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Study of the Chemical Components, Bioactivity and Antifungal Properties of the Coffee Husk

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Abstract

Coffee husk is a fibrous mucilaginous material representing the major residues from the handling and processing of coffee. Currently, coffee husk is discarded which contributes to environmental pollution. Dry and semi-dry coffee husk were extracted by hot infusion and the polyphenol profile was studied by UHPLC and Q-Orbitrap mass spectrometry. Free radical scavenging activity in the infusion was between 26.61 at 31.33 mmol Trolox equivalent antioxidant capacity (TEAC)/kg, as measured by the assay with ABTS and DPPH radical. The results indicated that coffee husk contained 367.45 and 396.04 g/kg of total dietary fiber in dry and semi-dry sample, respectively. While the infusions contain exclusively soluble dietary fiber 0.64 and 0.98 g/L. The methanol extracts of coffee husk presented antimicrobial activity of *P. camemberti*, *P. expansum* and *P. roqueforti*, *A. flavus* and *A. niger*, many of their mycotoxins producers. The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) values for the *Penicillium*s strains ranged from 6.3 to 50 g/L, whereas on the *Aspergillus* strains the data detected ranged from 25 to 50 g/L, respectively. This study indicates the possible exploiting of this coffee by-product as potential natural health promoting and preservative ingredient.

Keywords: bioactive compounds, polyphenol, antifungal properties, HRMS-Orbitrap

1. Introduction

Coffee is the most preferred morning beverage throughout the world due to its pleasant flavor and stimulating properties. In 2016, the total production of coffee was of 151.624 million of 60 kg bags, and the Brazil is largest producer (ICO, 2016); its production, indeed, causes the generetion of an enormous amount of wastes and by-product (Andrade et al., 2011). It was observed that coffee-derived wastes and by-products represent a source of serious contamination, in main coffee producer countries. Particularly, caffeine (12 g/kg), tannins (63 g/kg) and polyphenols (Franca, Gouvea, Torres, Oliveira, & Oliveira, 2009; Rathinavelu & Graziosi, 2005; Andrade et al., 2011), have been recognized the main substances involved in this environmental problem. (Franca, Gouvea, Torres, Oliveira, & Oliveira, 2009; Rathinavelu & Graziosi, 2005; Andrade et al., 2011),

Coffee husk is a fibrous mucilagenous material obtained during the processing of coffee cherries by dry and semi-dry process, respectively (Pandey et al., 2000). It is the main residue in the production of coffee and currently it was used especially in ruminant feed. Several studies, proposed coffee husk as silage, aerobic composting, animal feed, vermiculture, production of biogas, vinegar, biopesticides, enzymes, single-cell protein and probiotics (Fan, Soccol, Pandey, & Soccol, 2003). However, this residue has low commercial use and it is returned to the soil or burned, could be a serious threat to environmental pollution.

Recently the use of by-products for further using as food additives on the production of natural food ingredients with high nutritional value have gained increasing interest because these are high-value products and their recovery may be economically attractive (Murthy & Naidu, 2012a)

Coffee husks can be an innovative resource of phytochemicals for the food and pharmaceutical industries, because it contains high levels of compounds with antioxidant activity, caffeine and dietary fiber (Esquivel &

Jiménez, 2012; Mennini, 2013).

Furthermore caffeic and quinic acid derivatives showed a remarkable antifungal activity. In particular, recently was provide the evidence that caffeic and caffeolquinic acids are able to inhibit the fungal growth by blocking the glucan synthase in cell wall. (Ma & Ma, 2015; Villarino, Sandín-Espana, Melgarejo, & De Cal, 2011).

The objectives of this study were a) to investigate the qualitative and quantitative profile of the bioactive compounds in coffee husk (raw material and infusion) using UHPLC-High resolution mass spectrometry b) the antioxidant activity through the ABTS and DPPH assays c) Determination of Total, Soluble, and Insoluble Fiber and d) the antifungal activity in solid and liquid medium (MIC-MFC).

2. Materials and Methods

2.1 Samples and Treatments

Two types of coffee husk derived from coffee Arabica (coffee husk 1 and coffee husk 2) which will be called from now CH1 and CH2, were obtained from Caffè do Brasil S.p.A. (Naples, Italy). CH1 and CH2 were obtained by dry and semi-dry processing of coffee cherries, respectively. The procedure adopted for the preparation of coffee husk infusion (CHI) was as follows: 100 mL boiling water was poured onto 4 g of the coffee husk, covered and left to infuse for 4 min. After filtration with Whatman No. 1 paper, the volume and dry matter yield were measured. Subsequently, infusions were filtered through a 0.2 μm nylon filter and immediately frozen and stored at -18 ℃ until analysis. For the preparation of coffee husk polyphenol extract (CHPE), each sample was weighed (0.5 g), reduced to a fine powder in a blender and extracted with 25 mL of CH_3OH:H_2O (80:20 *v/v*) by vortexing for 2 min followed by 10 min in an ultrasonic bath and 20 min on an horizontal shaker at 250 rpm. The solution was centrifuged at 4500g for 5 min and the supernatant was transferred to a 50 mL tube and the extraction repeated on the pellet with further 25mL of 80% aqueous CH_3OH, supernatants were combined, filtered through a 0.2 μm nylon filter and stored at -18 ℃ until analysis.

2.2 Total Antioxidant Activity

The free radical scavenging activity of the CHIs and CHPEs were evaluated using 2,2′ azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical cation decolorization (Re et al., 1999). The absorbance was calculated at 734 nm, using a UV-VIS spectrophotometer. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity of samples was determined using the method by Yen & Chen (1995). The antioxidant capacity based on the DPPH and ABTS free radical scavenging ability of the extract was expressed as mmol Trolox equivalents (TEAC) per kg of sample and all determinations were perfomed in triplicate.

2.3 Determination of Total, Soluble, and Insoluble Fiber

The amount total dietary fiber (TDF), insoluble dietary fiber (IDF) and soluble dietary fiber (SDF) was calculated according to a gravimetric enzymatic method AOAC Official Method 991.43 as the weight of residue minus the weight of protein and ash.

2.4 UHPLC-High Resolution Mass Spectrometry (HRMS-Orbitrap) Analysis of Polyphenolic Compounds

Qualitative and quantitative profile of polyphenols including caffeine has been obtained using Ultra High Pressure Liquid Chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T=25 ℃) Kinetex 1.7 μm Biphenyl (100x2.1 mm) column. Injection volume was of 2 μL. Eluent phase was formed as follow: phase A (H_2O in 0.1% formic acid), phase B (methanol in 0.1% formic acid). All metabolites were eluted using a 0.2 mL/min flow rate with a gradient programmed as follows: 0 min - 5 % of phase B, 1.3 min - 30% of phase B, 9.3 min - 100% of phase B, 11.3 min - 100% of phase B, 13.3 min - 5% of phase B, 20 min - 5% of phase B.

For the mass spectrometry analysis a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) has been used. An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative ion mode (ESI-) for all the analyzed compounds and in positive (ESI+) ion mode for caffeine. Ion source parameters in (ESI-) mode were: spray voltage -2.8 kV, sheath gas (N_2 >95%) 45, auxiliary gas (N_2 >95%) 10, capillary temperature 275 ℃, S-lens RF level 50, auxiliary gas heater temperature 305 ℃. Value for automatic gain control (AGC) target was set at 1×10^6, with a resolution of 70,000 FWHM (full width at half maximum), and a scan rate in the range between 100 and 1000 *m/z* in Full MS/Scan mode. Ion source parameters in (ESI+) mode used for alkaloides analysis were: spray voltage 3.3 kV, sheath gas (N_2 >95%) 45, auxiliary gas (N_2 >95%) 13, sweep gas 7, capillary temperature 275 ℃, S-lens RF level 50, auxiliary gas heater temperature 305 ℃. The

AGC target value was of 1×10^6, the resolution set at 70,000 FWHM, with a scan rate in the range between 100 and 1000 *m/z* in the Full MS mode.

The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked daily using a reference standard mixture obtained from Thermo Fisher Scientific. The linearity of the method was assessed at both low (LOQ-5 mg/kg) and high (5 mg/kg-120 mg/kg) concentration ranges, using six concentration levels in each calibration range. Calibration curves for all compounds were prepared in triplicate. Sensitivity was evaluated by limits of detection (LODs) and limits of quantitation (LOQs). Data analysis and processing has been performer using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific).

The peaks for the studied compounds in the samples were confirmed by comparing the retention times of the peak with those of standards as well as by recognizing both the precursor and product ions with a mass error below 5 ppm.

2.5 Microorganisms and Culture Conditions

The strains of *P. camemberti* CECT 2267, *P. expansum* CECT 2278, *P. roqueforti* CECT 2905, *A. parasiticus* CECT 2681, *A. niger* CECT 2088, *F. moniliformis* CECT 2982, *F. verticillioides* CECT 20926, and *F. graminearum* CECT 20490 were provided by the Spanish Type Culture Collection (CECT, Valencia, Spain). The strain of A. flavus ITEM 8111 was obtained by the Agro Food Microbial Culture Collection of the Institute of Science of Food Production (Bari, Italy), and were preserved in sterile 30 % glycerol at -80 ℃. Therefore, they were mainteined in PDB broth at 25 ℃ and inoculated in PDA plates.

2.6 Antifungal Activity Tests on Solid Medium

Agar well diffusion method used to evaluate the antimicrobial activity of the water and methanol extracts of coffee husks samples was performed according to (Madhyastha, Marquardt, Masi, Borsa & Frohllch, 1994). Ten microliters of extract were added on sterile discs (6-mm Whatman No. 1, Madrid Spain). All strains were maintained in PDA for 7 days at 30 ℃, and the spores were obtained at the time of analysis using 1 mL of distilled water added on the agar surface. After that, 100 μL of a spore suspension was introduced in plate containing 10 mL of liquid PDA at 45 ℃. Once the medium has solidified, the discs were placed on the agar surface. After refrigeration at 4 ℃ for 6 h, the plates were incubated 7 days at 30 ℃. After the fungal growth, the measurement of the inhibition halo diameter was carried out, being considered positive for antifungal activity halos larger than 8 mm around the disc.

2.7 Determination of Minimum Inhibitory Concentration and M ĺnimum Fungicidal Concentration (MIC-MFC)

The test was performed in liquid medium and 96 well clear polystyrene microplate, using the modified method of (Siah, Deweer, Morand, Reignault & Halama, 2010). A volume of 100 μl of water and methanol extracts of coffee husks at concentrations of 0.1, 0.5, 1, 5, 10 50, 100, 250 and 500 μg/ml was added in the wells. The wells were inoculated with the mycotoxigenic fungi using 100 μl of a 10^5 spores/ml suspension in PDB. The negative control consisted of inoculated medium without any treatment. The microplates were incubated at 25 ℃, on a rotary shaker at 140 rpm for 7 days. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the coffee husk extract, that prevents visible fungal growth. Four replicates of each micro-assay were carried out and the experiment was repeated three times. For the Minimum Fungicidal Concentration (MFC) assay, the concentration corresponding to the MIC and higher were sub-cultured on PDA plates. The MFC is the lowest extract concentration in which a visible growth of the subculture was prevented after 3 days of incubation at 25 ℃.

2.8 Statistics and Data Analysis

The experiments were done in triplicate. The results are given as mean standard deviation (SD). One-way analisys of variance (ANOVA) was applied to the data by the use of IBM SPSS (version 21). A difference was considered statistically significant when $p \leq 0.05$.

3. Results and Discussion

3.1 Determination of TDF, IDF and SDF

Epidemiologic evidence suggests that a higher intake of dietary fiber and phytochemicals is associated with a lower risk of cardiovascular diseases and cancer (Petersen, Flock, Richter, Mukherjea, Slavin, & Kris-Etherton, 2017). In this regard, the intake of dietary fiber in Western countries should increase to help prevent degenerative diseases (Fidanza & Perriello, 2002). The amount of dietary fiber contained in CHIs and CHs are shown in Table 1. CHs as raw material for preparation of the new infusions showed amounts of TDF ranging from 367.45 to 396.04 g/kg for dry and wet CH respectively, these results are according with the values reported in literature

(from 245 to 430 g/kg) (Murthy & Naidu, 2012b). The high content of SDF can be considered as a very positive aspect of CHs. The consumption of functional foods enriched with fiber-rich by-products from coffee, might increase the total dietary fiber intake, representing a nutritional strategy in reaching the recommended dietary allowance (RDA). CHI contain soluble food fibers (SDF) and phenolic compounds that represent compounds of nutritional value. The CHIs, in fact, contained exclusively soluble dietary fiber ranging from 0.64 to 0.98 g of SDF per liter (Table 1). The amount of SDF is comparable with that of Hibiscus sabdariffa L and may represent a significant nutritional and health promoting quality considering that SDF appear to profitably modify biomarkers of cardiovascular disease, including low-density lipoprotein cholesterol (LDL-C) and C-reactive protein (Ning, Van Horn, & Lloyd-Jones, 2014).

Table 1. Comparison of DF content in CHIs and dry matter of CH and Roselle Flower (Hibiscus sabdariffa L.). The results are expressed in g/kg dry weight and in g/L of SDF in beverages

		CH1	**CH2**	**Hibiscus sabdariffa L.**
TDF	Dry matter	367.45 ± 2.49^{a}	396.04 ± 3.18^{b}	339.02 ± 2.13^{c}
	Infusion	0.98 ± 0.12^{a}	0.64 ± 0.53^{b}	$0.66.14 \pm 0.33^{b}$
IDF	Dry matter	326.35 ± 1.49^{a}	350.26 ± 1.89^{b}	290.45 ± 4.15^{c}
	Infusion	ND	ND	ND
SDF	Dry matter	41.26 ± 0.49^{a}	46.67 ± 0.22^{b}	49.02 ± 0.33^{c}
	Infusion	0.98 ± 0.08^{a}	0.64 ± 0.22^{b}	$0.66.45 \pm 0.13^{c}$

Value are ± standard deviation of three independent determinations. The different superscripts in the same line with different letters are significantly different ($p<0.05$)

SDF = Soluble Dietary Fiber, IDF = Insoluble Dietary Fiber, TDF = Total Dietary Fiber, CH = Coffee Husk and ND = Not detectable

3.2 Total Antioxidant Activity

The antioxidant activity was evaluated by ABTS and DPPH assays on row materials (CH1 and CH2), infusions (CHI) and polyphenol extracts (CHPE). The results are reported in Table 2. In particular, all samples showed antioxidant values ranged from 28.17 to 31.33 (ABTS assay) and from 26.61 to 29.55 TEAC mmol/kg (DPPH assay). The results obtained with ABTS and DPPH assays showed a remarkable antioxidant activity of polyphenolic extract of two analyzed samples. The antioxidant activity linked to polyphenolic extract is found in the infusion, highlighting that most of the polyphenols with antioxidant activity are hydrophilic and therefore extracted during aqueous infusion. The procedure used for the CHIs preparation is able to exctract more than 90% of antioxidant capacity measured on CHPEs. Few reports are reported on coffee husk antioxidant activity. The authors reported that the higher free radical scavenging activity was exhibit by coffee husk extracts treated by low pressure methods, highlighting that ethanolic extraction using ultrasonic bath allows to obtain an extract with an antioxidant activity evaluated with ABTS radical of 161 ± 3 mmol TEAC/kg (Andrade et al., 2011). of 161 ± 3 mmol TEAC/kg (Andrade et al., 2011). This value could be associated to the different coffee varieties and to different conditions of technological process. Our results about antioxidant activity measured with ABTS assay are comparable to those reported in literature, which shows that the coffee by-products have an appreciable free radical scavenging activity measured with ABTS assay: silverskin has the highest antioxidant activity (21.2 mmol Trolox/kg) followed by spent waste (20.4 mmol Trolox/kg), cherry husk (18.4 mmol Trolox/kg), and coffee pulp (15.3 mmol Trolox/kg) among the coffee byproducts (Murthy & Naidu, 2012b). Data on DPPH antioxidant activity on coffee husk, in literature were expressed as AA% and this prevents the comparison with our results.

Table 2. Antioxidant activity in CHPE and CHI samples. The results are expressed in mmol TEAC/kg

	CH1		CH2	
Samples	**ABTS**	**DPPH**	**ABTS**	**DPPH**
CHPE	31.33 ± 0.49^{a}	29.55 ± 0.79^{a}	29.05 ± 1.02^{a}	29.41 ± 0.35^{a}
CHI	28.58 ± 0.52^{b}	26.89 ± 0.59^{b}	28.17 ± 0.59^{a}	26.61 ± 0.59^{b}

Value are ± standard deviation of three independent determinations. The different superscripts in the same column with different letters are significantly different ($p<0.05$)

CH – Coffee Husk sample

CHPE – Coffee Husk polyphenol extract

CHI – Coffee Husk infusion

3.3 Identification of the Bioactive Compounds in Coffee Husks and in Infusion by High-resolution Mass Spectrometry (HRMS-Orbitrap)

A Q Exactive Orbitrap LC-MS/MS has been used for the mass spectrometry analysis. An ESI source operating in Full MS/Scan and PRM mode, both in negative (ESI-) and in positive (ESI+) ion mode was used for all the analyzed compounds. Qualitative data regarding the phenolic compounds of coffee husk extracts are shown in Table 3. In this work, the bioactive compounds has been identified by using MS/MS experiments; accurate mass measurements and precursor ion scan. The isotopic patter and accurate masses were selected for to establish the element composition. The strategies for identification of polyphenols were based on observed MS/MS spectra than those in the literature. Table 3 reported the 30 compounds identified using triple-quadrupole MS/MS and Orbitrap experiments, in addition to theoretical and measured mass, accuracy, molecular formula, retention times, collision energy and the fragment ions exploited for the identification.

Table 3. Peak Numbers, Compound name, formula, Ionization mode, Theoretical and Measured (*m/z*), Accuracy, fragment ion, collision energy and retention time

Peak	Compound	Formula	Ionization mode	Theoretical (*m/z*)	Measured (*m/z*)	Accuracy (Δmg/kg)	Fragment ions (*m/z*)	CE (V)	RT (min)
1	Trigonelline	$C_7H_7NO_2$	$[M-H]^+$	13.805.496	13.805.514	1.30	11.006.006	30	1.44
2	Gallic Acid	$C_7H_6O_5$	$[M-H]^-$	16.901.425	1.690.147	2.66	125.02428; 169.01406	20	2.37
3	Protocatecuic Acid	$C_7H_6O_4$	$[M-H]^-$	15.301.933	15.301.953	1.31	10.902.933	20	5
4	3-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	35.308.781	35.308.817	1.02	191.05609; 179.03481; 135.04508	20	7.06
5	(+)-catechin	$C_{15}H_{14}O_6$	$[M-H]^-$	28.907.176	28.907.186	0.35	245.08187; 203.07126; 205.05025	20	7.79
6	5-*p*-Coumaroylquinic Acid	$C_{16}H_{18}O_8$	$[M-H]^-$	33.709.289	33.709.338	1.45	191.05598; 173.04520; 163.03989	20	7.94
7	5-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	35.308.781	35.308.796	0.42	191.05609; 179.03478	20	8.25
8	Caffeic acid	$C_9H_8O_4$	$[M-H]^-$	17.903.498	17.903.507	0.50	13.504.503	20	8.37
9	Theobromine	$C_7H_8N_4O_2$	$[M-H]^+$	18.107.200	18.107.208	0.44	110.07130; 163.06142; 135.06652;	30	8.55
10	procyanidin A type trimer	$C_{45}H_{36}O_{18}$	$[M-H]^-$	86.318.290	86.318.502	2.46	57.512.382	25	8.57
11	Gentisic Acid	$C_7H_6O_4$	$[M-H]^-$	15.301.933	15.301.949	1.05	10.902.936	20	8.66
12	Theofillyne	$C_7H_8N_4O_2$	$[M-H]^+$	18.107.200	18.107.209	0.50	69.04483; 124.05061; 96.05561	30	8.67
13	(−)-epicatechin	$C_{15}H_{14}O_6$	$[M-H]^-$	28.907.176	28.907.218	1.45	245.08163; 205.05034	20	8.7
14	4-*O*-caffeoylquinic acid	$C_{16}H_{18}O_9$	$[M-H]^-$	35.308.781	35.308.826	1.27	173.04537;191.05589; 179.03484	20	8.93
15	procyanidin B type trimer	$C_{45}H_{38}O_{18}$	$[M-H]^-$	86.519.854	86.520.035	2.09	577.13519; 287.05609; 289.07185	40	8.93
16	4-*O*-feruloylquinic acid	$C_{17}H_{20}O_9$	$[M-H]^-$	36.710.346	36.710.370	0.65	19.105.609	20	9.33
17	*p*-Coumaric Acid	$C_9H_8O_3$	$[M-H]^-$	16.304.007	16.304.037	1.84	11.905.014	20	9.56
18	5-*O*-feruloylquinic acid	$C_{17}H_{20}O_9$	$[M-H]^-$	36.710.346	36.710.357	0.30	191.05600; 173.04537	30	9.6
19	3,4-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	51.511.950	51.511.982	0.62	353.08744; 173.04527; 179.03477; 335.07697	25	9.93
20	quercetin-3-*O*-rutinoside	$C_{27}H_{30}O_{16}$	$[M-H]^-$	60.914.611	60.914.685	1.21	30.103.514	30	10
21	3,5-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	51.511.950	51.511.986	0.70	17.903.486	30	10.06
22	quercetin-3-*O*-glucoside	$C_{21}H_{20}O_{12}$	$[M-H]^-$	46.308.820	46.308.888	1.47	30.103.465	32	10.16
23	Ferulic acid	$C_{10}H_{10}O_4$	$[M-H]^-$	19.305.063	19.305.070	0.36	149.00352; 134.03721; 178.02727	20	10.16
24	4,5-*O*-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$	$[M-H]^-$	51.511.950	51.511.983	0.64	35.308.725		10.49
25	Luteolin-7-*O*-glucoside	$C_{21}H_{20}O_{11}$	$[M-H]^-$	44.709.328	44.709.363	0.78	28.504.044	30	10.6
26	Isorhamnetin 3-*O*-Rutinoside	$C_{28}H_{32}O_{16}$	$[M-H]^-$	62.316.176	62.316.241	1.04	31.505.104	30	10.64
27	Caffeine	$C_8H_{10}N_4O_2$	$[M-H]^+$	19.508.765	19.508.780	0.77	138.06617; 110.0713;	25	10.74
28	3-*O*-Feruloyl-4-*O*-caffeoylquinic acid	$C_{26}H_{26}O_{12}$	$[M-H]^-$	52.913.515	52.913.538	0.43	367.10296; 335.07703	25	10.85
29	4-*O*-Caffeoyl-5-*O*-feruloylquinic acid	$C_{26}H_{26}O_{12}$	$[M-H]^-$	52.913.515	52.913.594	1.49	367.10318; 335.07699	25	11.41
30	Naringenin	$C_{15}H_{12}O_5$	$[M-H]^-$	27.106.120	27.106.156	1.33	151.00355; 119.05012; 271.06118	20	12.08

The discrimination among isomers of CGA was done using their fragmentation pattern in MS^2 and their relative intensity, in accordance with the hierarchical scheme of identification described by (Clifford, Johnston, Knight, & Kuhnert, 2003). For the peaks 4, 7 and 14 mass spectral analysis showed the same molecular ion negatively charged $[M-H]^-$ at *m/z* 353.08781. The isomers produced different mass spectra in-source fragmentation of *m/z* 353. 08781 which allowed distinguish the different compounds.

These 3 isomers were differentiated by the MS^2 fragmentation pattern. The 4-CQA (peak 14) showed a MS^2 base peak at *m/z* 173.04537 ([quinic acid-H-H_2O]) due to the dehydration of the quinic acid ion, a feature of cinnamoyl group bonded to the quinic acid moiety at the position 4. The 3-CQA (peak 4) and 5-CQA (peak 7) showed the same MS^2 base peak at *m/z* 191.05609 ([quinic acid-H]) and they could be distinguished by comparing the relative intensity of the secondary ion from caffeoyl moiety ([caffeic acid-H]) at *m/z* 179.03498 (Rodrigues & Bragagnolo, 2013). Peak 6 showed MS^2 base peak at *m/z* 191.05609 ([quinic acid-H]).

Peaks 16 and 18 with $[M-H]^-$ ions at *m/z* 367.10346 indicating the presence of feruloylquinic acids. 4-*O*-feruloylquinic acid showed a single fragment MS^2 base peak at *m/z* 191.05605. Peak 18 generated fragment ion at *m/z* 191.05605 with additional minor ion at *m/z* 173.04537 in keeping with that previously described for Peak 16.

Peaks 19, 21, and 24 with $[M-H]^-$ ions at *m/z* 515.1195 were identified as di-CQA isomers according to the formula $C_{25}H_{23}O_{12}^-$. Dicaffeoylquinic acid isomers showed peaks at *m/z* 353 and 515, which confirmed the losses of two caffeic acid units. The analysis in the LTQ-Orbitrap confirmed their presence showing the deprotonated molecule $[M-H]^-$ (*m/z* 515), the ion $[M-H-162]^-$ (*m/z* 353), corresponding to the loss of a caffeic acid unit and the deprotonated caffeic acid (*m/z* 179). Peaks 28 and 29 showing a fragmentation pattern characterized by predominant ion $[M-H]^-$ at *m/z* 529.13515 this daughter ion is indicative of an *O*-caffeoyl-*O*-feruloyl quinic acid conjugated compound. Peaks 28 and 29 showed a MS^2 base peak at *m/z* 367.10296 and the fragmentation spectra showed an additional fragment ions at *m/z* 335.07703. This would suggest that these compounds are the 3-*O*-feruloyl-4-*O*-caffeoylquinic acid isomers.

The ionization in the positive mode generated the protonated molecule $[M+H]^+$ for alkaloids. Theobromine and theophylline are methyl xanthines, which are intermediates of caffeine metabolism. Peak 9 and 12 were differentiated by MS^2 fragmentation pattern. The fragmentation pathway was characterized by base peak at *m/z* 110.07130 and 69.04483, this suggest the loss of $C_3H_3NO_2$ (cyanoformate) and $C_4H_4N_2O_2$ (diisocyanatoethane), respectively. Trigonelline (Peak 1) *m/z* 138.05496 shows a fragmentation pattern with the parent ion producing a single fragment at *m/z* 110.06006. Caffeine (Peak 27) in MS^2 fragmentation shows the base daughter ion at *m/z* 138.06617 corresponded to loss of C_2H_3NO methylcarbamoyl.

For cinnamic and benzoic acids the spectra showed the deprotonated molecule $[M-H]^-$. For ferulic, caffeic, protocatechuic, gentisic, *p*-coumaric and gallic acid loss of CO_2 was observed, giving as a characteristic ion the $[M-H-44]^-$ (Rabaneda et al., 2003). Peak 5 and 13 showed the same MS^2 base peak at *m/z* 245.08187 this suggest the loss of CH_2CHOH group (Pérez, Revilla, González-SanJosé, & Beltrán, 1999).

For flavonol *O*-glycosides such as quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, luteolin-7-*O*-glucoside and isorhamnetin 3-*O*-rutinoside the spectra generated with a collision energy (CE) between -30 and -32 V revealed both the deprotonated molecule $[M-H]^-$ of the glycoside and the ion corresponding to the deprotonated aglycone $[A-H]^-$. For Peak 30 the fragmentation pathway was characterized by base peak at *m/z* 151.00355 and 119.05012 characteristic for naringenin and its derivatives (Rabaneda et al., 2003). For peaks 10 and 15 loss of catechin residue was observed, obtaining the base daughter ion $[M-H-288]^-$ at *m/z* 575.12382 and 577.13519, respectively.

3.4 Quantification of Major and Minor Compounds Found in Coffee Husks and in Infusion

The CGAs identified and quantified in CHPEs and CHIs showed in Table 4 and the results are expressed as mg/kg. In CHPEs the results showed amounts of CGA ranging from 1019.19 to 1564.68 for CHPE1 and CHPE2, respectively. The infusion contains an amount of CGA ranging from 370.22 to 608.11 for CHI1 and CHI2, respectively. In all samples the 5-*O*-caffeoylquinic acid was the major CGA present, in according with the work of (Mullen, Nemzer, Stalmach, Ali, & Comb, 2013). Even the 5-*O*-feruloylquinic acid was present in high quantities, no statistically significant differences among samples of the same type were observed. Dicaffeoylquinic acid isomers, 3-*O*-Feruloyl-4-*O*-caffeoylquinic acid and 4-*O*-Caffeoyl-5-*O*-feruloylquinic were not detected in CHIs.

Table 4. Quantification of Major Compounds in CHPEs and CHIs in mg/kg, LOD and LOQ value in μg/kg

Compound	**CHPE 1**	**CHPE 2**	**CHI 1**	**CHI 2**	**LOD**	**LOQ**
3-*O*-caffeoylquinic acid	24.41±0.09	53.34±0.88	8.34±0.19	19.01±0.93	0.07	0.20
5-*p*-Coumaroylquinic Acid	35.12±0.19	61.55±0.76	8.82±0.12	20.23±0.43	0.07	0.20
5-*O*-caffeoylquinic acid	654.72±2.49	1121.7±5.22	283.32±0.12	502.0±3.59	0.07	0.20
4-*O*-caffeoylquinic acid	7.62±0.04	11.12±0.49	<LOQ	<LOQ	0.07	0.20
4-*O*-feruloylquinic acid	15.21±0.11	14.72±0.77	5.32±0.12	5.34±0.12	0.07	0.20
5-*O*-feruloylquinic acid	170.32±3.29	171.82±1.43	64.42±0.43	61.53±0.34	0.07	0.20
3,4-*O*-dicaffeoylquinic acid	22.82±0.53	20.21±0.14	<LOQ	<LOQ	0.07	0.20
3,5-*O*-dicaffeoylquinic acid	62.42±0.23	65.51±1.12	<LOQ	<LOQ	0.07	0.20
4,5-*O*-dicaffeoylquinic acid	18.28±0.12	34.41±1.23	<LOQ	<LOQ	0.07	0.20
3-*O*-Feruloyl-4-O-caffeoylquinic acid	8.62±0.13	8.84±0.22	<LOQ	<LOQ	0.07	0.20
4-*O*-Caffeoyl-5-O-feruloylquinic acid	0.4±0.09	1.46±0.02	<LOQ	<LOQ	0.07	0.20
Total major compounds	1019.19	1564.68	370.22	608.11		

Value are ± standard deviation of three independent determinations.

CHPE – Coffee Husk polyphenol extract

CHI – Coffee Husk infusion

LOD - Limit of Detection

LOQ - Limit of Quantification

The quantitative data on the alkaloids compounds found in the CHPEs and CHIs are reported in Table 5. The results showed that caffeine and trigonelline were the most predominant alkaloids according to literature data (Mullen et al., 2003).

Table 5. Quantification of alkaloids in CHPEs and CHIs in mg/kg, LOD and LOQ value in μg/kg

Compound	CHPE 1	CHPE 2	CHI 1	CHI 2	LOD	LOQ
Trigonelline	1825.72 ±3.39[a]	1559.00 ±5.49[b]	558.02 ±1.55[c]	550.45 ±1.39[d]	0.011	0.02
Theobromine	5.33 ±0.21[a]	4.42 ±0.19[b]	2.15 ±0.03[c]	1.19 ±0.03[d]	0.008	0.03
Theofillyne	15.04 ±0.09[a]	8.6 ±0.24[b]	4.82 ±0.22[c]	3.05 ±0.11[d]	0.008	0.03
Caffeine	1327.39 ±5.41[a]	1334.5 ±4.23[a]	506.32 ±0.29[b]	553.25 ±1.28[c]	0.009	0.02
Total alkaloids	3173.48	2906.52	1066.49	1107.94		

Value are ± standard deviation of three independent determinations. The different superscripts in the same line with different letters are significantly different (p<0.05)

CHI – Coffee Husk sample

CHPE – Coffee Husk polyphenol extract sample

LOD - Limit of Detection

LOQ - Limit of Quantification

The total amount of caffeine and trigonelline extracted with the infusion is approximately 2-3 times lower than CHPEs. The caffeine content in coffee husk in literature ranged from 1300 to 8200 mg/kg, values significantly higher than all the analyzed samples (Mennini et al., 2013; Heeger et al., 2016; Mullen et al., 2003). Theophylline and theobromine (methyl xanthines, intermediates of the caffeine metabolism) were few represented both in CHPEs and CHIs, however there are no terms of comparison in literature.

Table 6 shown the quantitative data on the minor flavonoid compounds present in the samples. In particular, phenolic acids, flavan-3-ols and flavonoids were detected and quantified in the CHPEs whereas in CHIs, were found only six phenolic compounds (protocatecuic acid, caffeic acid, epicatechin, rutin, Isorhamnetin 3-*O*-rutinoside and naringenin).

Table 6. Quantification of minor compounds in CHPEs and CHIs in mg/kg, LOD and LOQ value in μg/kg.

Compound	CHPE 1	CHPE 2	CHI 1	CHI 2	LOD	LOQ
Gallic Acid	3.22 ±0.09[a]	1.90 ±0.01[b]	<LOQ	<LOQ	0.07	0.20
Protocatecuic Acid	48.94 ±0.22[a]	37.42 ±0.78[b]	10.62 ±039[c]	5.05 ±0.32[d]	0.07	0.20
(+)-catechin	1.55 ±0.09[a]	0.82 ±0.01[b]	<LOQ	<LOQ	0.06	0.18
Caffeic acid	6.12 ±0.05[a]	18.33 ±0.07[b]	0.75 ±0.01[c]	2.14 ±0.09[d]	0.06	0.18
procyanidin A type trimer	4.74 ±0.12[a]	0.62 ±0.02[b]	<LOQ	<LOQ	0.06	0.18
Gentisic Acid	77.13 ±1.12[a]	27.37 ±0.33[b]	<LOQ	<LOQ	0.06	0.18
(−)-epicatechin	24.95 ±0.33[a]	7.97 ±0.24[b]	0.19 ±0.03[c]	0.13 ±0.09[d]	0.06	0.18
procyanidin B type trimer	12.52 ±0.12[a]	3.27 ±0.07[b]	<LOQ	<LOQ	0.06	0.18
p-Coumaric Acid	0.41 ±0.01[a]	4.56 ±0.09[b]	<LOQ	<LOQ	0.07	0.20
quercetin-3-*O*-rutinoside	95.82 ±1.12[a]	114.95 ±5.12[b]	10.95 ±0.09[c]	9.85 ±0.09[d]	0.07	0.20
quercetin-3-*O*-glucoside	69.87 ±0.93[a]	67.06 ±0.89[b]	<LOQ	<LOQ	0.07	0.20
Ferulic acid	10.24 ±0.22	ND	<LOQ	<LOQ	0.07	0.20
Luteolin-7-*O*-glucoside	10.27 ±0.39[a]	5.74 ±0.09[b]	<LOQ	<LOQ	0.06	0.18
Isorhamnetin 3-*O*-Rutinoside	6.67 ±0.31[a]	5.23 ±0.02[b]	0.65 ±0.02[c]	0.57 ±0.29[d]	0.07	0.20
Naringenin	1.32 ±0.19[a]	1.94 ±0.01[b]	0.14 ±0.01[c]	0.18 ±0.09[d]	0.06	0.18
Total minor compounds	373.67	297.18	23.30	17.92		

Value are ± standard deviation of three independent determinations. The different superscripts in the same line with different letters are significantly different (p<0.05)

CH – Coffee Husk sample

CHPE – Coffee Husk polyphenol extract sample

LOD - Limit of Detection

LOQ - Limit of Quantification

3.5 Water and Methanol Coffee Husk Extracts Antifungal Activity, MIC-MFC

Coffee is the most preferred morning beverage throughout the world due to its pleasant flavor and stimulating properties. Natural preservatives have been developed as an alternative source of novel antimicrobials in encountering food-borne pathogens. In recent years, coffee has been associated with antimicrobial activity against a number of different bacteria and fungi (Daglia, Cuzzoni, & Dacarro, 1994).

The active components or the antimicrobial mechanism of coffee are not fully elucidated yet. In the current study, the eight major primary or secondary components of roasted coffee for which an antibacterial activity has been reported before, were not investigated. Coffee is composed by several compounds such as water, caffeine, lipids, tannic acids, mineral substances, glucides, theobromine, organic acids, alkaloids, and several vitamins, that could be responsible of its antimicrobial effects. Possible candidates include complex mixtures of phenolic compounds or, in the case of roasted coffee, Maillard reaction polymers such as melanoidins (Daglia et al., 1994; Rufián-Henares & De la Cueva, 2009).

The antimicrobial mechanism of action of the phenolic compounds is not clear. They can express their antimicrobial activity by changing the cytoplasmic membrane's structure, disrupting the active transport, the proton motive force and/or the electron flow. Additionally, the mechanism for phenolic toxicity may include enzymatic inhibition by the oxidized compounds, probably through interaction with sulfidryl groups or through more non-specific reactions with proteins (Lou, Wang, Zhu, Ma, & Wang, 2011).

In Table 7 are evidenced the data related to the antifungal activity on solid medium of the water and methanol extracts of the two coffee husks samples. In particular the water extracts of the two samples tested do not presented any antifungal activity against the mycotoxigenic fungi assayed, whereas both methanol extracts presented an antifungal activity quantified with a diameter clearing zone of 8 mm on the three strains of Penicillium, and also on A. *flavus* and A. *niger*. The antifungal activity evidenced by the methanol extracts of the two coffee husks samples was quantified through the Minimum inhibitory Concentration and the Minimum Fungicidal Concentration (MIC-MFC) evaluation calculated using the 96-well microplates assay. In particular as evidenced in the Table 8 the methanol extract of the sample 1 presented a MIC variable from 6.3 to 25 g/L, considering that the most sensible strains to this natural antimicrobial complex were P. *expansum* and P. roqueforti with 6.3 and 12.5 g/L. The MFC data observed for the antimicrobial complex produced through the methanol extract of the sample 1, ranged from 6.3 to 50 g/L, confirming that the strains most sensible to this treatment was the P. *expansum*. The MIC data evidenced for the methanol extracts of the sample 2, ranged from 6.0 to 50 g/L. The highest sensibility of this extract was observed on the strain of P. *expansum*, whereas the lowest sensibility was observed on the strain of A. *flavus* and A. *niger*. The data evidenced for the MFC of the sample two-methanol extract ranged from 12.5 to 50 g/L. Finally considering in general the two samples extracts tested, an important antifungal activity was observed on the P. *expansum* strains, concluding that this extract could be considered a good candidate for the biocontrol of this mycotoxigenic fungi in food and feed chain.

Table 7. Antifungal activity in solid medium evidenced by Coffee husk 1 and Coffee husk 2 water and methanolic extracts on the mycotoxigenic fungi tested. Calculation of antifungal activity: 8 mm diameter clearing zone (+), 10 mm diameter clearing zone (++), and more than 10 mm diameter clearing zone (+++).

Fungi	**CH1**		**CH2**	
	Water extract	Methanol extract	Water extract	Methanol extract
P. camemberti CECT 2267	−	+	−	+
P. expansum CECT 2278	−	+	−	+
P. roqueforti CECT 2905	−	+	−	+
A. parasiticus CECT 2681	−	−	−	−
A. flavus ITEM 8111	−	+	−	+
A. niger CECT 2088	−	+	−	+
F. moniliformis CECT 2982	−	−	−	−
F. verticilliodes CECT 20926	−	−	−	−
F. graminearum CECT 20490	−	−	−	−

CH – Coffee Husk sample

Table 8. Minimum inhibitory concentration and minimum fungicidal concentration (MIC-MFC) evidenced by Coffee husk 1 and Coffee husk 2 water and methanolic extracts on the mycotoxigenic fungi tested.

Fungi	CH1		CH2	
	MIC	MFC	MIC	MFC
P. camemberti CECT 2267	25.0	50.0	25.0	50.0
P. expansum CECT 2278	6.3	6.3	6.0	25.0
P. roqueforti CECT 2905	12.5	25.0	12.5	12.5
A. flavus ITEM 8111	25.0	25.0	50.0	50.0
A. niger CECT 2088	25.0	50.0	50.0	50.0

CH – Coffee Husk sample

MIC - minimum inhibitory concentration

MFC - minimum fungicidal concentration

This article is the first report on antifungal activity of the coffee husk extracts studied against the mycotoxigenic fungi, whereas many authors have studied the antimicrobial activity of other coffee components on other organisms, important in food safety. In particular coffee inhibited the growth of both Gram-positive and Gram-negative bacteria as well as of the fungus. Coffee samples were in general less active against Gram-negative bacteria than Gram-positive, with the exception of E. *faecalis*. The response of each microorganism to coffee samples might be influenced by a number of factors. These different activities may be attributable to structural differences in cell wall composition. Gram-negative bacteria possess a LPS component in their outer membrane that makes them more resistant to antibacterial agents.28 Besides, bacteria susceptible to coffee are influenced by coffee variety, independently of roasting (Daglia et al., 1994). E. *coli*, which was inhibited by six coffee samples, is a representative of the family of Enterobacteriaceae and one of the prevalent facultative anaerobic bacteria in the intestinal tract. It is therefore used as an indicator organism for the fecal content of water and food (Campos, Couto, & Hogg, 2003). E. *faecalis* was the least sensitive Gram-positive bacterium to coffee. E. *faecalis* is also a major member of the intestinal flora in the human body and so it is speculated that coffee does not significantly disturb the composition of gut flora. The pseudomonads are bacteria commonly found with low-temperature spoilage of foods such as meat and seafood. Under the tested conditions, coffee samples exerted a strong antibacterial effect against S. epidermidis (Li, Wang, Xu, Zhang, & Xia, 2014). On the other hand, C. *albicans* is a commensal organism of the mouth and GI tract, therefore coffee may have the potential to reduce its numbers, considering that it demonstrated a great sensitivity to the powder coffee solutions. The antimicrobial activity of coffee against other bacterial species was previously studied in the literature. Various organisms have been reported, such as Streptococcus, Bacillus, other enterobacteria, or Legionella species (Lou et al., 2011). The melanoidins showed antimicrobial activity against Gram-negative reference pathogenic bacterium E. *coli*, and the best results were obtained with the high-molecular-weight fraction of water-soluble melanoidins (>10 kDa). At MIC value, melanoidins caused irreversible cell membrane disruption, which was independent of the bacterial trans membrane potential (Rufián-Henares & Morales, 2008).

Antimicrobial activity of coffee depends primarily on the roasting of the coffee beans and more specifically on the roasted ingredients mediating its effects. The possible role for the microflora in the biology of the coffee plant remains enigmatic. The antibacterial properties of coffee suggest that it has a promising potential as natural food ingredient to extend and stabilize the shelf life of foods such as yoghurt, cookies, cake, or biscuits.

4. Conclusion

Coffee husk represents a great source of bioctive compounds showing a very high antioxidant and antimicrobial activity. This study indicates the possible exploiting of this coffee by-product as potential natural health promoting and preservative ingredient. By-products derived from the preparation of coffee for its marketing involves a great economic loss for that sector food industry. From this study, it could be concluded that there is a real possibility of using this coffee by-product for developing natural food ingredients, herbal infusions and supplements with functional properties. In future, could be interesting to evaluate the economic and enviromental impact the use of coffee husk as source of bioactive compounds

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