Recovery of phenolic compounds from agro-industrial by-products: evaluating antiradical activities and immunomodulatory properties

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Abstract

Grape Pomace (GP), Spent Coffee Grounds (SCG), Tomato Pomace (TP) and Red Corn Cobs (RCC) were collected and polyphenols were extracted by optimizing ethanol solvent extractions. Subsequently, extracted phenolic acids, flavonoids and anthocyanins were completely characterized by HPLC, HPLC-DAD and LC-ES-MS. Antiradical activity (DPPH) and anti-inflammatory assays (IL-8 cytokine gene expression in the Caco-2 cell line) were then investigated.

Results indicated a total polyphenol content for the by-products in the range: 4.64±0.31– 22.77±0.65 mg Gallic Acid Equivalent g^{-1} Dry Weight, and an antiradical activity in the range: 21.47±0.09 - 89.76±0.24 μM Trolox equivalent g^{-1} Dry Weight. The best anti-inflammatory performances were reported for SCG and GP and they were due to the high flavonoid contents, playing phenolic compounds a minor role.

Taking into consideration the results above reported and the information related to the by-products studied, i.e. geographical provenience, daily availability and biomass potential preservation, the agro-industrial wastes were ranked for their suitability to be potentially upgraded to industrial level in the following order: spent coffee grounds >> grape pomace > red corncobs >> tomato pomace.

Keywords: Agro-industrial wastes; Anti-inflammatory activity; Antiradical activity; Ethanol extraction; Polyphenols.

Abbreviation Footnotes first page: : Grape Pomace (GP), Spent Coffee Grounds (SCG), Tomato Pomace (TP), Red Corncobs (RCC), Total phenolic content (TPC), Milligrams of Gallic acid equivalents on each gram of dry weight material (mg GAE g^{-1} DW), antiradical activity (AA), Micromole equivalent of Trolox on each gram of dry weight material (μM TE g^{-1} DW), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Total Anthocyanin content (TAC), High Performance Liquid Chromatography (HPLC), Real-Time PCR analysis (PCR), Dulbecco's Modified Eagle Medium (DMEM), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), Interleukine-1 beta cytokine (IL-1β).

1. Introduction

By-products produced by industrial and food industries are usually fated to animal feeding, anaerobic digestion or compost production (Kucic et al., 2018). Nonetheless, these residues are still rich in health promoting bioactive molecules, such as fiber, phenolic acids, flavonoids and anthocyanins (Martins et al., 2017). As consequence of that the recovery of these high-added-value compounds from agro-industrial wastes become recently a hot topic (Kumar et al., 2017).

Grape Pomace (GP) represents the main by-products of the wine industry and it mainly consists of skin, pulp and seeds, which accounts for about 30% (w/w) of the material used. In 2017, 75 million tons of grapes were produced in the world (with the top five among the producers: China, Italy, USA, France and Spain) on about 7.5 million ha of land used (http://www.OIV.org/, 2017), getting about 281 million hectoliters of wine. On average, 1 ton of wine produces 200 kg of grape pomace that generally is used for energy production or to prepare fertilizer (Cáceres et al., 2012). Grape pomaces can be re-used as bioactive additives to food since these by-products contain large quantity of flavonoids, procyanidins and stilbenes (Sette et al., 2020; Marinelli et al., 2018; Martinez-Saez et al., 2017).

Coffee is one of the most popular consumed beverages worldwide. The main by-products from coffee production are Coffee Spent Grounds (CSG) and coffee silver skin (CS) (Jiménez-Zamora et al., 2015). About 10 million tons of coffee were produced in 2018 according to the International Coffee Organization (ICO) (http://www.ico.org/, 2019), representing about 7.9 million tons y^{-1} of coffee residue (ICO 2019). These residues are used as fertilizers, animal feeds, biofuel and activated carbon (Bravo et al., 2012). Nevertheless, these residues still contain active molecules (in particular hydrocinnamic acids), of various interest for different further applications (Martinez-Saez et al., 2017).

Tomato is one the most cultivated vegetable in terms of production volumes, with 182 million tons produced each year (www.statista.com) on 5 million ha of land; in Italy about 38 million tons are destined to industrial processing (http://www.ISMEA.it, 2017). Italy has been considered as the

world's leading exporter of pulp and peeled (>75% of total world exports), and pastes and concentrates (26% of the total). Almost 1 million ton of skins and seeds are produced from the tomato processing chain (38 million tons) which could be economically re-evaluated. Commonly these leftovers are destined to the production of compost, biogas and feed (Lu et al., 2019), although recently the Tomato Pomace (TP) has gained high attention due to the considerable quantity of the phenolic compounds in tomato skin that could be recovered in the food industries (Lu et al., 2019).

Corn production has been around 6.2 million tons in 2018 in Italy, therefore, a huge quantity of corncob is produced annually (http://www.statista.com, 2018). Corncobs are mainly wasted but recently they were used as substrate for energy productions, forage protein, activated carbons, biofuel and charcoal production (Li et al., 2015). Colorful corn represents an interesting category of corn comprising high quantity of phenolic compounds in different parts of the plant (Iametti et al., 2019). It has been proved that anthocyanins are more concentrated in the corncob than in the kernel (Hernández et al., 2018). Corncobs have been usually re-utilized as animal feed and tissue coloring, although, this by-product crop is a promising source of phenolic compounds. Pigments extracted from colorful corncob have been used in the food industry as additive colorants in beverages (Jing and Giusti, 2005).

Valorization of bioactives from agro-industrial by-products is interesting from both environmental and economic perspectives, since it contributes to the reduction of negative environmental impacts due to their disposal, and because of the recovery of high-added value compounds having many biotechnological applications (Kumar et al., 2017; Sette et al., 2020). Optimizing the extraction conditions can improve the quality of final products, reducing both energy and solvent application (Zalazar-García et al., 2020).

Polyphenols has been demonstrated great benefits on human health (Shahidi and Ambigaipalan, 2015;) including anti-inflammatory activity (Di Donato et al., 2018; Jiménez-Zamora et al., 2015).

Several studies demonstrate that inflammatory pathways are critical targets in the treatment and prevention of various diseases such as endothelial dysfunction, atherosclerosis and vascular diseases (Siti

et al., 2015), since these compounds are proved to control the inflammation through modulating intracellular processes convoluted in inflammation (Vendrame and Klimis-Zacas, 2015).

This study aims at the investigation of phenol contents and their profile in extracts obtained from different well-diffused agro-industrial by-products in view of their possible uses. This work led getting maximum yields in terms of both total polyphenol content and antiradical activity by optimizing polyphenols extraction methodology. In addition, the obtained extracts were tested for their anti-inflammatory properties.

2. Materials and Methods.

2.1 Agro-industrial by-products

The Grape Pomace (GP) was obtained from industrial vine making (red Merlot from Franciacorta and Poncarale, Brescia, Italy). The Spent Coffee Ground (SCG) was obtained from the Agriculture Faculty canteen of University of Milan (UNIMI), composed of different coffee varieties. Tomato Pomace (TP) comes from tomato processing industry (OPOE Gruppo Cavicchi Scarl, Dodici Morelli, FE, Italy). Red Corn Cob (RCC) (Genotype R5412 of pigmented inbred line corn (*Zea mays L.*) carrying B (Booster 1) and Pl (Purple plant) regulatory genes, was used as a plant material in the study. Corn was harvested at the experimental farm of the University of Milan (Landriano, Milan province, Italy) (GPS Coordinates, N 45°180', E 9°150').

2.2 Sample preparation and characterization

Samples taken were brought to the laboratory; a portion of each sample (500 g) was placed in a vacuum oven at 37˚ C and were dried to constant weight. The dry samples were then smashed to fine powder with a laboratory mill (Thomas Wiley, Thomas Scientific, Swedesboro, NJ, USA) and then they were kept in refrigerator (at 4˚C) for further analysis.

Dry matter weight (DW) in by-product samples was evaluated by drying each sample (15 g) at 98–100 ˚C until a constant weight was achieved (AOAC, 2002). For each sample three replicates were considered.

2.3 Extraction method, total polyphenol content quantification and antiradical activities determination

Samples were extracted with ethanol 70% in water (v/v) at a solid-solvent ration of 1:100 (w/v), at different times according to the Table 1 with continuous stirring on shaker covered with aluminum foils to protect from the light. Following agitation, the slurries obtained were centrifuged at 4,500 rpm for 5 min, and then filtered (0.45 µm filter). Filtrate was stored at 4 °C in a dark place. The extraction method was selected according to literature review performed on the solvent type and conventional extraction techniques from the GP, SCG, TP and RCC. Ethanol–water 70 % mixture has been recommended because it allows both good extraction yield and the absence of toxicity for human consumption (Wasz-kowiak et al., 2016).

The Folin-Ciocalteu method was used for total phenolic content (TPC) detection and results were reported as mg of gallic acid (GA) equivalents g^{-1} DW (Abbasi-Parizad et al., 2020).

The antiradical activity (AA) was assessed by using DPPH radical scavenging method and results were expressed as µmol equivalents Trolox g^{-1} DW of samples (Abbasi-Parizad et al., 2020). DPPH (Prot. N. D9132, Sigma Aldrich, Dermstadt, Germany) solution (125 µM) in methanol was prepared and added to sample extracts at different concentrations at a ratio of 1:40 (v/v) and the color changes were recorded for 90 min at 10 min intervals. The absorbance was then measured at 517 nm by a spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA, USA).

2.4 Phenolic compounds characterization

2.4.1. Quantitative and qualitative characterization of phenolic compounds by HPLC

Phenolic acids and flavonoids were analyzed by HPLC (Agilent 1260 Infinity, Agilent Technologies, Santa Clara, California, United States) according to method described by Abbasi-Parizad et al., (2020) (full method description is available in the supplementary data).

2.4.2. Quantitative and qualitative characterization anthocyanins compounds

The total anthocyanin content was quantified by spectrophotometric approach according to pH differential method indicated by Giusti and Wrolstad (2001), with some minor modification. Briefly,

two buffer solutions were prepared at pH of 1 (using potassium chloride buffer 0.03 Mole L^{-1}) and pH of 4.5 (using sodium acetate buffer 0.4 Mole L^{-1}). Diluted samples (10 times) were prepared with the two buffers to a final volume of 2 mL, and then the absorbance was measured at 520 nm and 700 nm (for the correction) by using UV/visible Varian Cary 60 spectrophotometry; distilled water was used as blank. The concentration of total anthocyanin content was expressed as Cy-3-glc equivalents, and was calculated according to the formula: $A*MW*DF*10^3/\varepsilon*1$, in which: A is the absorbance measured at the different wavelength, i.e. $[(A520nm - A\ 700nm)_{pH\ 1} - (A520nm - A700nm)_{pH\ 4.5}]$; MW is the molecular weight for cyanidin-3-glucoside (cy-3-glc) (449.2 g $Mole^{-1}$); DF is the dilution factor; l is the path length (cm); Ɛ is the molar extinction coefficient for cy-3-glc (26,900 L $Mole^{-1}$ cm^{-1}), and 10^3 is factor for the conversion of g to mg. In the case of GP, the MW of Malvidin-3-glucoside (MW=528.9 g $Mole^{-1}$ and Ɛ:28,000) was used in the formula and the results reported as μg of Mal-3-glc equivalents g^{-1} DW.

The anthocyanins characterization was performed by both HPLC-DAD and LC/MS. HPLC-DAD was conducted by LC-20AD Series Shimadzu Prominence liquid chromatography (Kyoto, Japan). The device was equipped with SPD-M20A diode-array detector, and the separation was carried out by Luna® Omega C18 column (Torrance, USA) 3 μm, 3.0 × 150 mm. The aqueous solution of 1% (v/v) formic acid (solvent A) and 100% acetonitrile (solvent B) constructed mobile phase. The gradient was set up as follow: 10% B for 1 min, 15% B at 10 min, 20% B at 20 min, 45% B at 40 min, then returning to 10% B. Where it was needed, the samples were diluted in the solvent A. The injection volume was of 10 μL, flow rate of 0.4 mL min^{-1} and the column temperature of 30 ºC. The anthocyanin compounds were detected at 520 nm and 280 nm by using a diode-array detector set up for an acquisition in the range 200-600 nm and an acquisition rate of 1.25 scans s^{-1} (peak width 0.2 min). To control the HPLC system and for data processing, LabSolution software version 5.90 was used (Shimadzu Corporation, Kyoto, Japan).

LC/MS was performed by the LC/MS system 6130 Series from Agilent Technologies (Agilent Technologies, CA, USA) equipped with an Electro Spray Ionization (ESI) source and liquid chromatography sprayer and operated in the positive-ion mode. Analyses were performed by using both the same chromatographic separation conditions and column above described; injection volume was of 2 µL. Mass spectra was obtained under positive ion conditions using total ion scan (SCAN) from m/z 100 to 1000 and selected ion monitoring (SIM) modes. The MS settings were optimized for CyG signal-to-noise ratio. The capillary voltage was of +5.0 kV, the nebulizer pressure of 1.7 bar, the drying gas flow of 9 L min^{-1} and the drying temperature 350 °C. Compounds were detected as positive ions ($[M–H]^+$) at different channels: m/z 449 CyG, m/z 535 (cyanidin-3-malonyl glucoside, CyMG), m/z 433 (pelargonidin-3-glucoside, PlG), m/z 463 (peonidin-3-glucoside, PnG). For data processing ChemStation software Rev.B.04.03 (Agilent Technologies, CA, USA) was used.

2.5 Anti-inflammatory assay and cell viability

The anti-inflammatory properties of the extracts were assessed according to the method previously described (Abbasi-Parizad et al., 2020). To do the experiments, Caco-2 cells (ECACC 86010202, Public Health England) at confluence were treated, for each biomass. Two total polyphenol concentrations, i.e. 15 µg mL^{-1} and 25 µg mL^{-1} (5 µg mL^{-1} and 10 µg mL^{-1} in case of SCG) have been considered in the presence of IL-1β in the medium. The IL-1β treated cells have been considered as positive control, which allowed setting 100% of inflammation induction in the experimental study. Comparable concentrations of phenolic acids used as standards (Ferulic acid, Chlorogenic acid and Naringenin) were considered comparing with the tested by-products samples.

For each sample, the treatment was performed by two biological replications.

Expression of IL-8 cytokine by Real-Time PCR analysis (qPCR) was performed such as previously reported (Abbasi-Parizad et al., 2020; Livak and Schmittgen, 2001) (for method details please see-the Supplementary data).

Cell viability was evaluated using the MTT assay (Martins et al., 2017), with some modifications. Caco-2 cells ($1 * 10^5$/well) were seeded in 24-well plate for 24 h in Dulbecco's Modified Eagle Medium (DMEM). The medium was then removed prior to treat the cells with sample extracts.

A concentration of 25 µg mL $^{-1}$ TPC, was prepared and then dissolved in DMEM. The treated cells were incubated for 24 h; at the end of incubation time, the medium was removed and 0.5 mg mL of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) in DMEM was added into the plate and it was incubated for 3 h at 37 ˚C. The formed formazan crystals were then dissolved in 200 µL DMSO and the absorbance was measured at 570 nm using a FLUOstar OPTIMA plate reader (BMG Labtech, Durham, NC, USA). Untreated cells treated were considered as the control.

The cell viability assessed by diphenyl tetrazolium bromide (MTT) assay, did not reduce after 24 h exposure to 25 µg mL $^{-1}$ of each by-product extracts (Figure 1a), indicating the absence of toxicity of the phenolic compounds at the concentration tested.

2.6 Statistical analyses

Statistical analysis was performed by analysis of variance (ANOVA) (Duncan post-test) to assess differences between means data. For the inflammatory biomarker, with gene expression fold and dose of total phenolic content as the independent variables, ANOVA was performed to analyse statistical significance, followed by the Tukey post-hoc. A p-value $\leqslant 0.05$ was considered statistically significant. The Pearson's correlation test was used to analyse the correlation between variables (Martins et al., 2017).

3. Results and discussion

3.1 Characterization of total polyphenol content and antiradical activity detection

The total phenolic contents (TPC) for different extraction time are reported in Table 1. In general, a TPC increase was observed as consequence of the prolongation of the extraction time to 24 h (1,440 min); extending this time over 24 hours (1,440 min) did not lead to any extra phenolic compound's extraction (data not showed).

Table 1. Total phenolic content (TPC) and total antiradical activity (AA)

	Grape Pomace (GP)		Spent Coffee Ground (SCG)		Tomato Pomace (TP)		Red Corn Cob (RCC)	
Extraction time (min)	TPC (mg GAE g^{-1} DW)	AA (µmol TE g^{-1} DW)	TPC (mg GAE g^{-1} DW)	AA (µmol TE g^{-1} DW)	TPC (mg GAE g^{-1} DW)	AA (µmol TE g^{-1} DW)	TPC (mg GAE g^{-1} DW)	AA (µmol TE g^{-1} DW)
5	14.42 ± 0.17[a]a	54.06 ± 0.15a	8.89 ± 0.55a	77.55 ± 0.36c	3.49 ± 0.25b	18.93 ± 0.37ab	6.77 ± 0.43a	15.32 ± 1.53a
10	15.52 ± 0.41a	76.39 ± 0.22b	10.05 ± 0.44b	89.76 ± 0.24e	2.65 ± 0.41a	18.75 ± 0.92ab	7.33 ± 0.33a	17.33 ± 0.35a
20	15.85 ± 0.60a	74.55 ± 0.52b	10.46 ± 0.63bc	87.60 ± 0.23d	3.79 ± 0.47bc	19.17 ± 0.83b	9.80 ± 0.37b	37.54 ± 3.72bc
30	16.93 ± 0.55a	82.58 ± 0.46c	11.04 ± 0.51cd	88.12 ± 0.03d	3.69 ± 0.31b	19.16 ± 0.65b	9.70 ± 1.32b	32.79 ± 7.73b
60	20.05 ± 0.25b	85.50 ± 0.55c	11.96 ± 0.63d	75.66 ± 0.30b	4.17±0.6bc	18.75 ± 0.17ab	11.99 ± 0.28c	47.59 ± 0.26d
120	21.63 ± 0.96b	83.02 ± 0.58c	15.79 ± 0.76e	75.89 ± 0.45b	4.5 ± 0.50cd	17.51 ± 1.00a	13.70 ± 0.37c	39.01 ± 5.32c
240	22.79 ± 0.65bc	87.84 ± 0.88d	16.20 ± 0.94e	74.98 ± 0.80b	4.64 ± 0.31d	21.47 ± 0.09c	12.00 ± 1.27c	48.92 ± 0.21d
960	24.75 ± 0.84c	88.64 ± 0.11d	17.85 ± 0.65f	75.33 ± 0.36b	5.42 ± 0.90e	20.16 ± 0.15bc	16.14 ± 0.25d	40.41 ± 0.36c
1440	31.03 ± 0.77d	96.12 ± 0.71e	21.87 ± 0.50g	73.67± 0.32a	6.79 ± 0.44f	21.27 ± 0.34c	27.13 ± 1.50e	39.23 ± 3.81c

[a]Data are the mean (n = 3) ± SD. Different letters in the column are statistically different (ANOVA, p <0.05, post-test Duncan).

The vinery by-product extracts (GP) showed the highest polyphenol content among the by-products extracts, i.e. 31.03 ± 0.77 mg GAE g^{-1} DW. These values were similar to those reported by Hogan et al., (2010), which stated a polyphenol content equal to 30.4 ± 11 mg GAE g^{-1} DW. However, Sette et al., (2020) have reported total polyphenol content of 11.40 ± 0.23 mg GAE g^{-1} DW extracted by methanol from grape marc pomace, being this value lower than that obtained in the present work using ethanol extraction.

Spent Coffee Ground (SCG) showed after 1,440 minute of extraction the maximum TPC yield, i.e. 21.87±0.47 mg GAE g^{-1} DW (Table 1), being this value higher than those reported in literature, e.g. 16 mg GAE g^{-1} DW (Mussatto et al., 2011) and 19.66 mg GAE g^{-1} DW (Zuorro and Lavecchia, 2012). Anyway, total phenolic content has been reported to be quite variable in Spent Coffee Grounds, i.e. from 17 mg GAE g^{-1} DW to 35 mg GAE g^{-1} DW, because of the use of different coffee blends, roasting degree and solvent/biomass ratio used to perform phenol extraction (Panusa et al., 2013).

Red Corn Cob (RCC) showed highest TPC content in the extract after 1,440 min extraction, i.e. 27.13±1.49 mg GAE g^{-1} DW (Table 1), being this value higher than that previously reported by other Authors, i.e. 8.33+1.31 mg GAE g^{-1} DW (Kapcum et al., 2016).

Tomato pomace showed a low content of phenolic compounds, i.e. TPC ranged from 3.49 ± 0.25 mg GAE g^{-1} DW to 6.79 ± 0.44 mg GAE g^{-1} DW for extraction time between 5-1,440 min (Table 1). Total polyphenol content in tomato pomace can vary greatly depending on several factors including tomato fruit variety, size, country of origin, degree of ripening and industrial process procedures (Periago et al., 2002), i.e. literature reported a range of 0.7 ± 0.002 – 3.2 ± 0.09 mg GAE g^{-1} in tomato by-products (Valdez-Morales et al., 2014; Abbasi-Parizad et al., 2020).

Total antiradical activity (Table 1) did not follow the same trend of TPC. Grape Pomace (GP) showed the highest AA (AA of 96.12±0.71 µmol TE g^{-1} DW) after 1,440 min according to the highest TPC reported, but Tomato Pomace (TP) and Red Corn Cobs (RCC) showed maximum AA after 240 min extraction, i.e. 21.47±0.09 µmol TE g^{-1} DW and 48.92±0.21 µmol TE g^{-1} DW, in disagree with the highest TPCs obtained (Table 1). For Spent Coffee Ground, even, maximum AA was obtained after

only 10 minutes of extraction (Table 1). These results indicated that there was no significant correlation between TPC and AA. This behavior can be explained by both the possible decomposition of molecules during extraction procedure losing part of AA and/or the involvement of other non-phenolic compounds in the extracts (Sette et al., 2020). Since phenolic compounds are thermolabile, increasing the temperature or prolonging extraction time may cause phenolic oxidation and therefore their denaturation (Fontana et al., 2013).

Antiradical activity depends greatly on polyphenol chemical structure. It has been reported that polyphenols with the *o*-dihydroxyl group in the B ring of their chemical structure give the highest scavenging activity. Quercetin and catechin are examples with *ortho* 3',4' –dihydroxy moiety in the B ring of the flavonoid structure. The occurrence of *meta* 5,7-dihydroxy groups in the A ring in the flavonoids such as kaempferol and apigenin impart larger antioxidant activity (Rice-Evans et al., 1996). The substitutions of hydrogen atoms or hydroxyl groups in A, B and C rings with methoxyl groups could add up to an increase solubility of phenolic compounds. Phenolic compounds extracted after 10 minutes from Spent Coffee Grounds demonstrated strongest antiradical activity unless TPC content was about half of that reported after 1,440 min extraction. Further characterization of phenolic compounds in SCG revealed that it was characterized for high concentrations of apigenin, epicatechin, kaempferol and naringenin chalcone (Table 2), able to explain high AA.

Since no correlation was observed between the antiradical capacity and the polyphenol content, the former was considered as criteria to select time extraction. Therefore, extract obtained after 240 min for the GP, TP and RCC, and after 10 min for SCG (Table 1), were chosen to characterize phenolic compounds and to investigate anti-inflammatory properties.

3.2 Phenolic acids and flavonoids characterization by HPLC

The phenolic acids, flavonoids, stilbene, anthocyanins and caffeine were detected by HPLC and the profile was reported in Table 2.

Flavonoids were the main phenolic compounds in all the biomass analyzed in this study (Table 3). In the Grape Pomace (GP), flavonoids (17,186 ± 74 μg g^{-1} DW) counts for more than 95% of total

polyphenols, where quercetin and its glycosylated form, i.e. rutin, followed by apigenin and naringenin were amongst the most representatives. The phenolic acids (785 ± 24 μg g^{-1} DW) in GP were mainly constituted by gallic and ellagic acids (282 ± 5 μg g^{-1} DW, 247 ± 3 μg g^{-1} DW, respectively) (Table 2). Anyway, the distribution of diverse phenolic compounds in GP has been reported to be specific for grape varieties (Fontana et al., 2013).

Table 2. Phenolic acids, flavonoids, stilbene, anthocyanins and caffeine concentration found in the biomasses studied.

	Phenolic Acids (µg g^{-1} DW)										Stilbene (µg g^{-1} DW)	Total phenolic acids (µg g^{-1} DW)
	Gallic acid	**Chlorogenic acid (s)**	**Vanillic acid**	**Caffeic acid**	**Syringic acid**	**Ferulic acid**	**P-coumaric acid**	**Sinapic acid**	**Ellagic acid**	**Cinnamic acid**	**Resveratrol**	
Grape Pomace	282 ± 5[a]a	N.D.	17.2 ± 1.6a	111 ± 4b	16.4 ± 2.1a	62 ± 4b	13.6 ± 1.1a	N.D.	247 ± 3a	16.5 ± 1.5a	19.5 ± 2.1a	785±24
Spent coffee ground	272 ± 3a	592 ± 14b	N.D.	56.4 ± 5a	N.D	81 ± 6c	27 ± 4a	70.1 ± 6b	N.D.	N.D.	N.D.	1,098±38
Tomato pomace	259 ± 29a	195 ± 4a	31.5 ± 6.2b	53 ± 0.4a	12.9 ± 1.5a	21.3 ± 0.4a	59.7 ± 5.4b	32.3 ± 1.1a	N.D.	100 ± 7b	213± 15b	978±108
Red Corn Cob	256 ± 18a	1,233 ±73c	131 ± 5c	185 ± 16c	250 ± 16b	712 ± 4d	68 ± 7b	N.D.	N.D.	216 ± 7c	N.D.	3,050±147

Table 2. Continued

	Flavonols (µg g^{-1} DW)				Flavanols (µg g^{-1} DW)		Flavones (µg g^{-1} DW)	Flavanones (µg g^{-1} DW)	Chalcones (µg g^{-1} DW)	Total flavonoids (µg g^{-1} DW)
	Quercetin	**Rutin**	**Kaempferol**	**Myricetin**	**Catechin**	**Epicatechin**	**Apigenin**	**Naringenin**	**Naringenin Chalcon**	
Grape Pomace	13,612 ± 22c	926 ± 11d	427 ± 7b	132 ± 5c	N.D.	299 ± 14a	949 ± 9c	841 ± 6b	N.D.	17,186 ± 74
Spent coffee ground	15 ± 3a	361 ± 7b	1,693 ± 9d	N.D	286 ± 9a	2,037 ± 16c	1,469 ± 10d	N.D.	N.D.	5,861 ± 128
Tomato pomace	12 ± 3a	112 ± 10a	208 ± 13a	98 ± 5b	N.D.	N.D	14.7 ± 0.5a	734 ± 7a	704 ± 8b	1,883 ± 174
Red Corn-Cob	192 ± 2b	731.5 ± 2.3c	696 ± 59c	63 ± 2a	2,887 ± 623b	1,739 ± 58b	558 ± 48.8b	N.D.	643 ± 5.6a	7,510 ± 241

Table 2. Continued

	Anthocyanin (µg g^{-1} DW)												Total Anthocy-anin content (µg g^{-1} DW)
	Cyn-3-O-G	**Pel-3-G**	**Pn-3-G**	**Cyn-3-O-mal-G**	**Pel-3-mal-G**	**Pn-3-mal-G**	**Mv-3-G**	**Cpymv-3-G**	**Mv-3-cm-G**	**Dp-3-G**	**Pt-cm-G**	**Pn-cm-G**	
Grape Pomace *ab*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	259 ± 3c	282 ± 2d	734 ± 108e	154 ± 6b	157 ± 25b	125 ± 23a	1,711±167
Red Corn Cob	1,528 ± 347b	262 ± 28a	492 ± 84a	1,727 ± 189b	340 ± 22a	400 ± 17a	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4,749 ± 687
									Caffeine (µg g^{-1} DW)				
Spent coffee ground									2,877 ± 7				

[a]Data are the mean (n = 3) ± SD. Different letters in the column indicate statistically significant differences between means ($p < 0.05$) according to Duncan post-test. (Not Detected (N.D.): The compounds were not present or in lower quantity to be detected)

[b]Cyn-3-O-G: cyanidin-3-O-glucoside, Pel-3-G: pelargonidin-3-O-glucoside, Pn-3-G: Peonidin-3-O-glucoside; Cyn-3-O-mal-G: cyanidin-3-O-malonyl-Glucoside, Carboxy-pymv-3-G (Vitisin type A): carboxy-pyranomalvidin-3-glucoside, Mv-3-cm-G: Malvidin-3-p-comaroyl-glucoside, Dp-3-G: Delphinidin-3-O-glucoside, Pt-cmG: Petunidin-p-coumaroyl-Glucoside, Pn-cm-G: Peonidin-p-coumaroyl-glucoside. [a] Pigments identified in GP by mass spectroscopy and are expressed as the Malvinidin-3-o-glucoside equivalent g $^{-1}$ DW. [b]Different letters within the same raw are statistically different (ANOVA, p <0.05, post-test Duncan) for the biomass.

In Spent Coffee Ground (SCG), gallic acid and chlorogenic acids (272 ± 3 μg g^{-1} DW, 592 ± 14 μg g^{-1} DW, respectively) were in the highest concentrations (Table 2); however, the quantified total chlorogenic acids was lower than those previously reported by other authors, i.e. 2.12 - 7.65 mg g^{-1} DW in different coffee residue varieties (Cruz et al., 2012),

The caffeine concentrations quantified was of 2.87 ± 0.007 mg g^{-1} DW, roughly lower than ranges earlier reported from various coffeemakers (3.59 mg g^{-1} to 8.09 mg g^{-1} DW) undoubtedly due to the mix coffee powder used in coffee brews (Bravo et al., 2012). The main part of the phenolic compounds in Spent Coffee Grounds (SCG) was presented by flavonoids such as epicatechin, kaempferol and apigenin (Table 2).

Phenolic acids extracted from Tomato Pomace (TP), i.e. 978 ± 108 μg g^{-1} DW, were in higher concentrations respect to other biomass (34%) and mainly consist of cinnamic acid, *p*-coumaric and caffeic acids (Table 2). Flavonoids represented 65% of TPC in Tomato Pomace, and naringenin and naringenin chalcone were the most represented ones (Table 2) according, also, to the literature (Ćetković et al., 2012).

Red Corn Cob recorded a phenolic acid content (3,050 ± 147 μg g^{-1} DW) that accounted for 29% of total polyphenols, being chlorogenic acid and ferulic acids as the most abundant, i.e. 1,233 ± 73 μg g^{-1} DW and 712 ± 4 μg g^{-1} DW, respectively (Table 2). On the other hand, among flavonoids, that represented about 70% of TPC, catechin and epicatechin were the predominant, i.e. 2,887 ± 623 μg g^{-1} DW and 1,739 ± 58 μg g^{-1} DW, respectively (Table 2).

Table 3. Total phenolic content (TPC), total anthocyanins (TAC) content and total antioxidant activity (AA)

	TPC [a] (FCR)	TPC [b] (HPLC)		AA[c]	TAC [d] (pH differential method)
		Phenolic acids	Flavonoids		
	µg GAE g^{-1} DW	µg g^{-1} DW	µg g^{-1} DW	µM TE g^{-1} DW	µg C-3-G E g^{-1} DW
Grape Pomace	22,790 ± 650	785 ± 24 (4.3%)	17,186 ± 74 (95.6%)	87.8 ± 0.9	1,914 ± 15
Spent coffee ground	10,050 ± 440	1,098 ± 38 (15.8%)	5,861 ± 128 (84.2%)	89.8 ± 0.2	-
Tomato pomace	4,650 ± 310	978 ± 108 (34.2%)	1,883 ± 174 (65.8%)	21.5 ± 0.0	-
Red Corn Cob	12,000 ±127	3,050± 147 (28.9%)	7,510 ± 241 (71.1%)	48.9 ± 0.2	5,226 ± 67

[a]TPC (FCR): Total polyphenol content measured by Folin-Ciocalteu Reagent method.
[b]TPC (HPLC): Total polyphenol content measured by HPLC.
[c]AA: Antioxidant activity.
[d]TAC: Total anthocyanin content measured by pH differential method, expressed as µg Mal-3-glc equivalents g^{-1} dry weight in grape pomace (GP) and µg cyanidin-3-O-glucoside equivalents on grams of dry weight for red corncob (RCC).

3.3. Qualitative and quantitative characterization of anthocyanins in RCC and GP.

Red Grape Pomace showed anthocyanin content of 1,914 ± 15 µg g^{-1} DW) (Table 2). Malvidin and its derivatives were identified as the main anthocyanins in GP (Table 2). Anyway, literature reported a great variability in anthocyanin contents depending on grape variety, pretreatment and conservation modality (Sette et al., 2020; Gonçalves et al., 2017).

Red Corn Cob showed a total anthocyanin content (TAC) of 5,226 ± 67 µg g^{-1} DW, that was higher than values reported previously by Kapcum et al. (2016), i.e. 2,024 ± 296 µg g^{-1} DW and 3,105 ± 345

μg g^{-1} DW, respectively for RCC varieties from Thailand. Cyanidin-3-G and Cyanidin-3-Mal-G were the anthocyanin predominant in RCC (Table 2).

3.4 Anti-inflammatory properties

To investigate the anti-inflammatory property of GP, SCG, TP and RCC extracts, IL-8 modulation has been evaluated by quantitative PCR. IL-8 is a cytokine produced through NF-κB pathway by different types of cells including macrophages, neutrophils and epithelial cells during inflammation. The cytokine IL-8 expression was increased by 9-fold with respect to the control cells as result of IL-1β stimulation (Figure 1b).

The incubation of IL-1β-stimulated cells with phenolic standard molecules or phenolic-rich cocktail (by-product extracts) reduced significantly ($p < 0.05$) the IL-8 gene expression (Figure 1b), suggesting a general positive effect of phenolic compounds in contrasting inflammatory status.

All phenolic compounds used as standard exhibited anti-inflammatory activity with the best results reported for chlorogenic acid dosed at 25 μg L^{-1} (Figure 1b). The by-products extract also induced anti-inflammatory activities that were variable depending on extract types (by-products origin) and total polyphenols concentration (TPC). In particular, the best performant extracts resulted those coming from Grape Pomace (GP) and Spent Coffee Ground (SCG) which activities were statistically different from those measured for Tomato Pomace (TP) and Red Corn Cob (RCC). A dose-dependent response of IL-8 was also observed in response to TPC concentrations for GP, SCG and TP (***$p < 0.001$) (Figure 1b). No dose effect was registered for RCC.

To simplify the comparison between by-product extracts, phenolic molecules used as standards and control, the anti-inflammatory inhibitory activities were reported, also, as percentage of inflammatory inhibition assuming IL-1 beta as 0 % inhibition (100% inflammation) (Table 4).

The largest inhibition (Figure 1b) was achieved with extracts from GP and SCG, at 25 μg mL^{-1} and 10 μg mL^{-1} TPC, respectively, i.e. 85.6% and 80.6% inhibition (Table 4), followed by RCC, i.e. 69.4 % inhibition and TP, i.e. 62.8% inhibition (Table 4), both tested at 25 μg mL^{-1}. Differences in the

anti-inflammatory activity could be ascribed to the diverse content of phenolic compounds of the extracts (Table 2).

HPLC profiling revealed that flavonoids such as quercetin, rutin and apigenin accounted for major part of phenolics in GP (Table 2), being quercetin and its glycosylated form, rutin, reported having strong anti-inflammatory activity *in vivo* (Romier et al., 2008; Mascaraque et al., 2014). Nonetheless, anthocyanin like cyanidin, malvidin and their glycosylated forms, have also demonstrated strong anti-inflammatory properties both *in vitro* and *in vivo* studies (Huang et al., 2018), suggesting, probably, a contribution to the total anti-inflammatory activity of GP extracts.

Spent Coffee Grounds displayed similar anti-inflammatory activity to the Grape Pomace, despite different phenols profile. SGC extract was tested at a concentration lower (10 μg mL^{-1}) than those considered for the other extracts (25 μg mL^{-1}) because inhibition effect was similar for both doses tested (data not showed). SCG resulted rich, above all, in flavonoids such as epicatechin, kaempferol and apigenin (Table 2) that have shown anti-inflammatory effects in different studies (Zhang et al., 2014; Devi et al., 2015). Epicatechin at concentration of 7.14 mg g^{-1} have been reported modulating the secretion of inflammatory cytokines (IL-8 and IL-1β) (Martins et al., 2017). The main phenolic acid in the Spent Coffee Grounds was identified as chlorogenic acid (Table 2) that was previously reported displaying anti-inflammatory activities (Huang et al., 2015). The activities of phenolic acids were demonstrated, also, in the present work. Testing the anti-inflammatory activity of chlorogenic acid (used as standard) at 25 μg mL^{-1}, resulted in 83.6% inhibition of cytokine expression (Figure 1b). Anyway the very low phenolic acid contents in SCG with respect to flavonoids contents (Table 2), suggested a minor role of these class of coumpounds in determining anti-inflammatory activity.

Red Corn Cob (RCC) resulted third, after GP and SCG, to reduce the inflammation (Figure 1b). The TPC concentration of 25 μg mL^{-1} demonstrated about 69.4% inhibition of the cytokine IL-8 expression (Table 4). Again, flavonoids were present at high concentration in RCC, and they were represented by rutin, apigenin, kaempferol and naringenin chalcon, being all these reported having anti-inflammatory properties (Romier et al., 2008; Zhang et al., 2014; Devi et al., 2015; Huang et al.,

2015). Flavonols, such as catechin and epicatechin, were, also, presented in relatively high concentrations that have been reported as flavonoids with anti-inflammatory properties (Martins et al., 2017; Parizad et al., 2019). Phenolic acids, above all chlorogenic and ferulic acids, were present at much lower concentrations than flavonoids and flavonols (Table 2), and although their anti-inflammatory properties have been previously described (Huang et al., 2015; Chen et al., 2017) and demonstrated, also, in this work (Figure 2b), probably their contribution was minor because of their low concentration.

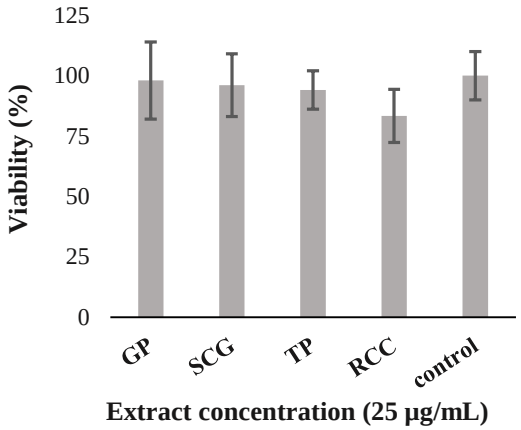
Tomato pomace (TP) demonstrated 62.8% inhibitory activity at 25 μg mL^{-1} with respect to the control (Table 4). Gallic acid, for which the ability to suppress the IL-1β secretion in Caco-2 cells was reported in literature (Romier et al., 2008) and flavonoids, i.e. kaempferol, naringenin and chalcone naringenin (Table 2), for which anti-inflammatory activities were previously discussed, were responsible for anti-inflammatory activity registered.

From the above discussion made it was not possible to correlate the anti-inflammatory activities to phenolic compounds profile, although it seemed that by-products extracts containing a high concentration of flavonoids (i.e. GP and SCG) determined the highest anti-inflammatory activities. Nevertheless, this trend was not confirmed for RCC that although showed a high content of flavonoid compounds (similar to SCG) resulted in a lower anti-inflammatory activity than GP and SCG (Figure 1b) (Table 3).

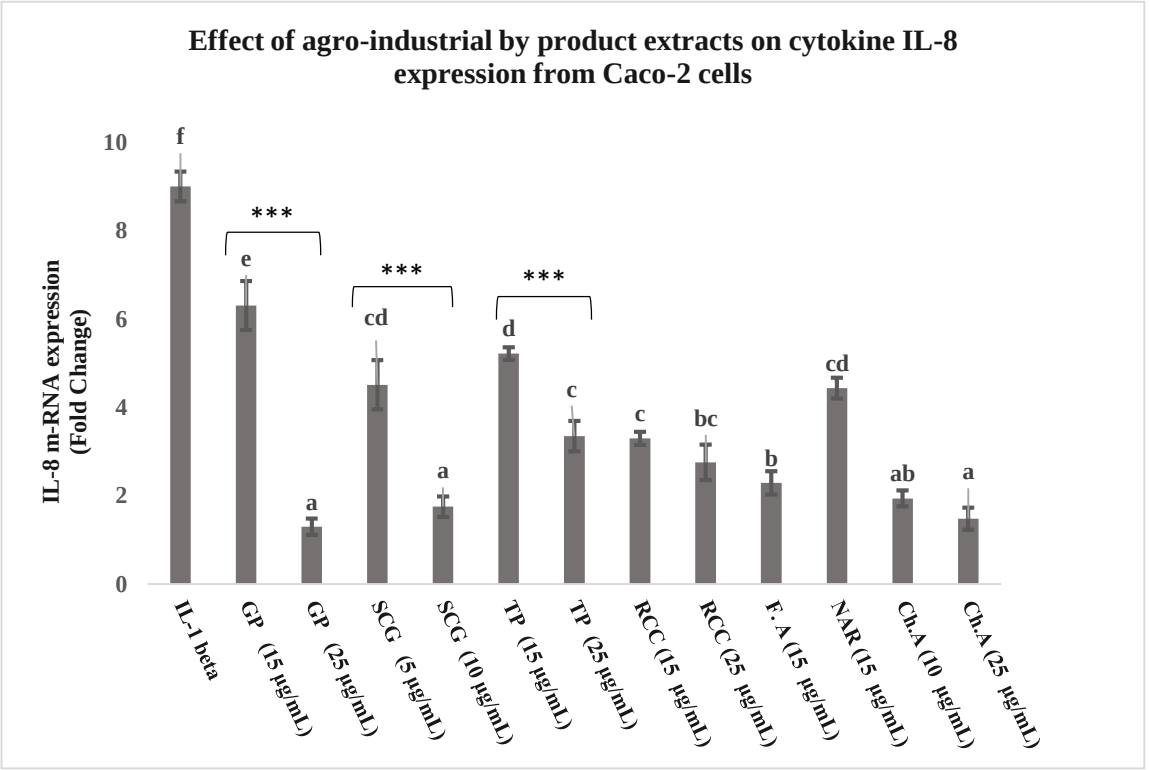
Hernández-Rodríguez et al., (2019), reported that the anti-inflammatory property of phenolic compounds was related to their antioxidant capacity. In this work, the antiradical activity was measured and reported as a rough approximation of the antioxidant activities (Table 3). Comparing antiradical activities (Table 3) with those for anti-inflammatory properties of by-products extracts (% inhibition) (Figure 4), a linear correlation was observed ($r = 0.97$, $p < 0.05$, $n = 4$), underlying the importance of antiradical activities in determining anti-inflammatory activities. This aspect should be further better investigated.

Figure 1. (a) The effect of incubating industrial by-products phenols-rich extracts at the concentration of 25 µg mL $^{-1}$ for 24 h on cell viability (expressed as % of control) of Caco-2 cells. (b) Effect of industrial by-products extracts on cytokine IL-8 expression in Caco-2 cells.

(a)



(b)



GP: Grape Pomace; SCG: Spent Coffee Ground; TP: Tomato Pomace; RCC: Red Corncob. Cells were treated with two doses of 15 µg mL $^{-1}$ and 25 µg mL $^{-1}$ determined based on the total polyphenol content (TPC). Standards of Ferulic acid (F.A) at 15 µg mL $^{-1}$, Naringenin (NAR) at 15 µg mL $^{-1}$ and Chlorogenic acid (Ch.A) at both 10 µg mL $^{-1}$ and 25 µg mL $^{-1}$ were used as control. Interleukin-1 beta pre-treatment was used to stimulate the inflammatory markers.

Fold change in IL-8 cytokine expression treated with IL-1 β (20 ng mL^{-1}) in Caco-2 cells is expressed as mean ± SD of three individual assays. Statistical significance between treatments were tested using ANOVA. The effect of increased concentrations of phenolic compounds in by-product extracts on IL-1β in Caco-2 cells was analyzed by using ANOVA and post-hoc Tukey HSD test ($p \leqslant 0.05$). *** Increased Concentrations is statistically significant at p value = 0.001).

Table 4. Anti-inflammatory inhibitory activity (%)

By-products	Inhibition (%)
GP (15 µg mL $^{-1}$)	30[a]
GP (25 µg mL $^{-1}$)	85.6
SCG (5 µg mL $^{-1}$)	49.9
SCG (10 µg mL $^{-1}$)	80.6
TP (15 µg mL $^{-1}$)	42
TP (25 µg mL $^{-1}$)	62.8
RCC (15 µg mL $^{-1}$)	63.4
RCC (25 µg mL $^{-1}$)	69.4
Ferulic Acid (15 µg mL $^{-1}$)	74.6
Naringenin (15 µg mL $^{-1}$)	50.7
Chlorogenic Acid (10 µg mL $^{-1}$)	78.5
Chlorogenic Acid (25 µg mL $^{-1}$)	83.6

[a]The percentage of anti-inflammatory inhibitory property was calculated according to the value given by IL-1β. GP: Grape Pomace; SCG: Spent Coffee Ground; TP: Tomato Pomace; RCC: Red Corncob, cells were treated with two doses of 15 µg mL^{-1} and 25 µg mL^{-1} determined based on the total polyphenol content.

3.5 From research to full-scale application

This study reported that by-products could be useful recovered by extracting bioactive molecules contained, which exhibited anti-inflammatory activity. Nevertheless, these results were not enough to prefigure a potential industrial application.

Therefore, it was interesting to study and compare the different by-products making a first attempt at indicating the more suitable by-products for possible full-scale upgrading, taking into consideration results of this work, i.e. phenol contents, properties, and anti-inflammatory performance, and adding

information related to by-products provenance and diffusion, daily by-products availability and preservation. Doing so the by-products studied in this work were ranked vs. suitability to be upgraded, in the following order: spent coffee grounds >> grape pomace > red corncobs >> tomato pomace (Table 5). Obviously, moving to a possible utilization of the results obtained at full-scale, additional data related to energy and chemical consumption, economic data etc., which were not among the goals of this work, are needed. In this way, previous studies, e.g. Zalazar-Garcìa et al. (2020), which studied phenolic extraction from biowaste performing mass and energy balances as well as providing information to optimize extraction processes, can be used as a model.

Table 5. Qualitative approach in ranking suitability in producing anti-inflammatory extract from different by-products

Biomass	Provenience and diffusion in the world	Day delivery	Preservation	Total yield of phenolic compounds	Total antiradical activity	Immunomodulatory properties
Grape pomace	+++	+/-	+	++++	++++	++++
Spent coffee grounds	++++	++++	+++	+++	++++	++++
Tomato pomace	+++	+/-	+	+	+	++
Red corn cob	++	+/-	++++	++	++	++

Rank: +/- = neutral; + and ++ = low-average (e.g. local diffusion); +++ and ++++ = high-very high (e.g. wide diffusion).

4. Conclusions

The results of this work confirmed the potential for using ethanol extracts from agro-industrial by-products rich in phenolic compounds as anti-inflammatory preparations. In this way, this work has set up the molecular basis for the explanation of anti-inflammatory activities of common agro-industrial residues.

Further investigations need to move from lab-scale to industrial-scale application by integrating scientific data with energy and economic data. In this way the interest should be focused on the most promising by-products, i.e. spent coffee grounds, grape pomace and red corncobs.

Acknowledgment

This work was supported by Gruppo Ricicla labs, University of Milan (UNIMI), Italy, project N. 14135 RV_PRO_RIC16FADAN02_M.

All the experiments involving cultured Caco-2 cells have been carried out in the DeFENS Cell Culture Laboratory, a core facility of the Department of Food, Environmental and Nutritional Sciences University of Milan (UNIMI), Italy.

Appendix A. Supplementary data

Appendix A. Supplementary data to this article can be found online at

Conflicts of Interest: The authors declare no conflict of interest.

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