Coffee industrial waste as a natural source of bioactive compounds with antibacterial and antifungal activities

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Coffee is one of the most popular and consumed beverages in the world, which leads to a high contents of solid residue known as spent coffee grounds (SCG). As is known, coffee beans contain several classes of health related chemicals, including phenolic compounds, melanoidins, diterpenes, xanthines and carotenoids which are associated with therapeutic and pharmaceutical effects, due to antimicrobial, antioxidant, anti-infectious and antitumour activities. Considering that this coffee industrial waste has no commercial value and are currently disposed as a solid waste or employed as fertilizers, we intend to highlight the use of SCG as a raw material with potential interest to the food and pharmaceutical industries. Moreover, this work seems to be valuable to promote the use of SCG as natural and an inexpensive food supplements or pharmaceutical additive. The phytochemical compounds content among the crude aqueous extracts of SCG followed this order: phenolics > flavonoids > carotenoids (mg/ g dry waste), respectively. Caffeine content found in SCG was ~ 0.82 g/ 100 g dry waste, 70 % lower than coffee roasting beans. Coffee ground extracts showed inhibition to *S. aureus* and *E. coli* growth for concentrations of 1.0 mg/ mL and a stronger inhibition was also observed against *C. albicans*, *C. krusei* and *C. parapsilosis* growth using lower concentration (0.5 mg/ mL).

Keywords: Spent coffee grounds; Aqueous extracts; Natural antioxidants; Antibacterial activity; Antifungal activity.

1. Introduction

Coffee is a rich source of dietary antioxidants, and this property, coupled with the fact that it is one of the world's most popular beverages, has led to the understanding that coffee is a major contributor to dietary antioxidant intake. Natural bioactive compounds from coffee industry by-products have been receiving increasing attention, having in view the sustainability of the processes [1,2]. Spent coffee grounds (SCG) are a by-product generated during espresso beverages or soluble coffee production. Food by-products are focus of great interest in scientific community, once they may provide natural antioxidant and antimicrobial substances. In addition, valorization and re-use of food by-products minimizes industry wastes, with higher impact in sustainability and economic concepts. *Coffea Arabica* (Arabica) and *Coffea canephora* var. robusta (Robusta) are the two main species of the genus *Coffea* usually cultivated for commercial production. Nowadays, Arabica accounts approximately 75 % of the world production and, is considered to be favored to Robusta due to its milder and more flavorful taste, while Robusta is mostly used by the instant coffee industry for the manufacturing of soluble coffee [3].

It is acknowledged that coffee beans contain several classes of natural health chemicals, such as phenolics, melanoidis, diterpenes, xanthines, and vitamin precursors [4,5]. Considering their health beneficial, several studies reported that the consumption of phenolic acids and flavonoids provide *in vivo* protection against free radical damage and reduce the risk of degenerative diseases usually associated with oxidative stress [6,7]. Caffeine, the major xanthine presented in coffee beans, is the most studied coffee component due of its well-established psychoactive effects and promotion of energy metabolism [8]. Other compounds also provide health benefits, including carotenoids and chlorogenic acids, which are formed by esterification of one molecule of quinic acid and one to three molecules of *trans*-hydroxycinnamic acids, mainly caffeic, ferulic, and *p*-coumaric. In addition, the main chlorogenic acids present in coffee are highly bioavailable, being easily absorbed and metabolized throughout the gastrointestinal tract [9].

Moreover, the indiscriminate use of antimicrobial agents resulted in the emergence of drug-resistant bacteria, fungi and viruses. Various populations in developing countries are using natural plant products against infectious diseases by accidental discovery, and trust in the benefit of their use. To overcome the increased resistance of pathogenic microbes, researchers are using traditional knowledge as source of development of new drugs with high antimicrobiotic potential. The use of phytochemicals as natural antimicrobial agents, commonly called "biocides", is gaining popularity. There is a growing interest among the medical proprieties of natural resources in terms of antibacterial activity. In the ongoing search for better antibacterial compounds, plant-derived products are gaining ground [10]. The *in vitro* antibacterial activity of coffee beans against Gram-positive and Gram-negative bacteria has also been reported. Furthermore, different natural active chemicals in coffee of low molecular weight such as trigonelline, caffeic acid and 5-caffeoylquinic acid have shown activity against the growth of *Legionella pneumophila*, *Enterobacteria* and *S. mutans* [11,12].

In view of these findings, the present study was undertaken to further assess the potential applications as a way of adding value to SCG extracts from Arabica and Robusta species by the recovery of total phenolics, flavonoids, carotenoids and caffeine quantification. Additionally, this study aimed to investigating the antibacterial efficacy and antifungal activity of crude aqueous of SCG to promote the use of this coffee by-product as a natural and an inexpensive food supplements or pharmaceutical additive.

2. Chemical characterization, *in vitro* antioxidant activity and antibacterial and antifungal activities of spent coffee grounds

2.1 Phytochemicals composition

Fine grinded spent coffee grounds Veracruz® constituted by ~ 80 % Arabica and ~ 20 % Robusta coffee beans (*Coffea canephora* var. robusta and *Coffea arabica*, respectively), 100 % Guatemala Los Volcanes Arabica Sanzala® and 100 % Uganda SCR18 Robusta Sanzala® were obtained by an Elen® express machine ma/ c/ 3 gr, gently provided by a local commercial source (Café Vera Cruz). Spent coffee grounds were sterilized in an autoclave JSM 250 at 134 °C during 40 min, until 5 % moisture content and stored in airtight bottles for further analysis. For the analysis of total phenolics and flavonoids, a simple aqueous extraction was conducted according to Costa et al. [1]. Briefly, 5 g of ground sample and 50 mL of 100 % water was mixture (600 rpm) at room temperature for 60 min. The final extracts were filtered and stored at -25 ºC to prior analysis. Total phenolics contents (TPC) of spent coffee grounds extracts were determined spectrophotometrically according to a modified method of Costa et al. [1]. Briefly, 500 µL of each extract were mixed with 2.5 mL of the Folin-Ciocalteu reagent (1:10) and 2 mL of a sodium carbonate solution (7.5 %). After 1 h incubation the absorbance was measured at 765 nm and results were expressed as mg of gallic acid equivalents (GAE)/ g of sample.

Total flavonoids contents were determined according to Barroso et al. [13]. Aliquots of 1 mL of each extract were mixed with 4 mL of distilled water and 300 µL of 25% sodium nitrite. After 5 min, 300 µL of 10% $AlCl_3$ were added and 1 min after 2 mL of sodium hydroxide (1 M) and 2.4 mL of ultrapure water. The absorbance was recorded at 510 nm and final results were expressed as mg catechin equivalents (CE)/ g of sample.

Chlorophyll a, chlorophyll b, β-carotene and lycopene were determined, in triplicate, according to Vinha et al. [14]. Briefly, samples were extracted with acetone/hexane (2:3, v/v), and supernatants absorbance were measured at 453, 505, 645, and 663 nm. The contents of chlorophyll a, chlorophyll b, β-carotene and lycopene were calculated according to the following equations: Chlorophyll a (mg/ g) = $0.999A_{663} - 0.0989A_{645}$; Clorophyll b (mg/ g) = $-0.328A_{663} + 1.77A_{645}$; Lycopene (mg/ g) = $-0.0458A_{663} + 0.204A_{645} + 0.372A_{505} - 0.0806A_{453}$; β-Carotene (mg/ g) = $0.216A_{663} - 1.22A_{645} - 0.304A_{505} + 0.0452A_{453}$.

For caffeine extraction coffee spent grounds (5 g) were stirred with ebullition water (150 mL) for 30 minutes. The resulting mixture was filtered and extracted with chloroform: isopropanol (3:1, w/w) (4 x 50 mL). The organic extract was dried with $CaCl_2$, filtered and evaporated to dryness [15]. The same procedure was used for caffeine determination in commercial ~ 80 % Arabica and ~ 20 % Robusta Veracruz® fresh grounds. The resulting extracts were quantitatively dissolved in ultra-pure H_2O and caffeine was quantified by HPLC with an UV detector (214 nm), using benzoic acid as internal standard in the same concentration as the standards [16]. Standard solutions of 20, 40, 60, 80, 100, 120 and 160 mg/ L in caffeine and 100 mg/ L in benzoic acid in ultra-pure H_2O were prepared. An aliquot (50 µL) of each sample was injected and its concentration was determined using linear regression analysis. Standards and samples were measured in triplicate and the mean peak height values were used for data acquisition. Identification of caffeine and benzoic acid was performed by comparison with retention time of the respective standards. Areas under the curve were calculated and calibration curves were obtained by plotting area caffeine / area benzoic acid versus caffeine concentration.

2.1.1 Antioxidant activity

The free radical scavenging activity of the samples was measured *in vitro* by 2,2 - diphenyl-1-picrylhydrazyl (DPPH) assay according to the same author described earlier [14]. Briefly, 14 µL of diluted extract of each sample (1:10) were mixed with 186 µL of a freshly prepared $DPPH^{\bullet}$ solution (9.3×10^{-5} mol/ L in ethanol). The decrease of the $DPPH^{\bullet}$ was measured in equal time intervals of 10 min by monitoring the decrease of absorption at 525 min, in order to observe the kinetics reaction. The reaction endpoint was attained in 40 min. A calibration curve was prepared with trolox (10–2000 µg/ L, r = 0.9995) and $DPPH^{\bullet}$ scavenging activity was expressed as mg of trolox equivalents (TE)/ g of sample.

2.2 Antibacterial and antifungal activities of spent coffee grounds

SCG antimicrobial activity was tested against Gram positive (*S. aureus*) and Gram negative (*E.coli* and *P. aeruginosa*) bacteria and yeasts (*Candida* sp.) since treatment of infectious diseases is, from ever, a challenge to the science. Besides the number and diversity of antimicrobial drugs available, microorganisms were throughout the times capable of

developing resistance mechanisms to the therapeutic options as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin intermediate *Staphylococcus* (VISA), Vancomycin-resistant *Staphylococcus aureus* (VRSA) or extended-spectrum β-lactamases (ESBLs) producers [17].

Antifungal resistance is also becoming a problem. *Candida albicans* is the major cause of candidosis, but *C. krusey* (intrinsically resistant to fluconazole) and *C. parapsilosis* causes medically important infections, difficult to treat [18].

2.2.1 Chemicals, Reagents and Microorganisms

Mueller-Hinton II (MH II) broth and Sabouraud dextrose agar (SDA) were from Liofilchem (Italy). Microorganisms stocks were kept at -20 ºC and cultures at 4 ºC. Bacteria (*E. coli* ATCC25922, *P. aeruginosa* ATCC27853, and *S. aureus* ATCC29213) and yeasts (*C. albicans* ATCC 10231, *C. krusei* ATCC6258 and *C. parapsilosis* ATCC2209) were cultured in MH II agar and SDA, respectively, 24 h just prior to the assays.

2.2.2 Antibacterial and antifungal activity assays

The lyophilized spent coffee ground extracts (commercial mixture) were solubilized in sterilized distilled water (10 mg/ mL) and stock solutions kept at -20ºC before analysis.

Minimal inhibitory concentration (MIC) of the extract was determined using the CLSI Reference Microdilution Method for Antimicrobial Susceptibility Testing for Aerobic Bacteria M7-A6 [19]. Briefly, serial dilutions (1:2; ranging from 1.0 to 0.25 mg/ mL) of the stock extract on MH II broth were added to the test bacteria (5 x 10^5 UFC/ mL) and were incubated for 24 h at 37 ºC. Positive controls (microorganism in culture media), negative controls (culture media), and extract control (extract in culture media) were included in all the experiments. MIC was defined as the lowest concentration that completely inhibited bacterial growth by visual lecture.

MIC of the extract was determined using the CLSI Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts M27-A3 [20]. Briefly, serial dilutions (1:2; ranging from 1.0 to 0.25 mg/ mL) of stock extract on RPMI-1640 broth medium supplemented with MOPS were added to the test yeast (0.5-2.5 x 10^3 CFU/ mL) and were incubated for 48 h at 37 ºC. Positive controls (microorganism in culture media), negative controls (culture media), and extract control (extract in culture media) were included in all the experiments. MIC was defined as the lowest concentration that completely inhibited yeast growth by visual lecture.

3. Results and Discussion

Spent coffee grounds of the three samples were extracted with an environmentally friendly procedure (water) and analyzed to evaluate the recovery of relevant natural antioxidants for further possible use as nutritional supplements, foods, cosmetic additive, or for pharmaceutical applications. The phytochemical composition of the spent coffee grounds extracts is given in Table 1, Table 3 and Figure 2.

Table 1 Phytochemical composition and *in vitro* antioxidant activity of the aqueous extracts obtained from three different coffee grounds (100 % Arabica, 100 % Robusta, and commercial mixture: 80 % Arabica + 20 % Robusta).

	Spent Coffee Grounds[*]		
Phytochemicals	**Arabica**	**Robusta**	**Commercial mixture**
Phenolics	14.97±0.09[b]	22.56±1.02[a]	11.04±0.17[c]
Flavonoids	2.12±0.04[b]	3.49±0.46[a]	1.09±0.16[c]
Chlorophyll a	0.0494±0.002[a]	0.0280±0.001[c]	0.0393±0.002[b]
Chlorophyll b	0.0873±0.004[a]	0.0042±0.000[c]	0.0653±0.004[b]
Lycopene	0.0079±0.000[b]	0.0011±0.000[c]	0.0258±0.001[a]
	Antioxidant activity		
	81.57±2.32[b]	88.16±1.16[a]	73.7±1.44[c]

*[a,b,c]Different letters stand for significant differences ($p < 0.01$) in mean value, according to the LSD post-hoc test (ANOVA).

Table 2 Correlation [r (*p*-value)] of the phytochemical composition and *in vitro* antioxidant activity of the aqueous extracts obtained from three different coffee grounds (100% Arabica, 100% Robusta, and commercial mixture: 80 % Arabica + 20 % Robusta).

	Flavonoids	Chlorophyll a	Chlorophyll b	Lycopene	Antioxidant activity
Phenolics	0.966 (<0.001)	-0.847 (0.004)	-0.819 (0.007)	-0.900 (0.001)	0.941 (<0.001)
Flavonoids		-0.773 (0.015)	-0.747 (0.021)	-0.919 (<0.001)	0.927 (<0.001)
Chlorophyll a			0.998 (<0.001)	0.552 (0.123)	-0.698 (0.037)
Chlorophyll b				0.511 (0.160)	-0.656 (0.055)
Lycopene					-0.950 (<0.001)

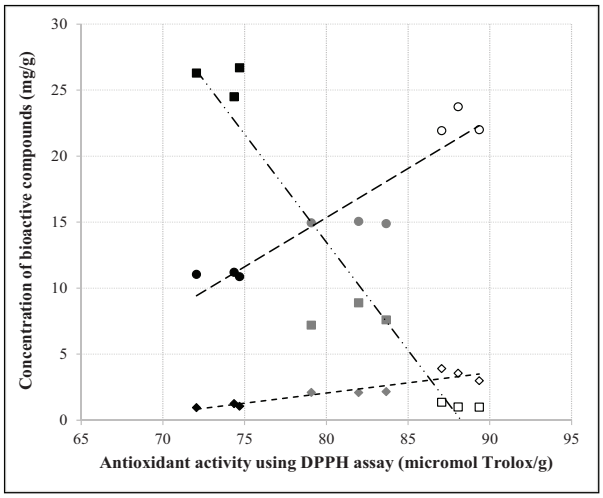

Fig. 1 Relation between the concentrations of bioactive compounds (■ stands for Lycopene (multiplied by a 1000 factor); ● stands for Phenolics and ♦ stands for Flavonoids. Black, grey and white represents SCG commercial mixture, SCG Arabica and SCG Robusta, respectively) and antioxidant activity. Lines were used to show the tendency observed in the relations between compounds and their antioxidant activity.

SCG is the main by-product of the brewing process and a potential source of bioactive compounds, mainly phenolic acids easily extracted with water. Knowledge of the total content of phenolic compounds in spent coffee extracts is crucial for their potential use as functional ingredients by the food and pharmaceutical industries. For comparison, the commercial spent coffee grounds presented lower contents of total phenolics and flavonoids (11.04 mg GAE/ g, 1.09 mg CE/ g, respectively) than SCG coffee grounds of 100 % Arabica and 100 % Robusta extracts. However, antioxidant activity showed significant differences ($p < 0.001$) in the 3 formulations, with the commercial mixture having the lowest and Robusta the highest antioxidant activity (Table 1). The total phenolics and flavonoids were strongly and positive correlated with the DPPH scavenging activity (r = 0.941, $p < 0.001$ and r = 0.927, $p < 0.001$, respectively, Table 2), suggesting that an increase in phenolic and flavonoid compounds are mainly responsible for the increase in antioxidant activity (Figure 1).

The antioxidant potential of plant extracts and pure compounds was measured using more than one *in vitro* assay, once each of these assays is based on one feature of antioxidant activity, such as antiradical ability or to inhibit lipid peroxidation. Carotenoids are known to be very efficient physical and chemical quenchers of singlet oxygen ($^1O_2$), as well as potent scavengers of other reactive oxygen species (ROS), however, in the present study, regarding their antioxidant activity, a different correlation was observed, showing an inverse significant correlation, with lycopene and chlorophyll-a increase being strongly associated with a decrease in antioxidant activity (r = -0.950, $p < 0.001$ and r = -0.698, $p = 0.037$, respectively, Table 2).

In addition to the compounds quantified in this work, other biologically active coffee components with potential beneficial health effects are nicotinic acid, trigonelline, quinolinic acid, tannic acid, and pyrogallic acid [5]. However, the presence of carotenoids in SCG can also be used as substrate for biotechnological, several applications in food industry (food colorants, antioxidants, animal feed supplements) and also in cosmetics and pharmacy. Generally, the market demand for carotenoids is expected to increase substantially, since those compounds exhibit significant anti-carcinogenic activities of alternative high value product. Thus, beyond the direct relationship between phenolic compounds and their antioxidant action, antioxidant properties such as reactive oxygen species (ROS) scavenging have also been recently proposed for caffeine, the most abundant alkaloid present in coffee beans. Caffeine content was analyzed by HPLC-UV and Figure 2 shows an illustrative chromatogram.

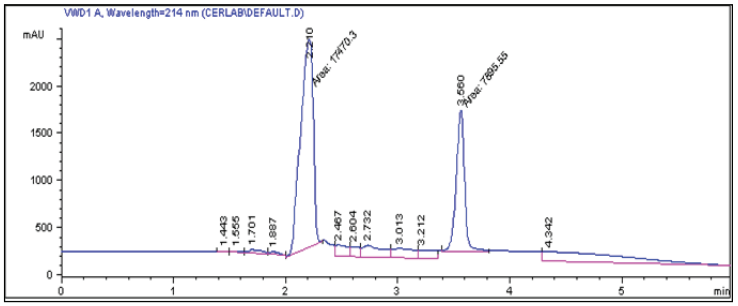

Fig. 2 Illustrative chromatogram of coffee grounds extracts. The 1st peak correspond to caffeine and the 2nd to benzoic acid (used as internal standard).

Caffeine retention time was 2.2 min, while benzoic acid takes 3.6 min to separate (Figure 2). The equation of the regression lines obtained was y = 0.0155x + 0.1865, being *y* the areas quotient and *x* the caffeine concentration. Naturally, the coffee grounds obtained from different varieties of coffee will contain different amounts of caffeine (Table 3). In this study that is demonstrated in the specific case of varieties *C. Robusta* and *C. Arabica*. Caffeine content in commercial mixture SCG was 0.82 ± 0.14 % (m/m), while in the fresh grounds was 2.43 ± 0.48 %, contain with the

humidity values of the samples. The results reveal that in the process of producing the so called "Espresso" approximately 30 % of the caffeine remains on the coffee spent.

Table 3 Caffeine content in % (m/m) of the several coffee grounds studied (Commercial fresh grounds, 100 % Arabica SCG, 100 % Robusta SCG, and commercial mixture 80 % Arabica + 20 % Robusta SCG).

	Caffeine content, % (m/m)
Fresh grounds	2.43±0.48
Arabica SCG	0.30±0.01
Robusta SCG	1.14±0.01
Commercial mixture SCG	0.82±0.14

Comparing with other results previously published, we have obtained quantities for SCG caffeine similar to the referred by Cruz et al. (2012) [21], and considering that coffee commercially more used in Portuguese coffee shops has 15-20 % Robusta and 80-85 % Arabica. They have obtained 194.0 to 787.7 mg caffeine/ 100 g (0.1940 % - 0.7877 %) on the SCG collected in bars of Oporto city, using 50 mL of distilled water to extract 5 g SCG during 5 minutes, with stirring and at boiling temperature [21]. Bravo et al. [22] have dried spent coffee, defatted with petroleum ether (1:11, w/v) for 3 h at 60 °C in a Soxhlet extraction system, using 24 g of SCG with 400 mL of water in a filter coffeemaker during approximately 6 min at 90 °C and referred ranges from 0.359 % for Arabica to 0.809 % for Robusta. Panusa et al. (2013) [5] published 6.00 ± 0.05 mg/ g (0.600 %) (water extract) and almost the same value for EtOH/H_2O (60:40) (v/v) extract (5.99 ± 0.16 mg/ g) for 2 g of dried SGC of coffee with 70 % Arabica, with 100 mL of the solvent, at 60 ºC, during 30 min.

The caffeine quantity differences can be explained by same factors: the ratio Arabica/Robusta, the extraction time, the ratio between the volume of the solvent and the mass of the sample, the extraction temperature and the grind of the coffee beans.

Spent coffee ground extracts showed to inhibit some Gram-positive and Gram-negative bacterial strains (Table 4). *S. aureus* and *E. coli* growth was inhibited for concentrations of 1.0 mg/ mL. At that concentration, no inhibition was observed for *P. aeruginosa*. A stronger inhibition of coffee ground extract was observed against *Candida* sp.. *C. albicans*, *C. krusei* and *C. parapsilosis* growth was totally inhibited for concentration of 0.5 mg/ mL.

Table 4 Antibacterial and antifungal activity (MIC, minimal inhibitory concentration) of spent coffee ground extracts.

	Microorganism strain	MIC (mg/ mL)
Bacteria	*S. aureus* ATCC29213	1.0
	E. coli ATCC25922	1.0
	P. aeruginosa ATCC27853	>1.0
Yeast	*C. albicans* ATCC10231	0.5
	C. krusei ATCC6258	0.5
	C. parapsilosis ATCC22019	0.5

*Results are obtained from at least 2 to 3 independent experiments performed in duplicate.

Our results suggest that spent coffee ground extracts are potentially useful in cosmetic formulations, due to their referred properties, including antifungal and antibacterial activities. The inclusion of those extracts on soaps or formulations to be applied in superficial infections caused by microorganisms, as in acne or superficial candidosis, could (i) improve the results of antimicrobial therapy, (ii) solve problems of antimicrobial resistance and (iii) improve therapeutics adhesion, being (iv) cheaper and lowering the cost/benefit of therapeutics.

4. Conclusions

The coffee industry has experienced a constant growth and, as a consequence, large amounts of residues are generated worldwide. One of the main coffee residues are spent coffee grounds (SCG), which are the solid residues obtained after preparation of the coffee beverages. References to its use as organic fertilizer in domestic cultures, especially in gardens, are common. However, scientific evidence of its effectiveness or even safety remains largely unknown. Different attempts to valorize this waste stream of coffee industry were made. Coffee is known as valuable source of biologically active phytochemicals such phenolic compounds and caffeine. New beneficial properties of the spent coffee grounds are being continuously discovered. The water as solvent showed to be a good option for an efficient extraction of bioactive compounds from SCG Additionally, SCG revealed to be a good source of antioxidant compounds, showing a direct correlation with the antioxidant activity. The caffeine content quantified in our samples may be used in pharmaceutical applications, depending on the site of action, dosage, and timing of drug exposure. Furthermore, SCG presented an inibitory activity against *S. aureus* and *E. coli*. A stronger inhibition was also observed against *Candida* sp. growth (*C. albicans*, *C. Krusei* and *C. parapsilosis*). This study allows us to understand whether the residues generated during coffee brewing procedure, produced in large amounts in cafeterias and restaurants, or at

domestic levels, can be considered as a source of natural antioxidants. These findings open up possibilities to evaluate SCG chemicals as bioactive compounds in different food and pharmaceutical applications.

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