

1 **Spent espresso coffee grounds as a source of anti-proliferative and**
2 **antioxidant compounds**

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1 **Abstract**

2 Disposal of spent espresso coffee grounds (SCG) is costly and leads to the loss of bioactive
3 compounds that could be fractionated, in several applications. This work aimed to investigate
4 phenolic profile, tocopherols, and antioxidant and anti-proliferative activities of SCGs ethanolic
5 extracts from coffee powders differing in coffee provenience and composition (arabica/robusta).
6 Tyrosol, detected for the first time in SCGs, was the most abundant phenolic measured (121-1,084
7 mg/kg in the extract), along with 4-hydroxybenzoic acid and vanillin (885-1813 and 340-1103 mg/kg,
8 respectively). Extract derived from 100% robusta from Guatemala (S7-R) showed the highest α - to
9 β -tocopherol ratio of 1.2 and the highest antioxidant potential as evidenced by RACI and GAS values
10 of -0.43 and 0.20, respectively. Moreover, S7-R showed a promising anti-proliferative activity toward
11 human lung carcinoma cells (A549), with IC_{50} value of 61.2 $\mu\text{g/mL}$ comparable to that given by the
12 positive control vinblastine (IC_{50} value of 67.3 $\mu\text{g/mL}$).

13

14 **Keywords:** coffee by-products; polyphenols; tocopherols; tyrosol; antioxidant properties; anti-
15 proliferative activity.

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1 **1. Introduction**

2 Coffee is one of the world's most widely consumed beverages. World coffee consumption is
3 estimated at 9714 million of kg in year 2017/18, 1.8% higher than previous year (2016/17). Colombia,
4 Vietnam, Indonesia, Ethiopia and Brazil are the major producers whilst Europe is responsible for the
5 highest consumption (one-third of total coffee worldwide consumption) (Brazilian Association of
6 Coffee Industry, 2018). Recently, the coffee-producing nations are also increasing their domestic
7 consumption. China, Panama, Kenya, Senegal and United States are considered the highest potential
8 markets in the coming years (International Coffee Organization, 2018).

9 The coffee industry has a significant impact on the environment, producing more than 2 billion
10 tons of coffee by-products (bio-waste) annually. These by-products are generated by the treatment
11 and processing of coffee cherries, the roasting of green coffee beans and the coffee-brewing
12 preparation. Due to the high organic load and the presence of compounds such as caffeine, tannins,
13 and polyphenols, coffee by-products can have negative effects on the environment. Thus, their
14 disposal needs to be properly managed. Following the policies introduced by European Commission
15 for food waste management (Directive 2008/98/EC, 2008; EU Publication (2012)], innovative
16 research is expected to improve the management of food by product resources and open new markets
17 in novel functional foods.

18 In this scenario, great attention has been devoted to find ways of reducing spent coffee grounds
19 (SCGs) environmental impact and /or transforming them into added-value products. SCGs consist in
20 solid residues left after the production of coffee beverages and instant coffee. Annually, the
21 worldwide production of SCGs are accountable for about 6 million tons (Getachew & Chun, 2017).
22 A total of 50% of SCGs come from the industrial preparation of instant soluble coffee and the
23 remaining 50% come from the worldwide production of different coffee brews in cafeterias,
24 restaurants and homes (Getachew & Chun, 2017). SCGs are characterized by fine particle size, high
25 organic load and moisture content. The composition of SCGs includes an oil fraction (7.9–26.4%),
26 crude fiber (19.7–22.1%), and several bioactive compounds (caffeine, trigonelline, phenolics,

1 minerals, lignin, and melanoidins) (Monente, Ludwig, Irigoyen, De Peña & Cid, 2015a; Cruz et al.,
2 2012). Due to their functional properties (*i.e.*, water-holding capacity, oil-holding capacity, and
3 emulsifying activity), raw SCGs have been applied as biosorbents and as fillers and additives for
4 polymer composites (Moustafa, Guizani, Dupont, Martin, Jeguirim, & Dufresne, 2017). Many works
5 have also investigated the possibility to reutilize raw SCGs as soil fertilizers, due the slow release of
6 nitrogen in the soil, and as supplements in animal feed (Cruz et al., 2012). However, the high caffeine,
7 tannin, and chlorogenic acid amounts found in SCGs result are toxic to plants and soil
8 microorganisms and confer unpleasant taste to animal feeds (Janissen, & Huynh, 2018).

9 In light of this, although direct utilization of raw SCGs can be seen as one of the economically
10 advantageous paths, fractionation of SCGs may enlarge their applicability. Fractionation of SCGs
11 allows secondary uses of the oil fraction, phenolic compounds, and carbohydrates, as well as
12 remaining residual materials. Carbohydrates and crude fiber isolated from SCGs are a good substrate
13 for fermentation technology (Ramalakshmi, Rao, Takano-Ishikawa & Goto, 2009). SCGs oil has been
14 also applied in biotechnological production of ethanol (Al-Hamamre, Foerster, Hartmann, Kröger &
15 Kaltschmitt, 2012).

16 The extraction of antioxidant compounds from SCGs would allow the recovery of bioactive
17 compounds useful for the food industry. SCGs polyphenol-rich extracts are exploited in food
18 manufacturing as shelf-life enhancers, stabilizers and for food fortification. Several authors (Campos-
19 Vega, Loarca-Pina, Vergara & Oomah, 2015; Monente, Bravo, Vitas, Arbillaga, De Peña & Cid,
20 2015b) reported that phenolic compounds, caffeine and melanoidins found in espresso SCGs water
21 extract are able to act as antimicrobial agents for extend food shelf-life, mainly acting against Gram-
22 positive bacteria and yeast. Kim, Ahn, Eun, & Moon (2016) evaluated the antioxidant effect of SCGs
23 ethanol and water extracts in oil and raw /cooked meat system. Ethanolic extract was effective in
24 preventing lipid oxidation in oil emulsion and raw meat systems, but was not effective enough to
25 prevent oxidative changes in cooked-meat packaged in oxygen permeable bags for more than 3 days.

1 Jiménez-Zamora, Pastoriza & Rufián-Henares (2015) evaluated the *in vitro* antimicrobial, prebiotic
2 and antioxidant activity of SCGs extracts and their melanoidins fraction. Authors underlined that
3 coffee melanoidins interfere with the prebiotic activity of SCGs. On the contrary, SCG-coffee
4 melanoidins fraction exerts an intense antimicrobial activity that could inhibit the growth of
5 pathogenic bacteria in food products. In addition, SCGs and SCG-coffee melanoidins were highly
6 antioxidant. More recently, Iriondo-DeHond *et al.* (2019) reported the chemical profile and the
7 antioxidant activity of coffee by-products obtained before and after the roasting (husk and parchment,
8 and silverskin, respectively) and suggest the use of these by-products as healthy food ingredients.

9 In this context, the present study was aimed to investigate the chemical and biological properties
10 of ethanolic extract from SCGs deriving from the preparation of espresso coffee. In order to evaluate
11 the potential application of SCGs extracts as suitable ingredient in formulations of
12 nutraceutical/functional foods, we have screened the ethanolic extracts for their polar phenolic
13 substances content and composition, α - and β -tocopherol content, antioxidant and anti-proliferative
14 activities. Taking into account that the presence of bioactive compounds in espresso SCGs, as well
15 as in espresso beverage, could depend on the coffee variety of the starting material, and different
16 espresso SCGs samples obtained from different coffee blend mixtures (arabica and robusta) but with
17 the same conditions for brewing process, were investigated. In this way, differences in terms of
18 composition and bioactivity among the SCGs samples could be determined, highlighting the
19 compositional variation presented by espresso SGCs.

20

21 **2. Materials and Methods**

22 **2.1 Chemicals and reagents**

23 All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical
24 Co. Ltd (Milan, Italy) and VWR International (Milan, Italy) and, unless specified otherwise, were
25 analytical grade or higher. Water (resistivity above 18M Ω *cm) was obtained from a Milli-Q SP
26 Reagent Water System (Millipore, Bedford, MA, USA). All the solvents and solutions were filtered

1 through a 0.45µm PTFE filter from Supelco (Bellefonte, PA, USA) before use. Cell culture and cell
2 culture materials were obtained from Sigma-Aldrich (Milan, Italy).

3

4 **2.2 Preparation of espresso coffee and collection of SCG samples**

5 The roasted coffee beans from *Coffea arabica* and *C. canephora* var. robusta and roasted coffee
6 blends were supplied by a local industrial coffee roaster (Caffè del Faro, Robin S.r.l., Montegranaro,
7 Italy). Arabica coffees were from Puerto Rico, Colombia, Brazil, Guatemala, Costa Rica and Ethiopia
8 whilst robusta coffee beans were from India, Vietnam and Guatemala (Table 1). Three 100% arabica
9 coffee samples, two coffee blends with different arabica/robusta ratio, one 100% robusta coffee
10 sample and one decaffeinated coffee blend sample were selected. Coffee samples were collected
11 during two different crop years in order to reduce the differences correlated to the harvest year and to
12 the blending lots. All coffee mixtures were roasted at 175°C for 15 minutes.

13 The espresso coffees were prepared by using a coffee machine VA388 Black Eagle Gravitech
14 (Victoria Arduino, Italy). The grinding was performed by using a grinder machine K30 Mahlkönig
15 (Hemro Manufacturing, Germany GmbH). Ground coffee (7 g) was inserted in a 1-cup filter holder,
16 water temperature was 92 °C and pressure, 9 atm. The total collected coffee volume was 20 mL. For
17 each coffee sample, the brew preparation was repeated in triplicate.

18

19 **2.3 Preparation of the SCG ethanolic extract**

20 After the brew preparation, the obtained SCGs were dehydrated at 65°C for 12 h and extracted
21 by maceration. Briefly, 7 g of dried SCG and 30 mL of anhydrous ethanol were transferred into glass
22 flasks and the mixture magnetically stirred in the dark, at 25°C for 12 h. Then, the top phase was
23 filtered through Whatman filter paper #4 (9.0-cm diameter) and the collected solution was dried in a
24 rotary evaporator.

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1 **2.4 Ultra high performance liquid chromatography - fluorescence (UHPLC-FLD) analysis of**
2 **tocopherols**

3 The tocopherols profile of the ethanolic extracts was determined following the procedure
4 reported by Giardinieri et al. (2019). Briefly, ethanolic extract (30 mg) was dissolved in 5 mL of
5 acetonitrile and UHPLC-FLD separation was performed on Ascentis® Express C18, (75 x 4.6 mm,
6 2.7 µm, from Supelco). The mobile phase was acetonitrile/methanol (90:10, v/v), at a flow rate of
7 0.45 mL/min. The injection volume was 1 µl. FLD was set with an excitation wavelength of 290 nm
8 and an emission wavelength of 330 nm. For the quantification, seven standard stock solutions of each
9 tocopherol in acetonitrile were prepared in the range 25-250.0 µg/mL and analysed to obtain the
10 calibration curve (correlation coefficients $R^2 = 0.996-0.999$).

11

12 **2.5 Ultra-high performance liquid chromatography (UHPLC) – high-resolution mass spectrometry**
13 **(HRMS) determination of phenolic compounds**

14 UHPLC-HRMS analysis of ethanolic extracts were carried out using an Accela UHPLC system
15 (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a quaternary pump. following the
16 procedure reported by Loizzo et al (2019). Briefly, Ascentis Express C18 (150 × 2.1 mm, 2,7 µm)
17 (Supelco, Milan, Italy) column was employed. The mobile phase was a mixture of solvent A (0.1%
18 formic acid in water) and solvent B (0.1% formic acid in acetonitrile) and the flow rate was 300
19 µL/min. Gradient elution program: from 0-1 min, 10 % B; 1-20 min, linear gradient from 10 to 95%
20 B; 20-23 min, isocratic step at 95% B; 23-25 min, back to initial conditions at 10% B, and 25-30 min,
21 isocratic step at 10% B for column re-equilibration. The UHPLC system was coupled to a Q-Exactive
22 quadrupole-Orbitrap (Thermo Fisher Scientific) mass spectrometer equipped with a heated
23 electrospray probe ionization source (H-ESI II). HESI-II was operated in negative ionization mode.
24 Nitrogen was used as a sheath gas, sweep gas and auxiliary gas at flow rates of 60, 0 and 10 a.u.
25 (arbitrary units), respectively. Heater temperature was set at 350 °C. Capillary temperature was set at
26 320 °C and electrospray voltage at– 2.5 kV. The HRMS instrument was operated in full MS scan

1 with a m/z range from 100 to 1500, and the mass resolution tuned into 70,000 full-width half-
2 maximum (FWHM) at m/z 200, with an automatic gain control (AGC) target (the number of ions to
3 fill C-Trap) of $5.0E5$ with a maximum injection time (IT) of 200 ms. Thirty-five polyphenols were
4 targeted, eighteen of which were detected and/or quantified in the samples, as discussed in section
5 3.3. Polyphenols that were not detected were: gallic acid, 2,5-dihydroxybenzoic acid, resveratrol,
6 ellagic acid, homogentisic acid, sinapic acid, morin hydrate, ursolic acid, rutin hydrate, quercetin,
7 myricetin, polydatin, *trans*-cinnamic acid, asiatic acid, genkwanin, rosmanol and rosmarinic acid.
8 Identification and confirmation criteria based on chromatographic retention time, accurate mass
9 measurements (with errors lower than 5 ppm), and isotopic pattern match, as well as comparison with
10 product ion scan spectra were employed.

11

12 **2.6 Antioxidant activity**

13 **2.6.1 Radical scavenging assays**

14 Radical scavenging activity of SCGs ethanolic extracts was investigated by using two different
15 spectrophotometric methods namely 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-
16 ethylbenzothiazoline-6-sulphonic acid (ABTS) test (Loizzo et al., 2015, 2019). Results of DPPH
17 radical scavenging activity was expressed as follow: scavenging activity = $[(A_0 - A_1)/A_0] \times 100$, where
18 A_0 is the absorbance of the blank, and A_1 is the absorbance in the presence of the spent coffee extract.
19 For the ABTS test a solution of $ABTS^+$ and potassium persulphate was prepared. After 12 hours the
20 solution was diluted with anhydrous ethanol until an absorbance measure of 0.70 at 734 nm by using
21 Perkin Elmer 40 UV-VIS spectrophotometer (Milan, Italy). The spent coffee extract and diluted
22 $ABTS^+$ solution were mixed and after 5 min the absorbance was read. The $ABTS^+$ scavenging ability
23 was calculated as follow: scavenging activity = $[A_0 - A]/A_0 \times 100$, where A_0 is the absorbance of the
24 control reaction and A is the absorbance in the presence of spent coffee extract. Ascorbic acid was
25 used as control in both assays and IC_{50} values are reported in Table 5.

26

1 **2.6.2 β -Carotene bleaching Test**

2 The protection of SCGs ethanol extracts on lipid peroxidation was assessed as previously described
3 (Loizzo et al., 2019). Briefly, 1 mL of β -carotene (2.5 mg in 5 mL of chloroform) was mixed with
4 linoleic acid (20 μ L) and 100% Tween 20 (200 μ L). The absorbance was measured at 470 nm against
5 a blank at $t = 0$ and successively after 30 minutes incubation. Propyl gallate was used as control and
6 IC_{50} value is reported in Table 5.

8 **2.6.3 Ferric reducing ability power (FRAP) Assay**

9 The FRAP assay was applied following the procedure previously described by Loizzo et al.
10 (2019). The FRAP value represents the ratio between the slope of the linear plot for reducing Fe^{3+} -
11 TPTZ reagent by SCG extracts compared to the slope of the plot for $FeSO_4$. Butylated hydroxytoluene
12 (BHT) was used as positive control and tested at concentration of 2.5 mg/mL (Table 5).

14 **2.6.4 Relative antioxidant capacity index (RACI)**

15 Standard scores derived from different antioxidant assays (ABTS, DPPH, β -carotene bleaching
16 test, FRAP) were used to calculate the Relative Antioxidant Capacity Index (RACI) (Loizzo et al.
17 2019). This statistical application integrates the antioxidant capacity values generated from different
18 *in vitro* methods and provides a ranking of antioxidant activity of different samples. The standard
19 score is calculated by using the following equation:

$$20 \quad (x - \mu) / \sigma$$

21 where x is the raw data, μ is the mean, and σ is the standard deviation.

23 **2.6.5 Global antioxidant score (GAS)**

24 T-scores was used to calculate the value of Global Antioxidant Score (GAS) following the
25 equation: $T - score = (X - min) / (max - min)$

1 where min and max, respectively, represent the smallest and largest values of variable X among the
2 investigated extract (Loizzo et al. 2019).

3

4 **2.7 Cell culture**

5 Seven cancer cell lines, adenocarcinomic human alveolar basal epithelial cells A549 (ECACC
6 No. 86012804), human caucasian breast carcinoma (MCF-7, ECACC N°:86012803), human cervix
7 epitheloid carcinoma (HeLa, ECACC N°:93021013), human caucasian breast adenocarcinoma
8 (MDA-MB-231, ECACC N°:92020424), human amelanotic melanoma (C32, ATCC N°:CRL-1585),
9 human caucasian prostate carcinoma (LNCaP, ECACC N°:891102011) and human caucasian
10 prostate adenocarcinoma (PC3, ECACC N°: 90112714) were used in our investigation. All media,
11 buffers, trypsin and dyes were filter-sterilized prior to use and warmed to 37 °C. The MCF-7, HeLa,
12 A549 and PC3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium while
13 MDA-MB-231, C32, and LNCaP cells were cultured in Roswell Park Memorial Institute (RPMI)
14 1640 medium. Both media were supplemented with 1% L-glutamine, 1% penicillin/streptomycin and
15 10% fetal bovine serum. The cell lines were maintained at 37 °C in a 5% CO₂ atmosphere with 95%
16 humidity. The cultures were passed once a week by trypsinization using trypsin-EDTA solution
17 (1:30). Cells counts and viability were performed using a standard trypan blue cell counting
18 technique.

19

20 **2.7.1 Sulforhodamine B (SRB) assay**

21 The anti-proliferative activity of SCGs ethanol extracts was performed by using the protein-
22 staining sulforhodamine B (SRB) assay as previously described (Lucci et al., 2015). Cells were
23 trypsinized, counted and placed in 96-well plates at optimal plating density. This cell density was
24 chosen for each cell line to ensure exponential growth throughout the experimental time and to ensure
25 a linear relationship between absorbance evaluated at 490 nm and cell number. In particular, 5 x 10⁴
26 cells were plated for MCF-7 and C32, 8 x 10⁴ cells for HeLa and MDA-MB-231, 15 x 10⁴ cells for

1 LNCaP and 15×10^4 for A549 and PC3. After 24 h the cells were treated with different concentrations
2 of SCGs ethanol extracts (5-200 $\mu\text{g}/\text{mL}$). After 48 h of exposure, 100 μL of ice-cold 40%
3 trichloroacetic acid (TCA) was added to each well, left at 4°C for 1 h, and washed with distilled water.
4 The TCA-fixed cells were stained for 30 min with 50 μL of 0.4% (w/v) SRB in 1% acetic acid. Plates
5 were washed with 1% acetic acid and air dried overnight. For reading plate, the bound dye was
6 solubilised with 100 μL of 10 mM tris base [tris(hydroxymethyl)aminomethane]. The absorbance
7 was read by using a Molecular Devices SpectraMax Plus Plate Reader (Molecular Devices, Celbio,
8 Milan, Italy) at 490 nm. Cell survival was measured as the percentage absorbance compared to the
9 untreated control. Vinblastine sulphate salt and doxorubicin were used as positive controls and IC_{50}
10 values are reported in Table 6.

11

12 **2.8 Statistical Analysis**

13 All experiments were carried out in triplicate. Data were expressed as means \pm standard
14 deviation (S.D.). The dose-response curve was obtained by plotting the percentage inhibition versus
15 concentration. The inhibitory concentration 50% (IC_{50}) was calculated by non-linear regression with
16 the use of Prism Graph Pad version 4.0 for Windows (Graph Pad Software, San Diego, CA, USA).
17 Differences of the analyzed compounds (tocopherols, polyphenols) among SCG samples were
18 calculated using one way analysis of variance (ANOVA) with Tukey's post hoc procedure, with a
19 level of significance at $p < 0.05$ (R Project for Statistical Computing; R Foundation for Statistical
20 Computing, Wien, Austria). Differences in terms of antioxidant and anti-proliferative activity within
21 and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's
22 test ($\alpha = 0.01$): **** $p < 0.0001$, *** $p < 0.001$ ** $p < 0.01$ compared to the positive controls. *Pearson's*
23 correlation coefficient (r) and linear regression, assessment of repeatability, calculation of average
24 and relative standard deviation were performed using Microsoft Excel 2010 software.

25

1 **3. Results and Discussion**

2 **3.1 Extraction yield**

3 Samples from arabica coffee mixtures provided higher ethanol extraction yield in comparison
4 to robusta variety samples (Table 2). SCG ethanolic extract amount ranged from 5.8 to 11.2 g/100 g
5 of dry powder , where the lowest value was found for the sample S7-R (100% robusta) and the highest
6 for S3-A (100% arabica). Extraction yields were lower as compared to values reported by Page,
7 Arruda, & Freitas (2017) for ethanol extract from espresso (capsule) spent coffee grounds dry waste
8 (20.8 g/100 g) largely due to the different extraction conditions: a cold extraction was performed in
9 our study (25°C for 12 h) and a warm extraction process (60°C for 30 min) in the study of Page et al.
10 (2017). The designation of samples is reported in Table 1.

11

12 **3.2. Tocopherol content**

13 Tocopherols are bioactive compounds able to inhibit lipid oxidation in foods and biological
14 systems.

15 Coffee beans are rich in tocopherols, but no literature has investigated the tocopherol profile of
16 SCGs. Consequently, our work examined the presence of tocopherols in espresso α SCGs.
17 Tocopherol and β -tocopherol predominated in all SCG samples. Our results were consistent with the
18 data reported for roasted coffee beans, where the total amount of tocopherols in the Arabica varieties
19 is almost three times higher than in the robusta varieties. The predominant tocopherol is β -tocopherol
20 in both coffee varieties. However, the concentration of β -tocopherol in relation to the α -tocopherol
21 is almost three times in Arabica, while it is nearly one in robusta (Górnaś, Siger, Pugajeva, Czubinski,
22 Waskiewicz & Polewski, 2014). It has been reported that α -tocopherol content in Arabica roasted
23 beans ranges between 2.4 and 6.9 mg/100 g of dry bean whereas it accounts for 0.3-4.9 mg/100 g in
24 dry robusta beans. The β -tocopherol content has been reported to range between 1.6 and 21.3 mg/100

1 g in dry Arabica beans and between 1.6 and 7.5 mg/100 g in dry robusta beans (Alves, Casal, Alves
2 & Oliveira, 2009).

3 In the present study, a wide range of concentration in the SCGs between 299 and 1508 mg/Kg
4 (Table 2) for the total α - and β -tocopherol was found with a ratio α -tocopherol/ β -tocopherol from
5 0.4 to 1.2. This makes SCGs extracts a valuable source of tocopherols, especially for β -tocopherol, if
6 considering that the typical sources, *i.e.* vegetable oils, contain much lower (Shaidi, 2000) or
7 comparable amount; *e.g.*, wheat germ oil, one of the richest sources of β -tocopherol, has been reported
8 to contain 106.5 mg/100 g of β -tocopherol (Woollard & Indyk, 2003).

9 S2-A (100% arabica from Colombia) resulted significantly richer ($p < 0.05$) in α and β -
10 tocopherol than all the other samples.

11 The lowest α and β -tocopherol amounts were found in the sample in S7-R (100% robusta from
12 Guatemala). Referring the values to the dry spent powder, the total tocopherol content ranged between
13 1.7 mg/100 g in S7-R and 13.2 mg/100 g in S2-A. Higher β -tocopherol levels are found in 100%
14 arabica samples, with significant differences ($p < 0.05$) as compared to blend and to 100% robusta
15 samples.

16

17 **3.3 Polyphenol profile of espresso SCG ethanol extracts**

18 Since the total polyphenol content of roasted coffee is mainly represented by phenolic acids,
19 and more specifically hydroxycinnamic acids (caffeic, chlorogenic, coumaric, ferulic and sinapic
20 acids), most of the literature focused on the investigation of these compounds in spent coffee grounds.

21 Especially chlorogenic acids and their related compounds (*i.e.* caffeoylquinic, feruloylquinic
22 and *p*-coumaroylquinic acids) were deeply investigated in the past. However, during coffee
23 processing, including roasting and brewing preparation, the formation of other phenolic compounds
24 with antioxidant activity (*i.e.* Maillard reaction products, chlorogenic acid degradation products) can
25 be promoted (Alongi & Anese 2018). Thus, in the present paper a detailed identification of the

1 phenolic constituents of SCGs ethanolic extracts was carried out by means of UHPLC-HRMS
2 analysis. As reported in Tables 3 and 4, the UHPLC-HRMS-based method allowed the identification
3 and quantification of eighteen selected phenolic substances belonging to different phenolic classes:
4 hydroxycinnamic, hydroxybenzoic, and methoxybenzoic acids, aldehydes, flavonoids and others.

5 By and large, the most abundant compound was 4-hydroxybenzoic acid in all ethanolic extracts
6 except in S5-A₁₀R₉₀, where the most abundant phenol was tyrosol (1048.8 mg/kg in extract
7 corresponding to 6.47 mg/100 g in dry SCG). Only S7-R extract presented comparable amounts of
8 CQA and 4-hydroxybenzoic acid. High value of tyrosol was found also in S3-A extract (1084.0 mg/kg
9 in extract corresponding to 11.85 mg/100 g in dry SCG). Moreover, the latter extract presented the
10 highest content of vanillic acid and vanillin. To the best of our knowledge, this is the first study
11 reporting the presence of tyrosol in coffee samples. The amount of tyrosol that has been found in S3-
12 A, S4-A₆₀R₄₀ and S5-A₁₀R₉₀ ethanolic extracts is very high, even if compared to the levels usually
13 encountered in matrices notoriously rich in tyrosol, such as olive oils for which European Food Safety
14 Authority (EFSA) has approved an health claim on polyphenols, that may be used for olive oil
15 containing at least 5 mg of hydroxytyrosol and its derivatives (*e.g.* oleuropein complex and tyrosol)
16 per 20 g of olive oil (250 mg/kg) (European Commission, 2012). In light of this, ethanolic extracts
17 from espresso spent coffee may represent valuable alternative sources of tyrosol, as well as of other
18 phenolic compounds, to be used in nutraceutical or dietary supplement formulations.

19 However, our results showed great variability of the phenolic profile among the SCG ethanolic
20 extracts without any clear relation with the coffee variety (arabica or robusta) and with the mixture
21 composition (arabica/robusta ratio). For instance, significantly higher ($p < 0.05$) value of
22 moncaffeoylquinic acids (CQA, chlorogenic acids isomers) was found in S7-R and the significantly
23 lower in S5-A₁₀R₉₀. These extracts presented also a level of 4-hydroxybenzoic acid statistically
24 different. Both SCGs samples derived from coffee mixture mainly formed by robusta variety.
25 Anyway, the geographical origin of robusta variety was different: S7-R contained 100% robusta from
26 Guatemala whereas S5-A₁₀R₉₀ contained 90% of robusta from Vietnam and India.

1 Comparing our results with those reported in literature, it is noteworthy to underline that the
2 investigated SCGs ethanolic extracts contained lower levels of CQA, than SCGs aqueous extracts
3 investigated by others. In our case, CQA range from 80.8 to 1262.4 mg/kg of ethanolic extract in S5-
4 A₁₀R₉₀ (10% Arabica /90 % robusta) and S7-R (100% robusta from Guatemala) samples, respectively.
5 Referring the values to dry spent powder, the CQA levels range from 0.5 to 7.33 mg/100 g dry powder
6 in S5-A₁₀R₉₀ and S7-R, respectively. According to Monente et al. (2015b), arabica SCG water extract
7 contained CQA at 82190 mg/kg in lyophilized extract whereas robusta SCG water extract contained
8 63280 mg/Kg in lyophilized extract. CQA and dicaffeoylquinic (diCQA) acids were also quantified
9 in SCG obtained from different coffeemakers (filter, espresso, plunger, and mocha) by Bravo et al.
10 (2012). Espresso SCG presented levels of total caffeoylquinic acids ranging from 7.49 to 11.05 mg/g
11 of robusta (from Vietnam) and arabica (from Guatemala), respectively. Cruz et al. (2012) found that
12 the total content of chlorogenic acids varying from 2.12 to 7.66 mg/g of espresso SGC, and Panusa
13 et al. (2013) found a total chlorogenic content ranging from 1.81 to 6.09 mg/g in dry espresso SCG.

14 Similarly, Lopez-Barrera, Vazquez-Sanchez, Loarca-Pina, & Campos-Vega (2016) also
15 reported higher levels of chlorogenic acid (from 1.8 to 5.6 mg/g SGC) compared with our study. The
16 observed differences might be related to the natural matrix variability and, also to the different solvent
17 and analytical procedure used for the spent coffee extraction. While in the present study a cold ethanol
18 extraction (25°C for 12 h) was performed, López-Barrera et al. (2016) conducted a microwave
19 extraction using 20% ethanol solution (1:9 solid/liquid ratio) whereas Bravo et al. (2013) extracted
20 spent coffee samples with water at 90°C. Such differences may affect the quali-quantitative profile
21 of phenolic fraction and explain such dissimilarities (Pettinato, Casazza & Perego, 2019).

22 **3.4. Antioxidant activity**

23 To counteract oxidative stress, the consumption of dietary antioxidants is important in order to
24 prevent the development of several diseases. Different *in vitro* assays were used to investigate the
25 antiradical and/or antioxidant effects of SCG extracts. A multiple approach to test the antioxidant
26 potential of sample is strongly recommended. The radical scavenging activity evidences the

1 of sample to react with free radicals, while with antioxidant activity researchers refer to all the other
2 mechanisms involved in the process (Tirzitis & Bartosz, 2010).

3 Generally, a concentration-effect relationship was found in all the tests, except in FRAP assay (Table
4 5). S7-R (100% robusta from Guatemala) sample showed the highest DPPH radical scavenging
5 potential with IC_{50} value of 1.5 $\mu\text{g/mL}$. In ABTS test promising results were obtained by S7-R, S6-
6 A₇₅R₂₅ alues of 1.5, 4.5 and 5.9 $\mu\text{g/mL}$, respectively. This finding could be related to the relevant
7 amount of CQA and it reinforces the results found by Xu, Hu, & Liu (2012) whose demonstrated that
8 both CQA and di-CQA are able to scavenge DPPH[·] and ABTS^{·+} radicals. The highest reductant
9 potential in FRAP assay was obtained with S5-A₁₀R₉₀ extract (FRAP value of 100.5 $\mu\text{M Fe (II)/g}$).
10 This value was quite higher than that reported for the positive control BHT (63.2 $\mu\text{M Fe (II)/g}$). It is
11 interesting to note that this sample showed a high amount of tyrosol. *Pearson's correlation coefficient*
12 (*r*) evidenced a positive correlation between this compound and FRAP assay (*r*= 0.59). The
13 antioxidant potential of SCG extracts was evaluated also using the β -carotene bleaching test, which
14 consists in the evaluation of the ability of the extract to inhibit the lipid peroxidation. Since no high
15 temperatures are required, the antioxidant activity of thermo-sensitive compounds may be determined
16 and quantitatively evaluated. Except for decaffeinated sample (S6-A₇₅R₂₅), SCG extracts showed a
17 promising protection of lipid peroxidation. The following samples exhibited the most promising
18 protection of lipid peroxidation with IC_{50} values of 10.7, 12.5, 13, and 13.4 $\mu\text{g/mL}$ for S1-A, S2-A,
19 S7-R, and S3-A, respectively.

20 Two indices, namely Relative Antioxidant Capacity Index (RACI) and Global Antioxidant
21 Score (GAS), were calculated in an integrated approach to evaluate and create a ranking clustering
22 of the antioxidant capacity of different samples. Based on both RACI and GAS values, S7-R extract
23 (100% robusta from Guatemala) showed the highest antioxidant potential. Differently from all the
24 other extracts, phenolic profile of S7-R sample was characterized by the highest CQA content and by
25 comparable amounts of CQA and 4-hydroxybenzoic acid. However, Pearson's correlation coefficient

1 calculation demonstrated that chlorogenic acid and hydroxybenzoic acid alone do not account for the
2 antioxidant activity. A possible synergism of action between SCGs components should be
3 hypothesized. Differently, ferulic and veratric acids positively correlated with ABTS test ($r= 0.69$).

4 A significant correlation was found also for tyrosol and ABTS with r value of 0.87. Ethyl gallate
5 positively correlate with FRAP assay ($r= 0.89$). No correlations were evidenced between tocopherols
6 content and antioxidant activities.

7

8 **3.5 Anti-proliferative activity**

9 In cancer, altered cellular pathways determine an abnormal tendency for uncontrolled growth.
10 Several phytochemicals able to targeting diverse oncogenic pathways can be used as
11 chemopreventive agent or in the treatment of this complex disease. In the present study, SCGs extracts
12 were screened against seven cancer cell lines. All investigated samples except S3-A showed a
13 concentration-dependent activity. Promising IC_{50} values were found with S7-R and S6-A₇₅R₂₅
14 extracts against human lung carcinoma cells (A549) with values of 61.2 and 61.3 $\mu\text{g/mL}$, respectively.
15 Both values are in the same order of potency of the positive control vinblastine (IC_{50} value of 67.3
16 $\mu\text{g/mL}$) (Table 6). The same samples evidenced a similar anti-proliferative activity against human
17 amelanotic melanoma cells with IC_{50} values 1.6-times higher than the positive control vinblastine
18 (IC_{50} values of 72.3 and 74.7 $\mu\text{g/mL}$, respectively). S1-A showed the highest anti-proliferative
19 activity against human uterine cervix adenocarcinoma (HeLa) and human prostate cancer (PC3) cells
20 with IC_{50} value of 39.7, and 40.0 $\mu\text{g/mL}$, respectively. Previously, Ramalakshmi et al. (2009)
21 evidenced that SCGs extracts obtained by arabica and robusta varieties were able to inhibit P388 cell
22 used as model of leukaemia. These extracts showed higher potency in cell viability inhibition in
23 comparison to the extracts obtained by green coffees. Chemically spent coffee extracts are
24 characterized by a lower content of polyphenols but are richest in melanoidins and phenolic polymers,
25 which are found able to protect cells from oxidative damage (Wen, Takenaka, Murata &

1 2004).

2 Except for ethyl gallate that correlated with HeLa cells ($r= 0.59$), no significant positive
3 correlations between the identified compounds and anti-proliferative activity were found. According
4 literature data, ethyl gallate exhibited a promising activity against several cancer cell lines including
5 IHH, PC3, Hep3B, HepG2, HeLa and CaSKi cells with IC_{50} values ranging from 38 to 211 μ M for
6 Hep3B and IHH, respectively. This compound could induce G2/M phase cell cycle arrest and display
7 interesting effect on microtubule stabilization in Hep3B cells. The activation of caspase 3/7 and
8 increase in the *ratio* of Bax/Bcl-2 expression was also observed (Sánchez-Carranza et al., 2017). The
9 anti-proliferative activity of ethyl gallate by apoptotic pathway was proved also against human oral
10 squamous carcinoma cell line KB (Mohan, Thiagarajan, & Chandrasekaran, 2015). Other identified
11 phytochemicals in SCGs samples are previously investigated as antitumor agents. Quiles et al. (2002)
12 evidenced the ability of tyrosol to reduce DNA oxidation and to increase the levels of glutathione
13 peroxidase and hydroperoxides in PC3 cell line. Chlorogenic acids are known for their antioxidant
14 and antitumor potential. Recently, Yamagata, Izawa, Onodera, & Tagami (2018) demonstrated that
15 chlorogenic acids are able to decrease proliferation of A549 human lung cancer cells by regulation of
16 apoptosis. In particular, among the most abundant identified phenols, the 4-hydroxybenzoic acid
17 showed anti-proliferative and pro-apoptotic activities by inhibition of histone deacetylases (HDAC)
18 whose pathological alteration determines the tumour progression (Seidel, Schnekenburger, Dicato &
19 Diederich, 2014). In fact, although the link between hyperacetylation and apoptosis is not completely
20 clarified, treatments with HDAC inhibitors usually lead to apoptosis induction (Marks & Xu, 2009).
21 With the same mechanism *p*-coumaric and ferulic acids act against cervical cancer cells (HeLa)
22 (Waldecker et al., 2008).

23

24 **4. Conclusions**

25 The present work investigated the composition and bioactivity of ethanolic extracts from spent
26 espresso coffee grounds obtained by using different arabica and robusta coffee mixtures and the

1 coffee brewing process parameters. The findings indicated that ethanolic extracts from espresso
2 SCGs contain valuable antioxidant compounds, especially of α - and β -tocopherols, CQA, 4-
3 hydroxybenzoic acid, vanillin and tyrosol, the last one to our knowledge identified for the first time
4 in this matrix. Among investigated samples, the extract from 100% robusta Guatemala coffee (S7-
5 R), showed promising anti-proliferative activity against A549 cell line with IC_{50} value (61.2 ± 3.5
6 $\mu\text{g/mL}$) comparable to that given by vinblastine ($67.3 \pm 2.0 \mu\text{g/mL}$). Moreover, S7-R was
7 characterized by a high phenolic content, particularly CQA and antioxidant potential. However, the
8 high variability in terms of chemical composition and bioactivities found in the different samples
9 investigated is to be considered if exploiting SCG as source of bioactive compounds. SCG is a
10 complex matrix containing phytochemicals differing for molecular size, polarity, and solubility,
11 parameters that may affect their bioavailability and distribution in different cells. Even if some
12 specific correlations between chemical composition and bioactivity have been highlighted, further
13 studies could better clarify these relationships and possible synergistic effects between the different
14 components.

15

16 **Acknowledgements**

17 We thank the roasting company “Caffè del Faro”, Montegranaro, Italy for providing the coffee
18 samples.

19

20 **Funding:**

21 This work was supported by Università Politecnica delle Marche (Ricerca di Ateneo 2017),
22 University of Calabria (Ricerca di Ateneo 2017), Spanish Ministry of Economy and Competitiveness
23 under the project CTQ2015-63968-C2-1-P and from the Agency for Administration of University
24 and Research Grants (Generalitat de Catalunya, Spain) under the project 2017SGR-310.

25

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19

1 **Table 1.** Coffee blend composition of roasted coffee mixture used as starting material for espresso
 2 coffee beverage preparation and spent coffee grounds (SCGs) obtained samples.

Composition of starting coffee blend	<i>Spent Coffee grounds</i> (sample code)
100% Arabica from Puerto Rico	S1-A
100% Arabica from Colombia	S2-A
100% Arabica (50% from Brazil, 20% from Colombia, 20% from Guatemala, 10% from Ethiopia)	S3-A
40% Robusta (15% from Vietnam, 25% from India) / 60% Arabica (20% from Brazil, 20% from Colombia, 10% from Guatemala, 10% from Costa Rica)	S4-A ₆₀ R ₄₀
90% Robusta (20% from India, 70 % from Vietnam) / 10% Arabica (from Brazil)	S5-A ₁₀ R ₉₀
75% Arabica (Unknown)/ 25% Robusta (Unknown) _ Decaffeinated coffee blend _	S6-A ₇₅ R ₂₅
100% Robusta from Guatemala	S7-R

3

4

5

1 **Table 2.** Ethanolic extraction yield and tocopherol contents in SCGs ethanolic extracts.

2

	S1-A	S2-A	S3-A	S4-A ₆₀ R ₄₀	S5-A ₁₀ R ₉₀	S6-A ₇₅ R ₂₅	S7-R
Ethanolic extraction yield (g/100g dry powder)	8.8±0.2 ^c	7.9±0.4 ^c	11.2±0.4 ^d	7.9±0.3 ^b	6.1±0.2 ^a	9.1±0.1 ^c	5.8±0.2 ^a
Total tocopherol content (mg/Kg ethanolic extract)	1142±49 ^e	1508±14 ^f	1005±10 ^d	913±3 ^c	822±17 ^b	905±36 ^c	299±1 ^a
α- tocopherol	345±4 ^b	516±12 ^d	309±2 ^b	344±5 ^b	417±24 ^c	414±22 ^c	165±1 ^a
β-tocopherol	796±45 ^f	991±14 ^g	697±8 ^e	569±3 ^d	405±85 ^b	492±31 ^c	134±1 ^a
α/β ratio	0.4	0.5	0.4	0.6	1.0	0.8	1.2

3 Results are expressed as the mean ± standard deviation (n=3). Values in the same row with different superscript letters are significantly different
4 (p<0.05).

5

6

Table 3. Polyphenols content (mg/kg ethanolic extract) in SCGs samples.

	mg/kg ethanolic extract						
	S1-A	S2-A	S3-A	S4-A _{60R₄₀}	S5-A _{10R₉₀}	S6-A _{75R₂₅}	S7-R
Hydroxycinnamic acids							
<i>p</i> -Coumaric acid	16.5±0.0 ^d	10.5±0.1 ^c	36.7±0.1 ^c	19.1±0.0 ^d	30.0±0.3 ^e	1.2±0.0 ^a	5.8±0.1 ^b
Chlorogenic acids*	281.8±7.6 ^d	203.4±4.5 ^{b,c}	118.7±1.0 ^b	99.8±0.6 ^b	80.8±0.3 ^a	143.0±4.3 ^c	1262.4±15.7 ^e
Caffeic acid	6.5±0.1 ^a	5.4±0.0 ^a	9.6±0.0 ^b	5.8±0.1 ^a	8.8±0.1 ^b	4.9±0.0 ^a	11.2±0.0 ^{b,c}
Ferulic acid	356.4±3.2 ^e	207.6±1.6 ^{b,c}	297.1±1.2 ^d	254.2±2.0 ^c	221.2±0.3 ^{b,c}	157.0±0.1 ^b	105.3±0.4 ^a
Hydroxybenzoic acids							
4-hydroxybenzoic acid	1812.7±8.5 ^{c,d}	1386.5±46.9 ^{b,c}	1789.1±14.2 ^d	1513.3±4.5 ^{b,c}	884.7±2.2 ^a	1011.7±12.2 ^b	1522.0±14.2 ^b
Procatechuic acid	9.7±0.1 ^b	nd	12.5±0.3 ^c	18.4±0.2 ^d	17.4±0.2 ^d	7.1±0.1 ^b	3.2±0.1 ^a
Vanillic acid	330.4±3.2 ^c	121.7±1.2 ^{a,b}	473.6±12.1 ^c	148.5±1.3 ^b	329.1±2.5 ^c	132.6±0.2 ^b	98.9±1.3 ^a
Syringic acid	129.8±0.4 ^b	58.9±0.4 ^a	219.5±4.5 ^d	135.7±0.6 ^b	148.9±0.4 ^c	152.4±7.2 ^c	125.9±0.4 ^b
Methoxybenzoic acids							
Veratric acid	419.9±3.2 ^e	147.9±0.2 ^b	461.8±7.9 ^e	410.7±2.2 ^e	346.3±3.0 ^c	322.8±3.8 ^d	61.9±0.7 ^a
Aldehyde							
Protocatechuic aldehyde	48.3±3.2 ^b	nd	47.6±0.6 ^c	40.3±0.2 ^d	23.4±0.1 ^a	26.8±0.3 ^a	40.8±1.4 ^b
Syringaldehyde	85.4±1.6 ^d	34.3±0.2 ^b	94.5±1.3 ^e	83.0±0.5 ^d	73.2±4.4 ^{b,c}	65.9±1.0 ^{c,d}	18.7±0.1 ^a
Vanillin	556.6±2.7 ^d	350.4±1.5 ^{a,b}	1103.4±1.6 ^e	339.8±2.0 ^{a,b}	728.9±4.7 ^{c,d}	357.0±1.6 ^{b,c}	341.9±2.0 ^a
Flavonoids							
(+)-catechin	2.7±0.0 ^b	1.1±0.1 ^a	2.5±0.1	2.3±0.1 ^b	2.1±0.0 ^b	1.3±0.1 ^a	1.4±0.0 ^a
Epicatechin	2.9±0.0 ^b	1.1±0.1 ^a	2.7±0.0 ^b	2.4±0.0 ^b	5.2±0.0 ^c	1.3±0.1 ^a	5.3±0.3 ^c
Other							
Ethylgallate	3.0±0.0 ^b	2.3±0.0 ^a	3.0±0.0 ^b	3.0±0.2 ^b	4.9±0.2 ^c	3.5±0.1 ^b	3.6±0.1 ^b
Tyrosol	192.3±0.5 ^c	121.1±0.7 ^b	1084.0±8.7 ^e	408.2±4.3 ^d	1048.8±3.0 ^f	212.6±0.6 ^c	143.4±0.8 ^a
D(-)-Quinic acid	61.8±0.1 ^c	39.0±0.4 ^b	43.8±0.6 ^b	20.6±0.5 ^a	41.6±0.3 ^b	22.3±0.2 ^a	nd
Homovanillic acid	119.4±0.8 ^c	48.7±0.3 ^b	133.2±1.9 ^d	114.2±2.3 ^c	102.4±2.7 ^b	97.1±5.0 ^c	27.7±0.1 ^a

Values in each row having different lowercase letters are significantly different at $p < 0.05$;

*Chlorogenic acids= sum of moncaffeoylquinic acids isomers (3-CQA+ 4-CQA+5-CQA, molecular weight = 354); n.d.: not detectable (<LOD); Limit of detection (LOD): 4-hydroxybenzoic acid: 10 µg/kg; *p*-coumaric acid: 15 µg/kg; D(-)-quinic acid: 25 µg/kg; chlorogenic acid: 25 µg/kg; syringaldehyde: 50 µg/kg; vanillin: 50 µg/kg; caffeic acid: 5 µg/kg; tyrosol: 50 µg/kg; vanillic acid: 40 µg/kg; homovanillic acid: 150 µg/kg; syringic acid: 50 µg/kg; catechin: 10 µg/kg; ferulic acid: 50 µg/kg; veratric acid: 70 µg/kg; protocatechuic aldehyde: 5 µg/kg; epicatechin: 10 µg/kg; ethyl gallate: 30 µg/kg; protocatechuic acid: 10 µg/kg.

Table 4. Polyphenols content (mg/100g dry powder) in SCGs samples.

	mg/100g dry powder						
	S1-A	S2-A	S3-A	S4-A ₆₀ R ₄₀	S5-A ₁₀ R ₉₀	S6-A ₇₅ R ₂₅	S7-R
Hydroxycinnamic acids							
<i>p</i> -Coumaric acid	0.14±0.03 ^{b,c}	0.09±0.02 ^b	0.40±0.02 ^d	0.15±0.01 ^c	0.19±0.0 ^c	0.01±0.01 ^a	0.03±0.01 ^a
Chlorogenic acids*	2.40±0.12 ^e	1.72±0.36 ^d	1.30±0.05 ^c	0.79±0.03 ^b	0.50±0.0 ^a	1.32±0.21 ^c	7.33±0.61 ^f
Caffeic acid	0.06±0.0 ^{a,b}	0.05±0.01 ^a	0.10±0.0 ^c	0.05±0.01 ^a	0.05±0.0 ^a	0.05±0.01 ^a	0.06±0.01 ^{a,b}
Ferulic acid	3.04±0.15 ^d	1.76±0.36 ^{b,c}	3.26±0.13 ^d	2.01±0.08 ^c	1.36±0.01 ^b	1.45±0.28 ^{b,c}	0.61±0.05 ^a
Hydroxybenzoic acids							
4-hydroxybenzoic acid	15.24±3.30 ^{c,d}	11.74±2.43 ^{b,c}	19.56±0.80 ^d	11.95±0.49 ^{b,c}	5.46±0.04 ^a	9.36±1.52 ^b	8.83±0.84 ^b
Procatecuic acid	0.08±0.0 ^a	nd	0.14±0.01 ^d	0.15±0.01 ^d	0.11±0.0 ^c	0.07±0.01 ^b	0.02±0.0 ^a
Vanillic acid	2.82±0.14 ^d	1.03±0.21 ^{a,b}	5.18±0.21 ^e	1.17±0.05 ^b	2.03±0.02 ^c	1.23±0.02 ^b	0.57±0.05 ^a
Syringic acid	1.11±0.05 ^{c,d}	0.50±0.1 ^a	2.40±0.1 ^e	1.07±0.04 ^{b,d}	0.92±0.01 ^{b,c}	1.41±0.23 ^d	0.73±0.06 ^{a,b}
Methoxybenzoic acids							
Veratric acid	3.58±0.17 ^d	1.25±0.26 ^b	5.05±0.21 ^e	3.24±0.13 ^d	2.14±0.03 ^c	2.99±0.48 ^d	0.36±0.03 ^a
Aldehyde							
Protocatechuic aldehyde	0.41±0.02 ^c	nd	0.52±0.02 ^e	0.32±0.01 ^d	0.14±0.0 ^a	0.25±0.04 ^b	0.24±0.02 ^b
Syringaldehyde	0.73±0.04 ^d	0.29±0.06 ^b	1.03±0.04 ^c	0.66±0.03 ^d	0.45±0.0 ^{b,c}	0.61±0.1 ^{c,d}	0.11±0.01 ^a
Vanillin	4.74±0.23 ^d	2.97±0.61 ^{a,b}	12.06±0.49 ^e	2.68±0.11 ^{a,b}	4.50±0.03 ^{c,d}	3.30±0.53 ^{b,c}	1.98±0.17 ^a
Flavonoids							
(+)-catechin	0.02±0.0	0.01±0.0	0.03±0.0	0.02±0.0	0.01±0.0	0.01±0.0	0.01±0.0
Epicatechin	0.02±0.0	0.01±0.0	0.03±0.0	0.02±0.0	0.03±0.0	0.01±0.0	0.03±0.0
Other							
Ethylgallate	0.03±0.0	0.01±0.0	0.03±0.0	0.02±0.0	0.01±0.0	0.01±0.0	0.01±0.0
Tyrosol	1.64±0.08 ^{a,b}	1.03±0.21 ^a	11.85±0.48 ^e	3.22±0.13 ^c	6.47±0.05 ^d	1.97±0.32 ^b	0.83±0.07 ^a
D-(-)-Quinic acid	0.52±0.11 ^c	0.33±0.07 ^{a,b}	0.48±0.02 ^{b,c}	0.16±0.01 ^a	0.26±0.0 ^a	0.21±0.03 ^a	nd
Homovanillic acid	1.02±0.05 ^c	0.41±0.09 ^b	1.46±0.06 ^d	0.90±0.04 ^c	0.63±0.0 ^b	0.90±0.14 ^c	0.16±0.01 ^a

Values are the mean±standard deviation of three replicates. Values in each row having different lowercase letters are significantly different at $p < 0.05$;

*Chlorogenic acids= sum of moncaffeoylquinic acids isomers (3-CQA+ 4-CQA+5-CQA, molecular weight = 354); n.d.: not detectable (<LOD);

Table 5. Antioxidant activity of SCGs ethanolic extracts.

<i>Extracts</i>	DPPH IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)	FRAP IC ₅₀ (µM Fe(II)/g) at 2.5 mg/mL	β-Carotene bleaching test IC₅₀ (µg/mL)	RACI	GAS
S1-A	49.7 ± 2.9****	5.9 ± 0.4	18.3 ± 2.5****	10.7 ± 1.4**	-0.34	0.33
S2_A	83.9 ± 4.7****	17.9 ± 1.1****	13.6 ± 2.1****	12.5 ± 1.9***	0.38	1.44
S3-A	301.8 ± 5.2****	164.8 ± 3.7****	17.3 ± 1.1****	13.4 ± 1.7****	0.43	2.79
S4-A ₆₀ R ₄₀	272.9 ± 6.3****	162.8 ± 2.9****	6.4 ± 0.3****	34.9 ± 2.5****	0.21	2.51
S5-A ₁₀ R ₉₀	48.9 ± 2.0****	185.7 ± 3.3****	100.5 ± 3.6****	36.8 ± 3.9****	-0.32	2.58
S6-A ₇₅ R ₂₅	182.9 ± 4.8****	4.5 ± 0.5	20.6 ± 2.0****	86.6 ± 4.0****	0.29	2.66
S7-R	10.1 ± 2.1	1.5 ± 0.9	22.8 ± 2.4****	13.0 ± 1.5***	-0.43	0.20
Positive controls						
Ascorbic acid	5.0 ± 0.8	1.7 ± 0.06				
BHT			63.2 ± 4.3			
Propyl gallate				1.0 ± 0.04		

Data are given as media ± S.D. (n = 3); DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation (ABTS⁺), β-Carotene bleaching test, Ferric ion reducing antioxidant power (FRAP); Relative antioxidant capacity index (RACI); Global antioxidant score (GAS). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by One-way ANOVA followed by a multicomparison Dunnett's test ($\alpha = 0.01$): **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ compared to the positive controls.

7 **Table 6.** Anti-proliferative activity of SCGs ethanolic extract (IC₅₀ in µg/mL).

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<i>Extracts</i>	MCF-7	HeLa	MDA-MB-231	C32	A549	LNCaP	PC3
S1-A	52.4 ± 4.0****	39.7 ± 2.8****	124.7 ± 3.3****	105.7 ± 3.1****	121.4 ± 3.8****	46.8 ± 4.2****	40.0 ± 2.9****
S2-A	82.4 ± 3.2****	43.2 ± 3.1****	143.2 ± 3.6****	85.8 ± 3.8**	78.8 ± 3.4**	81.1 ± 3.4****	75.3 ± 3.8****
S3-A	> 200	> 200	> 200	> 200	> 200	> 200	> 200
S4-A ₆₀ R ₄₀	> 200	> 200	> 200	> 200	145.2 ± 3.9****	> 200	> 200
S5-A ₁₀ R ₉₀	105.4 ± 3.8****	78.5 ± 3.4****	> 200	> 200	> 200	127.6 ± 3.9****	101.3 ± 3.6****
S6-A ₇₅ R ₂₅	184.7 ± 3.3****	79.9 ± 3.7****	191.5 ± 3.2****	74.7 ± 3.3****	61.3 ± 3.7	195.1 ± 3.7****	167.2 ± 2.7****
S7-R	75.5 ± 4.3****	71.9 ± 3.8****	100.2 ± 3.3****	72.3 ± 4.7****	61.2 ± 3.5	80.2 ± 3.6****	68.1 ± 3.1****
Positive control							
Vinblastine				45.5 ± 1.9	67.3 ± 2.0	29.3 ± 0.9	
Doxorubicin	4.04 ± 0.4	3.6 ± 0.3	14.9 ± 1.4				1.5 ± 0.1

9 MCF-7, human breast cancer cells; HeLa, human uterine cervix adenocarcinoma cells; MDA-MB-231, triple negative breast adenocarcinoma cells;
 10 C32, human amelanotic melanoma; A549, adenocarcinomic human alveolar basal epithelial cells; LNCaP, androgen-sensitive human prostate
 11 adenocarcinoma cells; PC3, human prostate cancer cell. Data are given as media ± S.D. (*n* = 3); [200 µg/mL] max concentration tested; Differences
 12 within and between groups were evaluated by One-way ANOVA followed by a multicomparison Dunnett's test (α = 0.01): *****p*< 0.0001, ***p* < 0.01
 13 compared to the positive controls (Vinblastine and Doxorubicin).
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