). Briefly, at the end of each experiments, SH-SY5Y cells were incubated with 10 μM DCFH-DA in DMEM 1% FBS w/o phenol red for 30 min. After removal of DCFH-DA, cells were incubated with 400 μM $H_2O_2$ in DMEM 1% FBS w/o phenol red for 15 min. Then, $H_2O_2$ was removed and replaced by PBS. Cells fluorescence was measured using 485 nm excitation and 535 nm emission with a microplate spectrofluorometer (VICTOR3 V Multilabel Counter, PerkinElmer). Data are expressed as % in respect to $H_2O_2$ treated cells. Peroxide treated cells are considered as 100% ROS production.

## 2.7. Antibacterial activity

### 2.7.1. Susceptibility testing

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

### 2.7.2. Anti-biofilm activity

The effect of the coffee silverskin extracts on the ability of the selected bacterial strains to form a mature biofilm was evaluated by measuring the biofilm biomass accumulated on the surface of polystyrene 96-well tissue culture treated cluster microtiter plates (Corning Inc., NY, USA). The biomass was quantified by the crystal violet method, with minor modifications. Briefly, two different media were used in independent experiments: Tryptone Soya Broth (TSB) supplemented with 1% (w/v) glucose and Muller-Hinton II broth (Oxoid, ). A two-fold serial dilution of the extracts was set in medium (concentration range 1.024 – 0.5 mg $L^{-1}$) and inoculated with 100 µL of a 1:100 dilution of a starting bacterial suspension prepared at 0.5 McFarland units. Biomass was evaluated after 24 h of incubation by removal of supernatant and gently washing with distilled water. Cells in the biofilm were stained with a 0.01% crystal violet water solution, left at room temperature for 15 min, washed three times with distilled water and incubated at 37°C to dryness. Two-hundred µL of 95% ethanol was then added to the wells and shaken for 10 min. 125 µL of the solution of each well were transferred in a new microtiter plate and the optical densities (OD) determined using ELISA microplate reader at 540nm wavelength. The test was performed in duplicate.

**2.8. Statistical analysis**

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

**3. Results and discussions**

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

The analytical method was validated by investigating the linearity, reproducibility and the sensitivity of the method for all the bioactive compounds considered in dynamic MRM mode (**Table 1**). The linearity of the analytical method was assessed by injecting standard solutions of different concentration ranges from 0.001 µg $mL^{-1}$ to 5 µg $mL^{-1}$ in order to build analytical curves of 8 points for each analyte. The coefficients of correlation obtained ranged from 0.993 to 1 confirming thus the high linearity of the method (**Table 1**). The reproducibility of the HPLC-MS/MS method was evaluated by determining the relative standard deviations (% RSDs) after three replicated injections of mix standard solution (0.5 µg $mL^{-1}$) on the same day (intraday precision) and on three consecutive days (interday precision). The intraday precision ranged from 0.4% to 7.6%, while the intraday precision was between 4.7% and 15.2% for all the targeted compounds. The sensitivity of the analytical method was validated by assessing the limits of detection (LODs) and the limits of quantification (LOQs) of each monitored bioactive compound. After the injections of standard solutions of known concentrations, the signal to noise ratios (S/N) were calculated. The LODs and LOQs were estimated as the concentrations of analytes giving the S/N of 3:1 and 10:1 respectively. The LODs obtained ranged from 0.0003 µg $mL^{-1}$ to 0.05 µg $mL^{-1}$, while the LOQs were between 0.001 µg $mL^{-1}$ and 0.2 µg $mL^{-1}$ (**Table 1**).

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

8 extraction procedures were tested to optimize the extraction of the 30 targeted compounds (**Table 1S**). The optimized HPLC-MS/MS method was used to analyze the different extracts. From these experiments, four ultrasound-assisted extraction methods with different extraction solvents (MeOH, water, MeOH/water 50:50, EtOH/water 70:30) were selected considering not only the extraction yields, the effectiveness of compounds extraction and recoveries but also the potentials nutraceutical applications of the extracts. The extraction yields of the four extarcts were respectively of 4.4%, 4.3%, 3.7% and 2.86% for MeOH, EtOH/water 70:30,

MeOH/water 50:50 and water, expressed as w/w dry weight. According to the effectiveness of compounds extraction, the four solvents have been chosen among all, also because the provided the highest total bioactive compounds concentration respectively of 745.4 mg $kg^{-1}$, 725.19 mg $kg^{-1}$, 724.17 mg $kg^{-1}$, and 714.26 mg $kg^{-1}$, for EtOH/water 70:30, MeOH, MeOH/water 50:50 and water. Interesting, the total concentration of bioactive compounds extracted through UAE with water (714.26 mg $kg^{-1}$) was higher with respect to non UAE (661 mg $kg^{-1}$) treatment (method 8 of Table 1S). The mean recoveries obtained by extracting the sample with ethanol:water (70:30) for the 30 bioactive compounds by spiking the sample at 0.25 and 25 mg $kg^{-1}$ were from 88.9-100.3%, with RSDs lower than 11.1% (**Table 3S**). The mean recoveries obtained spiking the sample at 0.5 and 50 (only for caffeine and chlorogenic acids) kg $L^{-1}$ were from 89.1-102.4%, with RSDs lower than 8.4% (**Table 3S**). Also matrix effect has been assessed through matrix-matched-calibration curve and it was negligible for all compounds (*Data not shown*).

The **Table 2** reports the concentrations (expressed as μg $g^{-1}$ of dry weight extract) of the 30 bioactive compounds in the 4 different extracts: E1 (MeOH extract), E2 (water extract), E3 (MeOH/water extract) and E4 (EtOH/water extract). 20 of the 30 monitored compounds were revealed in the analyzed extracts. As an example we reported an HPLC-MS/MS chromatogram of a standard mixture of the 30 bioactive compounds (**Figure 1**) and a chromatogram of CSS extract (**Figure 1S**). The difference of levels could be due to the polarities of the different solvents tested and their preferential interaction with specific classes of compounds.

**3.2.1. Alkaloids.**

Caffeine was the most abundant bioactive compound in all the extracts with content varying from 1.00% to 3.59% of dry weight of extract (dw). These levels are quite high considering that caffeine levels can range between 1.01% and 8.16% of dry coffee beans and dry coffee

extracts (Belay, Ture, Redi & Asfaw, 2008; Jeszka-Skowron, Sentkowska, Pyrzyńska, & De Peña, 2016). Nowadays, the market value of energy drink is considerably increasing. Considering that main component in most energy drinks is caffeine (Alsunni, 2015), CSS extracts could represent an ideal ingredient for the preparation of energy drinks and energy bars (Bondesson, 2015) given their high caffeine content and their low purchase cost. The level of caffeine extracted varied with the extraction solvent used. Indeed, the highest level of caffeine was observed in E4 (3.59%) using ethanol/water (70:30) as extraction solvent. These levels resulted higher than those reported by previous studies, in which the highest caffeine levels were: 1.42% of dw after UAE with water (Guglielmetti et al. 2017); 2.64% of dw after subcritical water extraction (Narita, & Inouye, 2012) and 2.4% using boiling water for extraction (Iriondo-DeHond et al., 2019). Good levels were also observed using water (E2) with caffeine contents closed to 2% of dry weight of CSS extract (**Table 2**). These results showed that UAE can be considered as a good extraction method for the production caffeine-enriched extracts from CSS. Moreover, the simplicity of UAE and the affordable cost of the extraction equipment could allow the application of the proposed method to small and medium-sized enterprises. Quinine was found in all the analyzed extracts with levels ranging from 0.23 μg $g^{-1}$ (E1) – 0.61 μg $g^{-1}$ (E4). Quinine is an alkaloid first isolated from the bark of cinchona tree and known as a potent antimalarial agent (Jones, Panda, & Hall, 2015). Its presence in coffee has been reported in various studies (Tongcumpou, Usapein, & Tuntiwiwattanapun, 2019). However, to our knowledge, this study is the first to reveal the levels of quinine in CSS extracts.

**3.2.2. Polyphenols.**

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

*Phenolic acids.*

Caffeoylquinic acids (3-CQA, 5-CQA and 3,5-diCQA) were the most abundant polyphenols in the different CSS extracts. Their total concentrations ranged from 3115.6 μg $g^{-1}$ to 5444.0 μg $g^{-1}$, corresponding to 78.5 – 96.8% of the total level of polyphenols detected in CSS. These results are in accordance with related studies on CSS phenolic composition (Regazzoni et al., 2016). However, contrary to previous studies, which stated that there is no unconjugated phenolic acid in CSS extract (Bresciani, Calani, Bruni, Brighenti & Del Rio, 2014), this study is one of the first to identify and quantify 7 unconjugated phenolic acids in CSS. Indeed, gallic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and trans-cynnamic acid were detected in CSS with a total concentration between 20.65 μg $g^{-1}$ (E1) and 899.39 μg $g^{-1}$ (E4). Just 3 unconjugated phenolic acids (gallic acid, p-coumaric acid and trans-cynnamic acid) were present in the methanolic extract (E1) while all the 7 were present in the other extracts with highest levels of vanillic acid (345.13 ± 50.11 μg $g^{-1}$), ferulic acid (226.23 ± 6.20 μg $g^{-1}$) and caffeic acid (212.38 ± 27.86 μg $g^{-1}$) in E4.

*Flavonoids.*

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

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

*Xanthone*

Furthermore, small quantities of isogentisin (0.03 – 0.50 μg $g^{-1}$) were found in all the CSS extracts. This compound, which is a polyphenol of the xanthone class, is an important bioactive compound present in *Gentiana lutea* L. Isogentisin is reported to prevent tobacco-induced endothelial diseases (Schmieder et al., 2007) and to inhibit monoamine oxidase (Mustafa et al., 2015). It is important to consider that this study is the first to report the presence and the levels of isogentisin in coffee by-product such as CSS.

*Secoiridoids.*

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

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

**3.3. Spectrophotometric analyses.**

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

**Figure 2** shows the TPC and TFC of the different CSS extracts**.** TPC measured spectrophotometrically provided an estimation of the total levels of polyphenols in the analyzed 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 dw of CSS extract, while the TPC varied according to the solvent of extraction with means levels between 40.4 mg of GAE $g^{-1}$ and 73.4 mg of GAE $g^{-1}$ of dw of extract. The levels obtained in the analyzed extracts were higher than the values reported in other studies using conventional solid-liquid extraction methods. For instance, Panusa et al., (2017) obtained CSS extracts with TPC ranging from 4.35 to 12.82 mg of GAE $g^{-1}$ of dw after conventional extraction with water at 60 °C for 30 min. EtOH: $H_2O$ (70:30) allow to obtain the highest TPC in CSS extracts (73.4 ± 6.6 mg of GAE $g^{-1}$ dw). As reported by Costa et al. (2014), 100% water resulted the worst solvent to extract phenolic compounds from CSS. In fact, the lowest TPC was observed in CSS extracts prepared with water as extraction solvent (40.4 ± 2.9 mg of GAE $g^{-1}$ dw). This result is in accordance with the levels observed after HPLC quantification. Nevertheless, the TPC of the aqueous extract (E2) resulted higher than those reported in previous studies, in which water was used as solvent for solid-liquid CSS extraction. Indeed, Conde, & Mussatto, (2016) obtained CSS extracts with TPC of 19.2 mg of GAE $g^{-1}$ of dw and TFC of 2.73 mg of RE $g^{-1}$ of dw after hydrothermal treatment at 120°C for 20 min. These results highlight therefore, the effectiveness of UAE in obtaining CSS extracts with high levels of polyphenols. Narita &Inouye (2012) obtained CSS extracts with highest TPC of 130 ± 6 mg GAE $g^{-1}$ of dw by using subcritical water extraction (240 °C, 3.2 MPa). These levels are higher than those obtained in this study. However, supercritical extraction requires significant investments in special equipment, unlike UAE, which provides high extraction efficiency while remaining affordable for laboratories and small industries.

The difference observed between the spectrophotometric and HPLC results can be explained by the higher selectivity of the HPLC analysis, which provided a quantification of specific

analytes. Therefore, considering both approaches is useful to understand the effect of solvent on bioactive compounds extraction.

### 3.3.2. Radical scavenging activity (RSA)

The radical scavenging activity of the investigated CSS extracts was determined by DPPH assays. The DPPH values varied in the different extracts, showing that the type of solvent affected the antioxidant capacity of CSS extracts (**Figure 2**). The methanolic extracts (E1) showed the highest RSA with an $IC_{50}$ of 101.7 ± 5.5 μg $mL^{-1}$, while the lowest RSA were observed in the aqueous extract (E2) with an $IC_{50}$ of 362.1 ± 65.7 μg $mL^{-1}$. It could be interesting to note that according to HPLC-MS/MS analyses results, E1 and E2 tended to be respectively the most and the least concentrated extracts in monitored polyphenols (**Table 2**). However, there was no evidenced correlation between the DPPH inhibition of the studied extracts and their respective TPC. This might suggest that the antioxidant activity of CSS extracts may be due to other compounds than polyphenols such as melanoidins and diterpenes which are present in CSS and possess relevant antioxidant activities (Costa et al., 2014; Mesías, & Delgado-Andrade, 2017). These antioxidant compounds are less soluble in water (Belandria et al., 2016) and this could explain why CSS extracts obtained from water (E2) and water containing solvents (E3 and E4) showed lowers DPPH inhibition than E1, which was obtained from 100% methanol. Moreover, DPPH is a lipophilic radical, which could limit the accessibility of hydrophilic antioxidant present in CSS extracts (Choi, & Koh, 2017).

### 3.4. Neuroprotective activity of silverskin extracts against $H_2O_2$-induced damage

To investigate the potential cytotoxicity of E1, E2, E3, and E4 extracts, differentiated SH-SY5Y cells were treated with different concentrations (1–200 μg $mL^{-1}$) of the four extracts for 24 h (**Figure 3**). Until 100 μg $mL^{-1}$ all the extracts did not reduce cell viability in respect to control cells indicating that they are not cytotoxic. Only E4 reduced cell viability at the highest concentration and for this reason 200 μg $mL^{-1}$ E4 has not been considered in the next

experiments. Of note, E1, E2, E3, and E4 significantly increased cell viability at 1-100 µg $mL^{-1}$. The observed increase in cell viability could be ascribed to an enhancement of mitochondrial respiration. In fact, MTT measures cell viability in terms of reductive activity as enzymatic conversion of the tetrazolium compound to water insoluble formazan crystals by dehydrogenases occurring in the mitochondria of living cells (Mosmann, 1983). On these bases, the MTT assay is dependent on mitochondrial respiration. In our opinion, the increased mitochondrial respiration could be related to the presence of caffeine. Caffeine has been shown to increase mitochondrial content by increasing the expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is a regulator of nuclear respiratory factors 1 and 2 (NRF1/2) (McConell, Ng, Phillips, Ruan, Macaulay, & Wadley, 2010; ; Ojuka, Jones, Han, Chen, Wamhoff, Sturek, & Holloszy, 2002; Schnuck, Gould, Parry, Johnson, Gannon, Sunderland, & Vaughan, 2018). PGC-1α and NRF1/2 modulate the expression of mitochondrial transcription factor A (TFAM), which regulates the expression of respiratory components of the electron transport chain (Li, Hou, & Hao, 2017) Interestingly, there is no a direct correlation between the content of caffeine in the four extracts and the increase in cell viability. This could be due to the presence in the extracts of compounds other than caffeine that could modulate both caffeine uptake and caffeine metabolism. In the future, it would be interesting to study the effect of pure caffeine on SH-SY5Y cell line to better clarify this point. Moreover, in order to verify that the increase in cell viability is not due to a de-differentiation we evaluated the expression of two well-known markers of neuronal differentiation: neuronal nuclear antigen (NeuN) and synaptophysin (SYP), (Borsani, Buffoli, Bonazza, Brunelli, Monini, Inchingolo, Ballini, Rezzani, & Rodella 2020). In vitro effects of concentrated growth factors (CGF) on human SH-SY5Y neuronal cells. *Eur Rev Med Pharmacol Sci*, 24, 304-314) (Figure 2S A, B). Interestingly, all the treatments did not modify the expression level of NeuN and SYP. Moreover, to further

demonstrate that the observed increase in cell viability is not due to an increased cell replication, we treated differentiated SH-SY5Y cells with 100 μg $mL^{-1}$ of each extract and after 24 h the number of cells was counted. Of note, the treatments maintain the number of cells to value comparable to control cells (data not shown). To study the potential protective activity of the extracts against oxidative stress, cells were treated with increasing concentrations (1–200 μg $mL^{-1}$) of the four extracts before the induction of oxidative stress by 700 μM $H_2O_2$ exposure for 1 h (**Figure 4**). This peroxide concentration has been chosen as it reduces cell viability by 50% with respect to control cells (**Figure 4**). Moreover, similar $H_2O_2$ concentrations have been recently used by Piras et al. (2016) in differentiated SH-SY5Y cells. At the lower concentrations, only E1 and E4 extracts showed a protective activity against peroxide exposure as they significantly increased cell viability in respect to $H_2O_2$ treated cells at 1-10 μg $mL^{-1}$. On the contrary, E2 and E3 increased cell viability in respect to peroxide exposed cells only at concentrations higher than 50 μg $mL^{-1}$. To verify if the observed protective effects could be related to a reduction of endogenous ROS levels, differentiated SH-SY5Y cells were treated with the four extracts at 1-200 μg $mL^{-1}$ prior to the addition of $H_2O_2$ and the level of intracellular ROS was determined using the peroxide-sensitive fluorescent probe DCFH-DA (**Figure 5**). In agreement with viability data, E1 and E4 were the most effective in reducing ROS levels. In particular, E1 significantly reduced ROS levels at 10 and 50 μg $mL^{-1}$, meanwhile E4 significantly reduced ROS levels at 50 and 100 μg $mL^{-1}$. E2 and E3 significantly decreased ROS levels only at the highest concentration. Of note, these data are in agreement with the RSAs of the 4 extracts obtained by DPPH assay, indicating that E1 and E4 extracts exert a higher antioxidant activity both in vitro and in cells. Interestingly, there is no correlation between the concentrations of E1 and E4 that significantly protected against peroxide and the concentrations that reduced ROS levels suggesting that the protective mechanisms could be not only ascribed to an antioxidant activity. Moreover, E1 greater

effectiveness in countering oxidative stress in respect to E2 and E3 seems to be related to a higher level of total polyphenols and in particular to a significantly higher concentration of 5-CQA and epicatechin. 5-CQA is an isomer of caffeoylquinic acid (CQA) that is the ester formed between one molecule of caffeic acid and one molecule of quinic acid (Liang, Dupuis, Yada, Kitts, 2019). Among CQA isomers, 5-CQA has been shown to possess the higher antioxidant activity in vitro (Liang, 2019). This compound has also been investigated in cell model system. In particular, 5-CQA lowered ROS production and recovered depleted GSH in endothelial EA.hy926 cells exposed to TNF-α. (Wang, Sarriá, Mateos, Goya & Bravo-Clemente, 2018). Similar results were observed in Caco2 cells challenged with the inflammatory and oxidative stress mediators PMA + IFNγ (Liang, & Kitts, 2018). Pre-treatment with 5-CQA ameliorated oxidative stress by reducing intracellular ROS, increasing reduced GSH, and activating Nrf2 signaling pathway. The main biological functions of epicatechin are the production of protein complexes, the scavenging of free radicals, and the reduction of lipid peroxidation, making it a great antioxidant (Bernatova, 2018). Rats pre-treated with epicatechin showed a reduced deterioration of spatial memory induced by the Aβ25–35, related to a reduction of oxidative stress and inflammation in the hippocampus (Diaz et al., 2019). A very recent paper demonstrated the protective effect of epicatechin against neuronal cell death via oxidative stress and ER stress induced by methamphetamin. In particular, epicatechin inhibited ROS generation as well as MAPK activity, and CHOP and DR4 expression, during methamphetamine-mediated apoptosis. Leonardo et al. (2013) showed that epicatechin protects mouse cortical neurons from oxygen/glucose deprivation trough the activation of Nrf2-mediated pathway and the upregulation of the antioxidant enzyme heme oxygenase 1. The ability of epicatechin to modulate Nrf2 pathway and to increase HO1 activity has also been demonstrated in vivo using Nrf2 and HO1 knockout mice (Shah et al., 2010). The same authors demonstrated that epicatechin protects embryonic

cortical neuronal cells against oxidative stress induced by $H_2O_2$ and tert-butyl hydroperoxide. On the bases of these studies we hypothesize that the protective effect showed by E1 could be related to its higher content of 5-CQA and epicatechin in respect to the other extracts. The specific composition of E4, characterized by a higher content of p-coumaric acid, ferulic acid, 3,5-diCQA, and quercitrin in respect to the other extracts, could be related to its protective activity against oxidative stress. All of these phenolic compounds have been demonstrated to be effective against oxidative stress in different cell systems. In PC12 neuronal cells, p-coumaric acid, a hydroxy derivative of cinnamic acid, strongly suppressed the accumulation of intracellular ROS and protected from Aβ toxicity (Hong, Jeong, & Jun, 2012). Ferulic acid is one of the most studied phenolic acid derivatives for its neuroprotective activity. In neuronal cells, ferulic acid counteracted oxidative/nitrosative stress caused by Aβ exposure or other radical initiators, with different mechanisms, including the activation of cell stress response (Picone et al., 2009). In SH-SY5Y cells 3,5-diCQA, a caffeoylquinic acid derivatives, attenuated the neuronal death and caspase-3 activation induced by $H_2O_2$ (Kim, Park, Jeon, Kwon & Chun, 2005). In addition, 3,5-diCQA restored $H_2O_2$-induced depletion of intracellular glutathione. Quercitrin is a glycosylated form of quercetin and the sugar bound to the aglycone portion increases solubility in polar solvents and consequently improves absorption of quercitrin in respect to quercetin (Gee, Dupont, Rhodes, & Johnson, 1998) Different studies evidenced quercitrin anti-oxidative and anti-inflammatory activity (Rattanajarasroj, & Unchern, 2010). Quercitrin antioxidant effect has also been demonstrated in ICR mice treated with carbon tetrachloride (Ma, Luo, Jiang, & Liu,2015). In particular a 4 weeks treatment with quercitrin suppressed the elevation of reactive oxygen species (ROS) production and malondialdehyde (MDA) content, reduced tissue plasminogen activator (t-PA) activity, enhanced the antioxidant enzyme activities and abrogated cytochrome P450 2E1 (CYP2E1) induction in mouse brains. In conclusion data obtained in SH-SY5Y cells showed

a higher effectiveness of E1 and E4 in protecting cells against $H_2O_2$-induced damage related to their specific pattern of phytochemicals. Moreover, these findings suggest a potential role of E1 and E4 extracts as preventive/protective agents against neurodegeneration due to their ability to counteract oxidative stress and maintain cell viability.

**3.5. Antimicrobial activity**

All silverskin extracts were not inhibiting the growth of anyone of the bacterial species included in this study. The minimal inhibitory concentrations (MIC) would have to be searched for at concentration above 512 mg $L^{-1}$, values which hamper the realistic utilization of the crude extracts as antimicrobials. Actually, the extracts contain many bioactive compounds with proven antimicrobial activities, such as 5-CQA, caffeic acid and caffeine (Dash et al., 2008). Besides their high relative amount into the extracts, their absolute amount is far below the MICs reported in the literature for the single compounds. For instance, MIC of caffeine against *S. aureus* and *E. coli* is > 200 mg $L^{-1}$ (Dash et al., 2008). To reach these concentrations, at least 5 mg $mL^{-1}$ of extract would have to be used. The inactivity at the tested concentrations additionally indicated that there was not a synergism by the complex and unique mixture of components, which is often an interesting feature of natural extracts. Also in this respect, the silverskin extracts did not show interesting profiles of antimicrobial activity. Additionally, given the importance of biofilms, a screening of the potential anti-biofilm activity of the silverskin extracts has been conducted by measurement of the biomass produced by the different species after 20 hours of growth in two different media. This widely used approach gives an estimation of the amount of mature biofilm formed and developed onto a surface. Overall the extracts did not show a strong inhibitory effect on biofilm formation and maturation. For *S. aureus* and *E. coli* the trend is toward inhibition (**Table 3**). However, the highest contributions to the obtained significant negative Pearson correlation coefficients (e.g. E2 and E3 vs *S. aureus* and E1, E2, and E4 vs *E.coli*) was given by the

higher extract concentrations (> 128 mg $L^{-1}$). In the case of *P. aeruginosa*, instead, the trend was opposite, that is the Pearson correlation coefficients were positive, indicating a general stimulatory effect of extracts on the biofilm biomass accumulation. Only the water extract E2 was an exception not showing any concentration dependent effect (r = -0.07). Considering the different average composition of silverskin methanol extract E1 in respect to the others, a paired mean differences comparison analysis was performed between E1 and E2, E1 and E3, E1 and E4 (**Figure 3S**). Results indicated that in the case of *S. aureus* E2 and E4 were more effective in decreasing the biomass formation, even if only E2 was doing it significantly (P = 0.006, two-sided, Wilcoxon test). *E. coli* biofilm biomass was not differentially influenced by the E1 in respect to the extracts obtained by the water based solvents, with the exception of E4 that was less effective, albeit at a low level of statistical significance (P = 0.029, two-sided, Wilcoxon test). At last, *P. aeruginosa* behaviour in the presence of different concentration of extracts was similar to that shown by *S. aureus* with E4 having instead the major effect on biofilm biomass reduction (P = 0.006, two-sided, Wilcoxon test). Overall, data indicated that silverskin extracts obtained by water-based solvents might deserve a deeper future investigation on biofilm-related activities, such as quorum sensing or virulence factors' expression.

**Conclusions.**

This study is one of the first to provide a deepen characterization of bioactive compounds in CSS. UAE, an affordable extraction technique, allowed to obtained CSS extracts with high content of caffeine, chlorogenic acids and flavonoids and the total of the 30 bioactive compounds monitored in this tudy represent 1.56 - 4.01% w/w of CSS extracts. Data obtained on SH-SY5Y cells showed a higher effectiveness of E1 and E4 extracts in protecting cells against $H_2O_2$-induced damage related to their specific pattern of phytochemicals, suggesting a potential role as protective agents against neurodegeneration due to their ability to counteract

oxidative stress and maintain cell viability. Moreover, data indicated that silverskin extracts obtained by water-based solvents might deserve a deeper future investigation on biofilm-related activities, such as quorum sensing or virulence factors' expression. The low cost of CSS and the biological activities of the obtained extracts, which are attributed to their phytochemical compositions, could suggest a possible application of CSS extracts as ingredients in food and pharmaceutical formulations.

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**Figures legend**

**Figure 1.** HPLC-MS/MS chromatogram of a standard mixture of the 30 bioactive compounds plotted as overlapped multiple reaction monitoring (MRM) transition of each compound.

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

**Figure 3.** Viability of differentiated SH-SY5Y treated with the extracts. Cells were treated with increasing concentration of E1, E2, E3, and E4 (1-200 μg $mL^{-1}$) and after 24 h cell viability was evaluated by MTT assay. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Dunnett's test. *$p<0.05$ with respect to CTRL.

**Figure 4.** Viability of differentiated SH-SY5Y treated with the extracts in the absence/presence of $H_2O_2$. Cells were treated with increasing concentration of E1, E2, E3, and E4 (1-200 μg $mL^{-1}$) for 24 h, exposed to 700 μM $H_2O_2$ for 1 h and cell viability was evaluated by MTT assay. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *$p<0.05$ with respect to $H_2O_2$.

**Figure 5.** Antioxidant activity of the extracts against $H_2O_2$ in differentiated SH-SY5Y cells. Cells were treated with E1, E2, E3, and E4 (1-200 μg $mL^{-1}$) after 24 h were exposed to $H_2O_2$. Intracellular ROS levels were measured with the peroxide-sensitive probe DCFH-DA. Data are expressed as percentage with respect to $H_2O_2$-treated cells. Each bar represents means ± SEM of at least four independent experiments. Data were analyzed by one-way ANOVA followed by Bonferroni's test. *$p<0.05$ with respect to $H_2O_2$;

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

| Compounds | Concentration range (µg ml$^{-1}$) | Regression Equation | $R^2$ | LOQs[a] | LODs[b] |
|---|---|---|---|---|---|
| Shikimic acid | 0.05-5 | y = 7937.8x +736.11 | 0.994 | 0.1 | 0.03 |
| Gallic acid | 0.01-5 | y = 13243x - 1329.5 | 0.993 | 0.05 | 0.02 |
| Loganic Acid | 0.01-5 | y = 6662x + 48.058 | 1 | 0.01 | 0.003 |
| 5-caffeoylquinic acid | 0.01-5 | y = 16814x - 1057.5 | 0.997 | 0.01 | 0.003 |
| Swertiamarin | 0.005-5 | y = 7685.1x + 15.302 | 1 | 0.005 | 0.002 |
| Gentiopicroside | 0.05-5 | y = 1706.4x - 1.6666 | 1 | 0.2 | 0.05 |
| (+) Catechin | 0.01-5 | y = 6481.3x + 31.934 | 1 | 0.01 | 0.003 |
| Delphinidin 3,5-diglucoside | 0.1-5 | y = 4483.2x - 433.35 | 0.998 | 0.1 | 0.03 |
| Sweroside | 0.01-5 | y = 2109.5x + 6.4729 | 1 | 0.01 | 0.003 |
| 3-caffeoylquinic acid | 0.005-5 | y = 29047x - 610.29 | 0.999 | 0.005 | 0.002 |
| Caffeine | 0.005-5 | y = 135892x + 1311.1 | 1 | 0.004 | 0.001 |
| Cyanidin 3-glucoside | 0.005-5 | y = 241096x - 15411 | 0.997 | 0.004 | 0.001 |
| Vanillic acid | 0.05-5 | y = 857.52x - 19.701 | 0.999 | 0.05 | 0.02 |
| Caffeic acid | 0.01-5 | y = 23436x - 537.21 | 0.999 | 0.01 | 0.003 |
| (-)Epicatechin | 0.01-5 | y = 6524.8x + 151.77 | 1 | 0.01 | 0.003 |
| Syringic acid | 0.005-5 | y = 2125.6x - 154.2 | 0.995 | 0.005 | 0.002 |
| P-coumaric acid | 0.005-5 | y = 28093x - 2.4157 | 0.999 | 0.005 | 0.002 |
| Ferulic acid | 0.01-5 | y = 5853.5x - 113.69 | 0.999 | 0.01 | 0.003 |
| 3,5-Dicaffeoylquinic acid | 0.005-5 | y = 22621x - 1430.1 | 0.996 | 0.005 | 0.002 |
| Quinine | 0.001-5 | y = 129606x - 3926.8 | 0.998 | 0.001 | 0.0003 |
| Naringin | 0.001-5 | y = 9379.7x + 110.96 | 1 | 0.001 | 0.0003 |
| Rutin | 0.001-5 | y = 15411x - 532.59 | 0.998 | 0.001 | 0.0003 |
| Hyperoside | 0.001-5 | y = 31512x + 325.65 | 1 | 0.001 | 0.0003 |
| Trans-Cinnamic acid | 0.005-5 | y = 54199x + 1479.1 | 0.999 | 0.05 | 0.02 |
| Resveratrol | 0.005-5 | y = 11091x + 297.04 | 1 | 0.005 | 0.002 |
| Amarogentin | 0.001-5 | y = 26116x + 212.77 | 0.999 | 0.001 | 0.0003 |
| Quercitrin | 0.001-5 | y = 24198x + 468.93 | 0.999 | 0.001 | 0.0003 |
| Kaempferol | 0.001-5 | y = 24224x + 500.82 | 0.999 | 0.001 | 0.0003 |
| Quercetin Dihydrate | 0.005-5 | y = 19350x + 913.34 | 0.997 | 0.005 | 0.002 |
| Isogentisin | 0.001-5 | y = 175952x + 19759 | 0.993 | 0.001 | 0.0003 |

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

**Table 2.** Quantificaction of the 30 bioactive compounds (expressed as μg $g^{-1}$ of dry weight extract) in different coffee silverskin extracts by HPLC-ESI-MS/MS analysis.

| 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 |
| **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 |

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

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

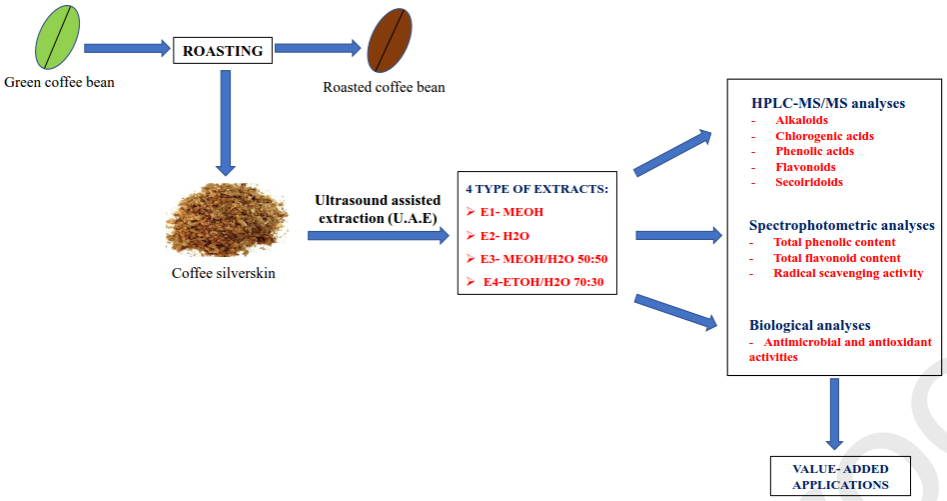
| Extracts | *S. aureus* | | *E. coli* | | *P. aeruginosa* | |
|---|---|---|---|---|---|---|
| | Pearson's *r* | P Value | Pearson's *r* | P Value | Pearson's *r* | P Value |
| E1 | -0.52 | .0852 | -0.71 | .0144 | 0.70 | .0108 |
| E2 | -0.88 | .0004 | -0.86 | .0007 | -0.07 | .8216 |
| E3 | -0.68 | .0148 | -0.24 | .4829 | 0.45 | .1437 |
| E4 | -0.33 | .2948 | -0.76 | .0069 | 0.84 | .0006 |

Highlights:

- Silverskin can be used as raw material for the recovery of functional compounds
- A new HPLC-MS/MS method has been developed for 30 bioactive compounds of different subclasses
- The monitored bioactive compounds represent 1.6-4% of silverskin extracts
- Silverskin extracts play a potential role as protective agents against neurodegeneration

GA

*Graphical Abstract



## Credit Author Statement:

**Franks Kamgang Nzekoue**: Conceptualization, Methodology, Writing- Original draft preparation; **Simone Angeloni:** Methodology, Validation, invesitgation; **Luciano Navarini:** Resources; **Cristina Angeloni:** Writing- Original draft preparation; **Michela Freschi:** Investigation; data curation **Silvana Hrelia:** Supervision; **Luca A. Vitali:** Software, Validation; **Gianni Sagratini:** Conceptualization; Data curation **Sauro Vittori:** Supervision; **Giovanni Caprioli**: Data curation, Supervision, Writing- Reviewing and Editing