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Characterization of polysaccharides extracted from spent coffee grounds by alkali pretreatment

Lina F. Ballesteros [a], Miguel A. Cerqueira [a], José A. Teixeira [a], Solange I. Mussatto [b,*]

[a] Centre of Biological Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal
[b] Department of Biotechnology, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

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ABSTRACT

Spent coffee grounds (SCG), obtained during the processing of coffee powder with hot water to make soluble coffee, are the main coffee industry residues and retain approximately seventy percent of the polysaccharides present in the roasted coffee beans. The purpose of this study was to extract polysaccharides from SCG by using an alkali pretreatment with sodium hydroxide at 25 °C, and determine the chemical composition, as well as the antioxidant and antimicrobial properties of the extracted polysaccharides. Galactose (60.27% mol) was the dominant sugar in the recovered polysaccharides, followed by arabinose (19.93% mol), glucose (15.37% mol) and mannose (4.43% mol). SCG polysaccharides were thermostable, and presented a typical carbohydrate pattern. Additionally, they showed good antioxidant activity through different methods and presented high antimicrobial percent inhibition against *Phoma violacea* and *Cladosporium cladosporioides* (41.27% and 54.60%, respectively). These findings allow identifying possible applications for these polysaccharides in the food industry.

1. Introduction

Polysaccharides are the main constituents of the coffee beans and play an important role in the final beverage. During the preparation of soluble coffee, the polysaccharides present in the roasted coffee are partially extracted with hot water and are responsible for the retention of coffee volatile substances, stabilization of foam, binding of aroma, formation of sedimentation, and increased viscosity of the extract (Arya & Rao, 2007; Nunes, Domingues, & Coimbra, 2005). Arabinogalactan, galactomannan and cellulose are the dominant polysaccharides in coffee beans (Arya & Rao, 2007; Fischer, Reimann, Trovato, & Redgwell, 2001). Arabinogalactans is the most significant group of polysaccharides extracted with hot water from green coffee (Arya & Rao, 2007; Nunes et al., 2005). Nevertheless, after roasting process, galactomannans become the most relevant polysaccharides in roasted coffee infusions. Thus, galactomannans and arabinogalactans are the most important coffee constituents after hot water extraction.

Recently, some researchers have exposed the great potential of polysaccharides presented in coffee, showing that they can provide enormous functional properties. Most of these polysaccharides are not degraded by human digestive enzymes; thus, they reach the colon and potentially serve as substrates for the colonic microbiota supporting the growth of bifidobacteria and other lactic acid bacteria that are considered beneficial for human health (Gniechwitz, Reichardt, Blaut, Steinhart, & Bunzel, 2007). Polysaccharides from coffee decrease the cholesterol levels in blood, controlling the blood glucose and insulin response and act against infectious and tumor diseases (Gniechwitz et al., 2007; Simões et al., 2009). Additionally, polysaccharides from natural sources, especially galactomannans are excellent stiffeners and stabilizers of emulsions that can be used in food, pharmaceutical and cosmetic industries since they do not have toxic substances (Cerqueira et al., 2009; Vieira, Mendes, Gallão, & de Brito, 2007). All these biological and physicochemical properties offered by coffee polysaccharides can be found in the main residue obtained during the processing of coffee powder with hot water to prepare instant coffee, known as the spent coffee grounds (SCG), which retain about 70% of total polysaccharides present in roasted coffee (Arya & Rao, 2007).

Nowadays, the market of the soluble coffee around the world is increasing, which in turn generates around 6 million tons of SCG per year (Mussatto, Machado, Martins, & Teixeira, 2011). Some studies have been proposed to exploit this residue, which is not only rich in polysaccharides but also in proteins, phenolic compounds among other components, being of great interest to chemical and food industries. SCG could be used, for example, to produce fuel for industrial boilers due to its high calorific power of approx.

* Corresponding author. Tel.: +31 015 278 8789; fax: +31 015 278 2355.
E-mail addresses: S.I.Mussatto@tudelft.nl, solangemussatto@hotmail.com (S.I. Mussatto).

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5000 kcal/kg (Silva, Nebra, Machado Silva, & Sanchez, 1998) and fuel ethanol (Mussatto, Machado, Carneiro, & Teixeira, 2012), to produce mannitol (Arya & Rao, 2007; Mussatto, Carneiro, Silva, Roberto, & Teixeira, 2011), and as raw material to obtain antioxidant phenolic compounds (Machado, Rodriguez-Jasso, Teixeira, & Mussatto, 2012; Mussatto, 2015; Mussatto, Ballesteros, Martins, & Teixeira, 2011) or to produce a distilled beverage with aroma of coffee (Sampaio et al., 2013). Despite these possible applications, SCG are still underutilized as valuable material for industrial processes.

Some studies have reported the extraction of polysaccharides from coffee (green and roasted) and SCG by different techniques such as solid–liquid extraction with organic solvents (Fischer et al., 2001; Simões et al., 2009; Simões, Nunes, Domingues, & Coimbra, 2010), dilute acid hydrolysis (Mussatto, Carneiro, et al., 2011) and microwave-assisted extraction (Passos & Coimbra, 2013), but to the best of our knowledge, there is no study in the literature that claims the characterization of polysaccharides extracted from SCG as exposed here. Therefore, the purpose of the present study was to perform a chemical and structural characterization, as well as to determine the antioxidant and antimicrobial properties of the polysaccharides extracted from SCG by alkali pretreatment.

2. Materials and methods

2.1. Raw material

SCG were provided by the Portuguese coffee industry Nova Delta-Comércio e Indústria de Cafés S.A. (Campo Maior, Portugal). The material was dried in an oven at 60 °C until 5% moisture content and stored for further extraction.

2.2. Alkali pretreatment

Polysaccharides extraction from SCG was carried out according to the method described by Simões et al. (2010) with some modifications. Briefly, previous to the extraction, the SCG (605 g) were defatted in a Soxhlet extraction system (Tecator, HT2, Netherlands) during 4 h using petroleum ether as solvent (1:5 (w/v)). The fat free SCG were dried at 60 °C until constant weight and stored for the further stages. The alkali pretreatment was then performed for polysaccharides extraction by using 4 M sodium hydroxide (4 L) at 25 °C overnight (0.02 M sodium borohydride was also added to prevent peeling reactions and alkaline oxidation of the polysaccharides). After this time, the produced alkali extract was centrifuged at 9700 × *g* for 15 min at 4 °C, filtered through Whatman filter paper and acidified to pH 5.0 with glacial acetic acid. Next, the filtrate was dialyzed at 4 °C with an 8000 Da membrane for 12 days, with several distillated water changes. After dialysis, the retentate into the membrane was centrifuged at the same conditions above mentioned and the supernatant was frozen and freeze-dried. Freeze-dried powder was stored at room temperature and protected from the light and humidity until further use.

2.3. Polysaccharide yield

Three different extraction yields of polysaccharides were determined (Y1, Y2, and Y3), which can represent important economic parameters of the process. Y1 represents the total yield of the extraction, expressed as g of lyophilized material per 100 g SCG; Y2 refers to the quantity of sugars extracted and was expressed as g of total sugars present in the lyophilized material per 100 g SCG; finally, Y3 represents the yield of the quantity of sugar extracted with respect to total sugars existent in the SCG, which is defined as g of total sugars in the lyophilized material per 100 of sugars from SCG.

2.4. Analytical methodology

2.4.1. Chemical characterization

Polysaccharides recovered from SCG were submitted to a dilute acid hydrolysis with sulfuric acid (120 mg H_2SO_4/g material lyophilized). The mixture was vortexed and sterilized at 120 °C for 20 min. Then, sugar concentrations were made by high performance liquid chromatography (HPLC) using an equipment LC-10 A (Jasco, Japan) with a Meta Carb 87P column at 80 °C, ultrapure water previously boiled and degassed in a ultrasonic bath as mobile phase, and a refractive index (RI) detector. The flow rate and the injection volume were adjusted to 0.4 mL/min and 20 μL, respectively. Glucose, arabinose, galactose and mannose were identified and quantified from standard curves made with known concentrations of each compound and expressed as % mol. The response of the RI detector was recorded and integrated using the Star Chromatography Workstation software (Varian).

2.4.2. Structural characterization

Crystalline phases of SCG polysaccharides were evaluated by X-ray diffraction (XRD) as described by Ballesteros, Teixeira, and Mussatto (2014). The chemical groups and bonding arrangement of constituents present in the polysaccharides were determined by Fourier transform infrared spectroscopy (FTIR) using a Perkin-Elmer 16 PC spectrometer (Boston, USA) equipped with a diamond-composite attenuated total reflectance (ATR) cell. The measurements were recorded with a wavenumber range from 4000 to 400 cm^{-1} and 16 scans per sample. Differential scanning calorimetry (DSC) and thermogravimetric analyses (TGA) were carried out as previously described (Ballesteros et al., 2014).

2.4.3. Antioxidant phenolic compounds characterization

For the analysis of total phenolic compounds and antioxidant activity of the polysaccharides extracted from SCG by alkali pretreatment, the lyophilized material was mixed with ultrapure water in a ratio of 1 mg/mL, vortexed for 1 min and then filtered through 0.22 μm filters. Additionally, two commercial antioxidant phenolic compounds (2,6-di-tert-butyl-4-methylphenol and tert-butyl-4-methoxyphenol, known as BHT and BHA, respectively) were used as standards to evaluate the antioxidant capacity of polysaccharides and Tukey's range test was considered to evaluate significant differences ($p < 0.05$) among samples.

2.4.3.1. Phenolic compounds. The total phenolic compounds obtained from SCG were determined using the Folin-Ciocalteu reagent method adapted to a 96-well microplate (Meneses, Martins, Teixeira, & Mussatto, 2013). The total content of phenolic compounds was expressed as milligram of gallic acid equivalent per g of dry material (mg GAE/g lyophilized).

2.4.3.2. Total antioxidant activity. The total antioxidant activity (TAA) of polysaccharides extracted from SCG was estimated as described by Prieto, Pineda, and Aguilar (1999) with some modifications. Briefly, 200 μL of sample was added to a glass tube containing 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were covered and maintained during 90 min in a water-bath at 95 °C and then, placed to cool at room temperature. The absorbance was measured at 695 nm using a spectrophotometer V-560 (Jasco, Japan) against a blank of distilled water. A calibration curve was prepared with a standard solution of α-tocopherol (25, 75, 125, 250, 375 and 500 μg/mL). TAA expressed as milligrams of α-tocopherol equivalent per mL of extract (mg TOC/mL).

2.4.3.3. Ferric reducing antioxidant power assay. The antioxidant activity of polysaccharides extracted from SCG by the ferric

reducing antioxidant power (FRAP) assay was determined according to the methodology described by Meneses et al. (2013). The FRAP values were expressed as milligrams of ferrous equivalent per mL of extract (mg Fe(II)/mL).

2.4.3.4. Free radical scavenging activity (DPPH assay). The DPPH radical scavenging activity of polysaccharides extracted from SCG was determined using the methods described by Fukumoto and Mazza (2000) and Silva et al. (2004) in combination and with some modifications. For each sample, a dilution series (four different concentrations) were prepared. The reaction was carried out in a 96-well microplate containing 25 μL of sample and 200 μl of 150 μM DPPH solution (2,2-diphenyl-1-picrylhydrazyl dissolved in 80% methanol to an absorbance value of 0.700 at 515 nm). The produced solutions were vortexed and allowed to stand for 1 h in the dark at room temperature. Then the absorbance was measured at 515 nm in a spectrophotometric microplate reader (Sunrise Tecan, Grödig, Austria) using methanol as blank. The control solution consisted in using methanol instead of the sample. The radical scavenging activity was calculated by using the Eq. (1), where A_c and A_s are the absorbance of the control solution and the absorbance of the sample solution, respectively. A calibration curve was prepared with a standard solution of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) diluted in methanol (40, 80, 100, 300, 400 and 600 μM). DDPH percent inhibition data were plotted as a function of antioxidant concentration to obtain DPPH inhibition concentration at 50% (IC_{50}). The IC_{50} values were expressed as milligrams of Trolox equivalent (TE) per mL of extract (mg TE/mL).

$$\%\, inhibition = (1 - As/Ac) * 100 \quad (1)$$

2.4.3.5. Radical cation decolorization assay. The radical cation decolorization (ABTS) assay of polysaccharides extracted from SCG was determined as described by Re et al. (1999) and Ozgen, Reese, Tulio, Scheerens, and Miller (2006) with some modifications. Each sample was diluted to four different concentrations such that the percent inhibition was between 20% and 80%. Assays were conducted by combining 130 μL of sample with 3 mL of ABTS radical cation solution. The resulting solutions were maintained during 30 min in darkness at room temperature, and the absorbance was then measured at 734 nm using a spectrophotometer V-560 (Jasco, Japan) being distilled water used as control solution instead of the sample. ABTS radical cation was prepared by mixing 7 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) dissolved in water with a 2.45 mM potassium persulfate solution. This mixture was vortexed for 2 min, set in ultrasonic bath during 20 min and then, left in the dark at 4 °C between 12 and 16 h for achieving a stable oxidative state. After this time, ABTS radical cation solution was diluted in a 20 mM acetate buffer (pH 4.5) solution to an absorbance of 0.70 ± 0.01 at 734 nm. A calibration curve was constructed using a standard solution of Trolox diluted in ethanol (50, 100, 200, 250, 300, 400 and 500 μM). The percent inhibition of ABTS radical cation was calculated using the same equation employed in the DPPH radical scavenging. The IC_{50} values were expressed as milligrams of Trolox equivalent (TE) per mL of extract (mg TE/mL).

2.4.4. Antimicrobial activity assays

Antimicrobial evaluation was performed against five food pathogenic fungi that drastically influence the quality and safety of postharvest fruits (Jasso de Rodríguez et al., 2011): *Alternaria* sp. MUM 02.42, *Cladosporium cladosporioides* MUM 97.06, *Phoma violacea* MUM 97.08, *Penicillium italicum* MUM 02.25 and *Penicillium expansum* MUM 02.14, being obtained from the collection of the Mycology Laboratory (MUM) of the University of Minho, Portugal. All the strains were cultured into potato dextrose agar (PDA) and incubated at 25 ± 2 °C during 15 days before antimicrobial test.

The determination of minimal inhibitory concentration (MIC) of polysaccharides extracted from SCG was performed using the micro-dilution methodology for filamentous fungi described by the Clinical and Laboratory Standards Institute (CLSI, 2002).

3. Results and discussion

3.1. Yield of extraction and chemical characterization of polysaccharides

Table 1 shows the monosaccharide composition and extraction yield of the recovered polysaccharides. SCG are a residue rich in sugars polymerized into cellulose and hemicellulose, which correspond to 51.50% (w/w) of its composition on a dry weight basis (Ballesteros et al., 2014). In the present study, the total sugar content extracted from SCG (lyophilized material) was 39%, while Y1, Y2 and Y3 were 6.05, 2.38 and 4.57% (w/w), respectively. Y1 is in agreement to the values obtained by Simões et al. (2009) when used 4 M NaOH to extract polysaccharides from SGC, in contrast toY2, which was almost 2-fold higher. Y3 was lower taking into account the high amount of polysaccharides present in the SCG (Ballesteros et al., 2014).

The chromatogram profile shown in Fig. 1a revealed glucose, galactose, arabinose and mannose as the only sugars present in SCG polysaccharide. The monosaccharide composition showed galactose (60.27% mol) as the dominant sugar, followed by arabinose (19.93% mol), glucose (15.37% mol) and mannose (4.43% mol). These results are in agreement with others studies which reported that polysaccharides in coffee wall are constituted by galactose, arabinose, mannose and glucose, forming mainly galactomannan, arabinogalactan and cellulose structures (Arya & Rao, 2007; Ballesteros et al., 2014; Mussatto, Carneiro, et al., 2011; Simões et al., 2009). However, the obtained sugar percentages revealed differences when compared with those works, but concurred with the results reported by Simões et al. (2009), who evaluated 4 M NaOH to extract polysaccharides from SGC. As a result, galactose and arabinose were the most representative sugars found in the supernatant, which is in agreement with the presented results. The quantity of mannose extracted in both cases was lower when compared with other methods used to extract polysaccharides from SCG (Ballesteros et al., 2014; Mussatto, Carneiro, et al., 2011), which indicates that a large proportion of mannose remains in SCG, suggesting that stronger conditions should be used for their extraction. For instance, mannose from SCG could be subjected to a chemical acetylation process (Simões et al., 2009, 2010) increasing thus the solubility of this sugar in water and other organic solvents, since the solvent plays an important role in the extraction process and should be chosen with respect to the organic compound of interest.

Additionally, the efficiency of the extraction depends of many factors such as solid/liquid ratio, solvent, temperature, extraction time, variety of the beans used and their degree of roasting, among others. Such parameters could be optimized, but this was not the objective of the present study.

3.2. Structural characteristics

3.2.1. X-ray diffraction

Fig. 1b displays the XRD patterns for SCG and the polysaccharide extracted from SCG. In order to evaluate the crystallinity of polysaccharides after alkali pretreatment, the XRD spectrum was compared with a XRD spectrum of SCG, obtained in a previous study (Ballesteros et al., 2014), in which the SCG did not suffer any chemical pretreatment before the analysis. As it can be seen, the unique

Table 1
Monosaccharide composition and extraction yield of the polysaccharides from spent coffee grounds.

Yield[a]			Monosaccharide composition (% mol)				Total sugars (%)
Y1	Y2	Y3	Arabinose	Mannose	Galactose	Glucose	
6.05	2.38	4.57	19.93 ± 1.74	4.43 ± 0.16	60.27 ± 0.51	15.37 ± 0.93	39.00 ± 0.19

Results of monosaccharide composition are expressed as mean ± standard deviation; $n = 3$.

[a] Y1: total yield of the extraction process with 4 M NaOH, expressed as g of lyophilized material per 100 g SCG; Y2: yield of the quantity of sugars extracted with 4 M NaOH, expressed as g of total sugars present in the lyophilized material per 100 g SCG; Y3: yield of the quantity of sugar extracted with respect to total sugars existent in the SCG, expressed as g of total sugars in the lyophilized material per 100 g of sugars from SCG.

crystalline peak in SCG corresponds to the cellulose (Ballesteros et al., 2014), while the polysaccharides extracted from SCG did not present any crystalline region. Although the chemical composition (Table 1) revealed glucose (cellulose) into the sugars present in the lyophilized material, the XRD spectra of polysaccharides suggests a glucose with amorphous structure, since it is known to have crystalline and amorphous regions, in contrast with hemicellulose that present an amorphous structure (Ragauskas & Huang, 2013). This result could be related to the fact that alkali pretreatment is more suitable to extract the hemicellulose structure, being more easily degradable and susceptible to chemical attacks than cellulose (Ragauskas & Huang, 2013).

3.2.2. Fourier transform infrared spectroscopy

Fig. 1c shows the FTIR analysis performed to polysaccharides extracted from SGC. The obtained spectrum corresponds to a typical carbohydrate pattern when compared with others' IR spectra of polysaccharides reported in the literature (Cerqueira et al., 2011; Ren et al., 2014; Zeng, Zhang, Gao, Jia, & Chen, 2012). The broad peak between 3600 and 3200 cm^{-1} was related to the hydroxyl group of O—H stretching vibration and the weak band between 3000 and 2800 cm^{-1} was attributed to C—H stretching vibration. The region between 1700 and 1500 cm^{-1} was related with carbonyl groups (C=O) asymmetrical and symmetric stretching vibrations (Ren et al., 2014). This band was also highly associated with chlorogenic acids and caffeine (Ribeiro, Salva, & Ferreira, 2010). Therefore, the peak at 1650 cm^{-1} could be attributed to a small absorption of these compounds, remaining from the SCG. The peak at 1374 cm^{-1} corresponds to C—H in plane bending vibration (Ren et al., 2014). The sharp band between 1194 and 925 cm^{-1} corresponds to stretching vibration of C—O in C—O—H bonds such as glycosidic bonds, and was related to polysaccharide sugars (Figueiró, Góes, Moreira, & Sombra, 2004). The peaks at 1155 and 1080 cm^{-1} resulted from the bending vibrational modes of C—O existing in the pyranose form (Figueiró et al., 2004), while the shoulder at 1024 cm^{-1} was indicated as C—O stretching (Ren et al., 2014). The peaks at 885 and 790 cm^{-1} were related to the presence of β-linked D-mannopyranose units and α-linked D-galactopyranose units, respectively. These glycosidic configurations were reported in most seed galactomannans (Cerqueira et al., 2011; Figueiró et al., 2004).

3.2.3. Thermal properties

DSC and TGA curves (Fig. 2) were performed in order to understand the thermal behavior and chemical changes of the polysaccharides extracted from SCG. DSC thermogram exhibited two events: an initial endothermic phase followed by an exothermic phase. Thus, an early endothermic event was detected with a peak at 80.43 °C with an associated enthalpy change of 167.30 J/g.

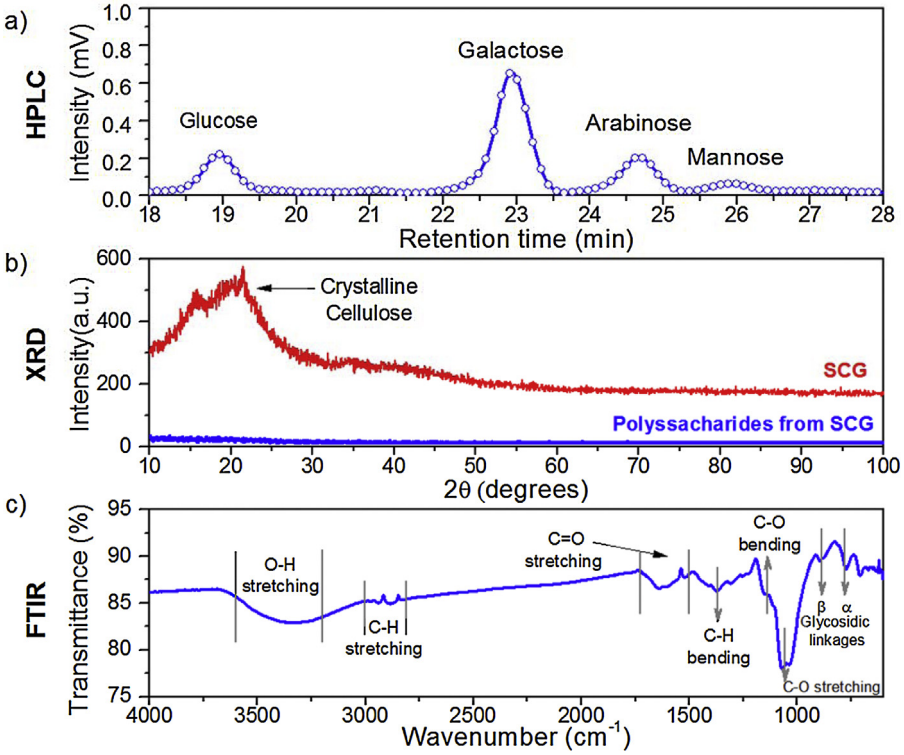

Fig. 1. Cromatogram profile of sugars solubilized (glucose, galactose, arabinose and mannose) from spent coffee grounds by alkali pretreatment and further acid hydrolysis (a). XRD diffractograms (b) obtained for spent coffee grounds and polysaccharides extracted from this residue. FTIR spectra (c) obtained for the polysaccharides extracted from spent coffee grounds using an alkali pretreatment.

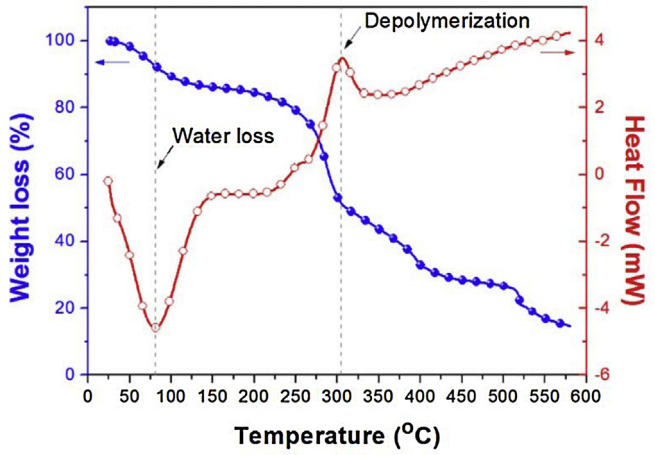

Fig. 2. TGA and DSC curves showing the thermal behavior, chemical changes and weight loss of the polysaccharides extracted from spent coffee grounds by alkali pretreatment.

This event was related to the presence of impurities in the sample and the vaporization of water (indicating the presence of hydrophilic groups), which occurs over a range of temperature. Enthalpy change in the first thermal transition was inferior when compared to those obtained for others polysaccharides (Cerqueira et al., 2011), associated to the low content of mannose:galactose ratio (Cerqueira et al., 2011; Chaires-Martínez, Salazar-Montoya, & Ramos-Ramírez, 2008), as reported in Table 1. The second event corresponds to an exothermic transition and was observed at 303.60 °C, accompanied with an enthalpy change of 39.96 J/g. This transition was related to the thermal depolymerisation and branching of the polysaccharides, occurring at temperature ranges varying between 220 and 310 °C (Sperling, 2006).

The TGA curve (Fig. 2) shows the weight losses of the polysaccharides when exposed to heating until 580 °C, with four defined mass loss stages. The first one started at approximately 80 °C and corresponded to weight losses of about 12.91%, resulting from the adsorbed and structural water evaporation (dehydration of the sample). The greatest transformation and mass losses occurred during the second stage, at approximately 300 °C. At this stage, the depolymerization and decomposition of polysaccharides occurred, providing weight losses of 37.61%, in agreement with the DSC thermogram. Finally, the third and fourth thermal stages started at approximately 400 °C and 520 °C, respectively, being related with the decomposition of the material and resulting in weight losses of 13.95% and 9.73%.

Table 2
Total phenolic compounds and antioxidant capacity of the polysaccharides extracted from spent coffee grounds by alkali treatment.

Assay method	Response
Total phenolic compounds (mg GAE/g lyophilized)	230.14 ± 1.43
Total antioxidant activity (mg TOC/mL)	0.19 ± 0.01
FRAP (mg Fe(II)/mL)	0.20 ± 0.11
DPPH IC_{50} (mg TE/mL)	0.11 ± 0.00
ABTS IC_{50} (mg TE/mL)	0.08 ± 0.00

Results are expressed as mean ± standard deviation; $n = 3$. FRAP: antioxidant activity by the ferric reducing antioxidant power assay; DPPH: antioxidant activity by the 2,2-diphenyl-1-picrylhydrazyl assay; ABTS: antioxidant activity by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt.

3.3. Antioxidant phenolic compounds

In order to evaluate the phenolic compounds and the antioxidant activity of the polysaccharides extracted from SCG, aqueous extracts were obtained by mixing the lyophilized material with ultrapure water to obtain a concentration of 1 mg/mL. The values obtained for the total phenolic compounds and the antioxidant activity determined by different methods are presented in Table 2. The content of phenolic compounds was higher (230 mg/g lyophilized) when compared with other studies, which employed a conventional solid–liquid extraction method using organic solvents such as ethanol (Zuorro & Lavecchia, 2012) and methanol (Mussatto, Ballesteros, et al., 2011) obtaining 16 mg GAE/g SCG and 21.56 mg GAE/g SCG, respectively. The high value obtained in the present study may be related with the additional stages carried out before and after the extraction process, such as the SCG defatting and the extract lyophilization.

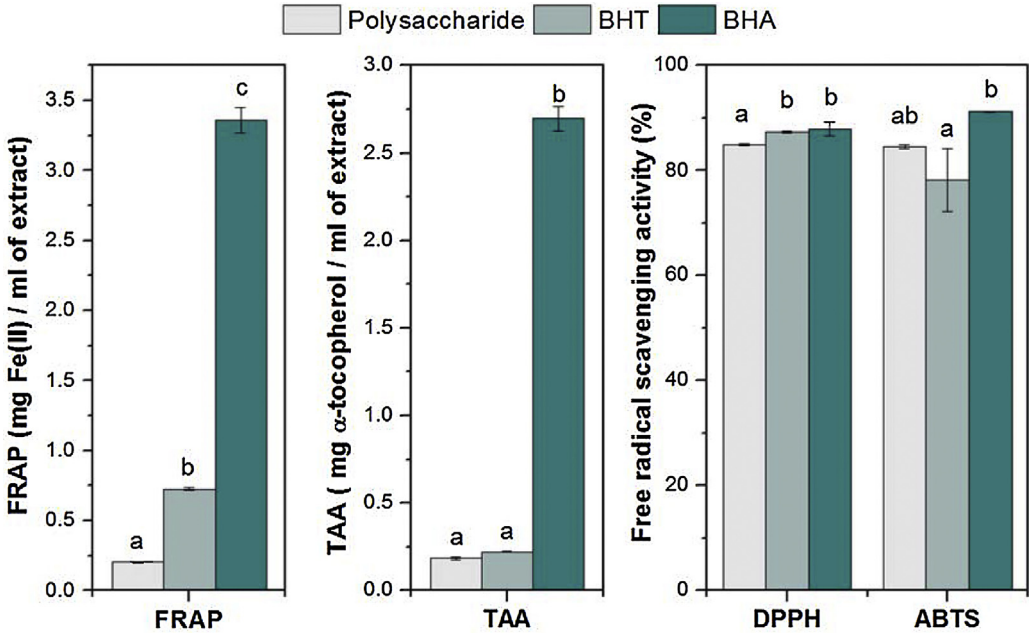

Fig. 3. Antioxidant activity of the aqueous extracts from SCG polysaccharide and two commercial antioxidant (BHT and BHA) evaluated by different methods including FRAP, TAA, DPPH and ABTS assays. Different letters within each method mean values statistically different at 95% confidence level.

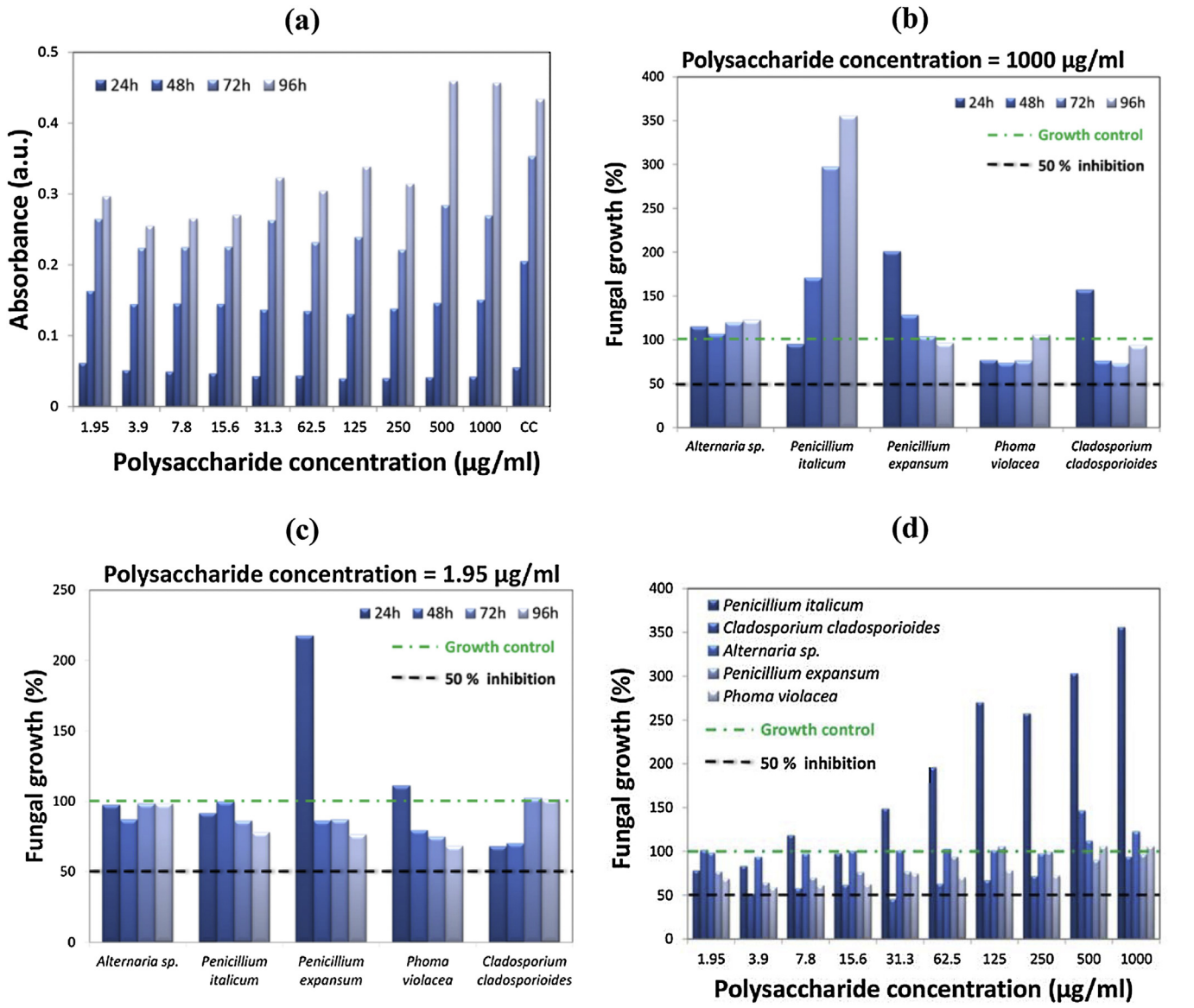

Fig. 4. Absorbance values at 530 nm for the different polysaccharide concentrations after 24, 48, 72 and 96 h of fungal inoculation with *P. violacea* (a) as an example of the all fungi behavior. Evolution of all microbial strains on two different polysaccharide concentrations, 1000 μg/mL (b) and 1.95 μg/mL (c), being the highest and lowest used concentrations, respectively. Fungal growth as a result of the effect of polysaccharide extracts at different concentrations on *P. italicum*, *C. cladosporioides*, *Alternaria* sp., *P. expansum* and *P. violacea* after 96 h of inoculation and incubation at 25 ± 2 °C (d), expressing the minimal inhibitory concentration (MIC) of polysaccharides extracts when compared with a growth control.

According to the current literature, different methods can be used to evaluate the antioxidant activity in food and biological systems. However, as each method is based on a different reaction, it is strongly advisable determining the antioxidant potential of a sample by different methods in order to better interpret the results. Fig. 3 shows the antioxidant properties of polysaccharides extracted from SCG, using three different methods. The obtained values were compared with standard antioxidant compounds such as BHT and BHA, which were analyzed under the same procedure and concentration than the extracted polysaccharides. Significant differences ($p < 0.05$) were obtained when comparing the values of extracted polysaccharides and the standards. When analyzed by FRAP assay the values of BHT were 3.5-fold higher than the values obtained for extracted polysaccharides. BHA was 15-fold higher in both FRAP and TAA assays, but the polysaccharides showed a similarly antioxidant potential for TAA when compared with BHT, which was 1.2-fold higher. On the other hand, the percentage of inhibition for all samples (at concentration of 1 mg/mL) when analyzed by DPPH and ABTS methods was much closer to the standards, clearly seen in Fig. 3. However, it is known that the scavenging activity of compounds is directly related with the concentration, and hence for the polysaccharide concentration showing the IC_{50} (at concentrations of 0.7 and 0.9 mg/mL, for DPPH and ABTS, respectively), BHA and BHT exhibited higher than 50% of inhibition, revealing the stronger antioxidant capabilities of the standards. Although in almost all antioxidant assays the standards showed to have higher values than polysaccharides extracted form SCG, the antioxidant activity obtained by DPPH assay revealed higher free radical scavenging activity compared with other works; e.g. polysaccharides extracted from edible mushrooms species, such as *Pleurotus australis*, *Ileodictyon cibarium*, *Hericium erinaceum* and *Hericium coralloides* need higher concentration of polysaccharide extract to achieve inhibitions at 50%, being 4.03, 5.78, 5.82 and 7.19 mg/mL, respectively (Ren et al., 2014). There are several factors that can influence the antioxidant activity of the extracts. For instance, defatting process, which is normally

used to remove fatty compounds in coffee before polysaccharides extraction (Bravo, Monente, Juániz, De Peña, & Cid, 2013; Nunes et al., 2005), may influence the antioxidant capacity of the samples since antioxidant compounds could be also removed (Bravo et al., 2013).

3.4. Antimicrobial activity

Polysaccharides extracted from SCG were screened for antimicrobial activity against five fungi using the micro-dilution methodology. All strains were evaluated as a function of the incubation time, assessing the growth rate after 24, 48, 72 and 96 h of incubation at 25 ± 2 °C. Fig. 4a shows the absorbance values at 530 nm obtained for *P. violacea* as an example of the absorbance values at 530 nm for different polysaccharide concentrations. The graph clearly depicts a normal development of *P. violacea* growth control (CC), while clear alterations to the fungi growth are noticed when different concentrations of the polysaccharides are tested. These changes were observed in all fungi tested and were discussed in more detail using the percentage of growth, plotted in Fig. 4b and c. The evolution of all microbial strains growth on two different concentrations, 1000 μg/mL (maxima condition) and 1.95 μg/mL (minimal condition) are displayed in Fig. 4b and c, respectively. Both polysaccharide concentrations properly represented two distinctive behaviors for low and high concentrations against all fungi. Thus, when the polysaccharide concentration was lower than 31.3 μg/mL, the trends of fungi growth with respect to time were similar and were presented in Fig. 4c, while for higher concentration the growth tendencies were presented in Fig. 4c.

Alternaria sp. exhibited very low inhibition growth for all the tested concentrations, reaching values no larger than 20% of inhibition. On the other hand, *P. italicum*, showed a particular behavior when compared to the growth of the control (CC), where higher polysaccharide concentrations promoted the fungal growth instead of inhibiting, attaining a growth almost 4-fold higher after 96 h of incubation. This behavior suggests that higher polysaccharide concentrations may act as a carbon source, stimulating the *P. italicum* growth in greater proportions than the culture media. On the contrary, for lower concentrations, the fungus growth was slightly inhibited up to value no greater than 25%. *P. expansum* exhibited higher growth rates for the first 24 h compared to the growth control. However, a monotonic reduction of fungus growth was observed when the experiment time increased for all the concentrations, achieving higher inhibition values for lower polysaccharide concentrations. The extracted polysaccharide presented the higher inhibition efficacy for *P. violacea* and *C. cladosporioides* among all the strains tested. At higher polysaccharides doses, the *P. violacea* showed a constant inhibition, reducing to 0% of inhibition after 96 h of incubation, indicating a short-term inhibition that may be due to the consumption of the inhibitory components present in the extracted polysaccharide. Nonetheless, for lower doses, an increase of the growth inhibition was observed. This behavior was also observed for *C. cladosporioides* at some concentrations, but with less defined tendencies in this particular strain. The differences between the high and low polysaccharide concentrations suggest a competition between the antimicrobial components in the extracts and the increased carbon source that the polysaccharide may offer to the microorganism, limiting the extracts function as an antimicrobial agent to low polysaccharide concentrations.

The previous behavior could be more clearly observed in Fig. 4d, where the growth percentage for all the strains at 96 h is plotted as a function of the polysaccharide concentration. This figure evidences that the increment of the polysaccharide doses reduced the inhibition of growth for the five different fungi strains. As previously mentioned, *Alternaria* sp. did not show significant changes among the concentrations studied, indicating the lack of interaction between the extract and the strain. *C. cladosporioides* exhibited the highest inhibition at 31.3 μg/mL (54.60%), as shown in Table 3. Concentrations of 3.9 μg/mL showed high percent of inhibition, being the concentration in which the five strains revealed higher antimicrobial activity. The evolution of the growth inhibition as a function of time is exposed in Fig. 5, confirming the facts previously described. Whereas the optimal conditions and percent inhibition of the polysaccharide extracts after 96 h of incubation are shown in Table 3 for the different microbial strains.

Table 3
Optimal conditions and percent inhibition of the polysaccharide extracts on growth of different microbial strains.

Microbial strains	Optimal conditions[a] (μg/mL)	Percent inhibition (%)
Penicillium italicum	1.95	22.04 ± 4.98
	3.9	17.03 ± 4.89
Cladosporium cladosporioides	31.3	54.60 ± 7.06
	3.9	48.63 ± 9.84
Alternaria sp.	3.9	6.62 ± 0.73
	7.8	2.78 ± 0.18
Penicillium expansum	3.9	36.08 ± 5.60
	7.8	30.48 ± 5.75
Phoma violacea	3.9	41.27 ± 6.95
	7.8	38.89 ± 4.49

[a] Results of the two better concentrations for each fungus. Percent inhibition was expressed as mean ± standard deviation; $n = 6$.

Additionally, tests with a known antimicrobial agent (fluconazole) revealed antimicrobial behavior (50% of growth inhibition or more) for concentrations larger than 50 μg/mL for *Alternaria sp* and *C. cladosporioides*, and 100 μg/mL for *P. italicum* and *P. violacea*, without evidence of the antimicrobial effect on the *P. expansusm*, where the growth inhibition was no higher than 30%.

Although the antimicrobial effect of extract rich in polysaccharides is not well understood, some authors have proposed that the polysaccharide may act as an external barrier, blocking the essential nutrients, impeding the microbial growth (Ren et al., 2014). Nevertheless, this barrier behavior should be increased as the polysaccharide concentration is increased, contradicting the results found in this report, where for higher concentration the antimicrobial effect is not evidenced. As a result, probably the antimicrobial effect of the extract may be due to residual components such as phenolic compounds (Jasso de Rodríguez et al.,

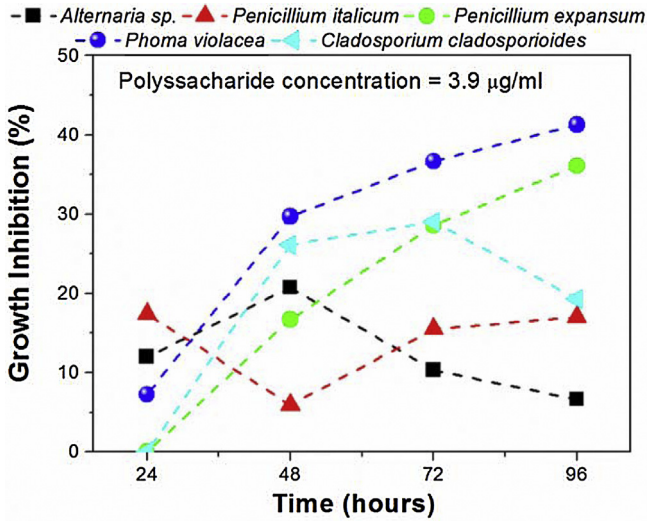

Fig. 5. Growth inhibition percentage of the polysaccharide concentration at 3.9 μg/mL, as a function of time, revealing higher antimicrobial activity against the five tested stains (*Alternaria* sp., *P. italicum*, *P. expansum*, *Phoma violacea* and *Cladosporium cladosporioides*).

2011) that are retained in the extract during the process, which compete between the polysaccharide as a carbon source for high concentrations.

4. Conclusions

The alkali pretreatment using 4 M NaOH as solvent showed to be a good option for an efficient extraction of polysaccharides from SGC. The most relevant sugars in SCG polysaccharides were galactose, followed by arabinose, glucose and mannose. Polysaccharides were thermostable in a large range of temperature, being therefore suitable for application in the manufacture of biomaterials and encapsulation products for several industrial purposes. Additionally, they revealed good antioxidant activity through different methods and presented high antimicrobial percent inhibition against *P. violacea* and *C. cladosporioides*. These findings open up possibilities to evaluate SGC polysaccharides as bioactive compounds in different food and pharmaceutical applications.

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