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PHENOLIC PROFILE OF CANE SUGAR DERIVATIVES EXHIBITING ANTIOXIDANT AND ANTIBACTERIAL PROPERTIES

ABSTRACT

Health beneficial effects of sugarcane have been attributed to antioxidant components present in the plant material, phenolic compounds having been identified mainly in the raw juice, culms and leaves. However, the presence of specific natural phenolic constituents in non-refined cane sugars and their potential impact on the diet as an alternative to refined sugar has not been completely evaluated. Phenolic constituents of six commercially available sugarcane derivatives (granulated jaggery, muscovado sugar, light and regular jaggery blocks, cane honey and brown sugar) were identified and quantified, in addition to their physicochemical, antioxidant and antimicrobial properties against cariogenic bacteria. Physicochemical and antioxidant properties of raw sugars were highly related to degree of refining of each product. Specific hydroxycinnamic acids (chlorogenic, caffeic, coumaric, ferulic) and flavones (apigenin, tricin, luteolin) were identified and quantified in sugarcane products. Tricin and apigenin were the most abundant phenolics in raw sugars, both considered important bioactive constituents of foods which postulate as nutraceuticals, antiproliferative and chemopreventive agents. Some derivatives and their extracts also exhibited antibacterial properties against *Streptococcus mutans* and *Streptococcus sobrinus*. Bioactive compounds identified in raw sugars make sugarcane natural sweeteners a healthier alternative to white sugar, to be used at home and industry. Granulated jaggeries postulate as the best substitutive due to their nutritional benefits and physicochemical attributes.

Keywords: sugarcane; non-refined sugars; antioxidant; hydroxycinnamic acids; flavones; anticariogenic.

INTRODUCTION

Sugar is a plant derived ingredient which has been related to several health problems such as metabolic disorders or a higher incidence of dental caries. Nevertheless, despite the growing concerns with regard to excess sugar intake, especially in high consuming countries, the average world level of per capita consumption is still expected to increase in the following years (OECD/FAO 2018). In fact, sugar continues to be an extensively used sweetener and additive, not only due to its sweetening properties but also to its technological properties and preservative capacity (Harish-Nayaka et al. 2009, Payet et al. 2005), both being of capital importance for the food industry. From a nutritional point of view, sugars mostly contribute to the energetic value of foods. Modern nutritional trends aim at reducing sugar content or replacing sugars by alternative sweeteners; however, health issues of intensive and extensive sweeteners have also been debated (Soffritti et al. 2006). In addition, this strategy does not consider the loss of technological properties for which formulation and processing conditions need to be adapted, e.g. the addition of preservatives to the formulation and/or the need for thermal treatments in order to reduce or limit microbial growth. In some cases, only partial replacement is possible.

Refined sugar (white), obtained either from sugarcane or sugar beet, is the sugar most widely consumed in Europe and North America, whereas non-refined alternatives (non-centrifugal sugar) are commonly consumed in the regions where sugarcane is cultivated (South America, Asia and Africa). Despite the availability of non-refined sugars has increased worldwide due to immigration and globalization phenomena (Seguí et al. 2015) worldwide consumption of these products is still reduced as compared to white sugar. Raw sugarcane and sugarcane juice is widely consumed in the above mentioned countries as a medicinal plant. Health beneficial effects of sugarcane have been attributed to the presence of antioxidant components in the plant material (Duarte-Almeida et al. 2006;

Guimarães et al. 2007; Harish-Nayaka et al. 2009; Kadam et al. 2008; Mujica et al. 2008; Payet et al. 2005; Seguí et al. 2015). Several investigations have demonstrated the effectiveness of sugarcane extracts in *in vivo* and *in vitro* models. Sugarcane extracts have shown antiproliferative properties against different cancer cell lines (leukemia, stomach, lung, colon or bladder), among other health-promoting properties such as stimulation and regulation of the immune system, protective effect against hepatic damage, recovery of intestinal function, anti-thrombotic and anti-stress properties, protective role against DNA damage, growth stimulator, prevention from hypertension and diabetes disorders, etc. (Abbas et al. 2014; El-Abasy et al. 2003, 2004; Jaffé 2012; Koge et al. 2001; Lo et al. 2005; Motobu et al. 2006; Noa et al. 2002; Singh et al. 2015; Yamauchi et al. 2006; Yoshimoto et al. 2008). Sugarcane extracts have even been suggested as prophylactic radio-protector and free radical scavenger against free radical generating agents including that by radiation exposure (Amer et al. 2004; Kadam et al. 2008).

On the other hand, sugarcane has also been claimed to exhibit a whitening and anticariogenic role. The anticariogenic effect of sugarcane was first suggested by Osborn et al. (1937a, 1937b) in the first half of the 20th century. In their studies, a lower incidence of decalcification of teeth maintained in sugar cane juice *vs.* a refined sugar solution was reported; accordingly, the authors suggested that the sugar naturally present in sugarcane was accompanied by a protective (not identified) factor against caries. Later, Jenkins (1970) included sugarcane as an enamel protecting food in a review paper. More recently, an epidemiological study of Singh (2006) associated a caries protective role to sugarcane chewing. Finally, Takara et al. (2007) demonstrated the presence or caries protective compounds in sugarcane derivatives, showing that some of the phenolic constituents extracted from sugar molasses have antibacterial activity against *Streptococcus mutans*

and *Streptococcus sobrinus*, microorganisms responsible for the development of dental caries.

Identification of phenolic compounds in sugarcane has been mainly performed in the raw juice, culms and leaves. High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) analysis of phenolic compounds from sugarcane have shown the presence of phenolic acids (sinapic, caffeic, coumaric, ferulic), flavones (apigenin, luteolin and tricin) and their derivatives (-*O*- and -*C*- glycosides). Among the flavones, the aglycone tricin and its derivatives account for a significant concentration (Colombo et al. 2006; Duarte-Almeida et al. 2011, 2006), and have shown a remarkable antiproliferative and antioxidant activities (Alves et al. 2016; Duarte-Almeida et al. 2007). Previous studies focus on identifying antioxidant components in sugarcane extracts and their potential health benefits; however, less efforts have been devoted to the characterization of non-refined commercially available sugars and their antioxidant constituents. Sugarcane extracts have been suggested as therapeutic agents, but the potential impact of non-refined cane sugars in the diet as an alternative to refined sugar has not been completely evaluated. In a previous study (Seguí et al. 2015) different kind of brown sugars (coated, boiled, light to dark), several jaggeries (light to dark, granulated or in block) and cane honey were evaluated in terms of physicochemical and antioxidant properties. Results confirmed that non-refined sugarcane products exhibit *in vitro* antioxidant activity which depend on degree of refining. In a recent study, other authors (Lee et al. 2018) have also confirmed this relationship between degree of refining and antioxidant potential of unrefined sugars. However, identification of phenolic constituents of such a variety of sugarcane derived products is still to be done. On the other hand, the presence of specific antibacterial compounds exhibiting a role against cariogenic bacteria in non-refined cane sugars has not been demonstrated to date.

100 Therefore, the objective of the present work is to extend the characterization of
101 antioxidant and anticariogenic properties of non-refined sugar cane products that have
102 been proved to exhibit *in vitro* antioxidant capacity, by identifying and quantifying
103 specific phenolic constituents by HPLC and evaluating their properties against the
104 cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*.

105 **MATERIALS AND METHODS**

106 **Non-refined sugarcane commercial products**

107 Based on previous results (Seguí et al. 2015), cane honey (CH), granulated jaggery (GJ),
108 muscovado sugar (MS), light jaggery block (LJB), regular jaggery block (RJB) and brown
109 sugar (BS) were selected for this study (Figure 1). Sugarcane products were purchased
110 from supermarkets and specialized stores in Valencia (Spain), and stored in dark and dry
111 conditions and at room temperature until analysis.

112 **Physicochemical characterization**

113 Moisture content (x_w) was calculated gravimetrically (ICUMSA, International
114 Commission for Uniform Methods of Sugar Analysis; De Whalley, 1964). Water activity
115 (a_w) was obtained with a hygrometer (Aqualab 4TE, Decagon devices, Pullman, WA,
116 USA); Total soluble solids (TSS) were measured on 1:10 water solutions of the sugars
117 using a thermostated refractometer (Abbe ATAGO 3-T, Atago Co. Ltd., Japan); Sugar
118 profile (sucrose, fructose, glucose) was obtained by ion exchange chromatography
119 (HPAEC-PAD) (high-performance anion-exchange chromatography with pulsed
120 amperometric detector (HPAEC-PAD) (Seguí et al. 2015). A 716 Compact IC Metrohm
121 system and a Metrosep Carb 1 250/4.6 column (250 mm L 9 4.6 mm ID) were used;
122 sodium hydroxide 0.1 M being the mobile phase (1 mL min^{-1}). Chromatograms were read
123 with ICnet 2.0 software (Mehtrom Ltd., Herisau, Switzerland). Samples were diluted in
124 deionized water at appropriate concentrations and further filtered (0.45 μm) before

125 chromatographic analyses. Standards (>99.5% purity) were from Sigma-Aldrich Quimica
126 (Spain). The ICUMSA official method was used for colour analysis (ICUMSA Units, IU)
127 (De Whalley 1964; Seguí et al. 2015).

128 **Antioxidant properties**

129 ***Total phenol and flavonoid content***

130 Phenols were obtained by a modified Folin-Ciocalteu colorimetric method (Singleton et
131 al. 1999; Wolfe et al. 2003). Samples were diluted in water in different proportions. 0.125
132 mL of sample were introduced in a cuvette in which 0.5 mL of bidistilled water and 0.125
133 ml of the Folin-Ciocalteu reagent were added. The sample was allowed to react in
134 darkness during 6 min and then 1.25 mL of a Na_2CO_3 at 7% (w/v) in bidistilled water,
135 together with 1 mL of water were added. Absorbance at 760 nm was measured after 90
136 min of reaction in the dark, using a Helios Zeta UV/Vis (Thermo Fisher Scientific,
137 Waltham, MA, USA) spectrophotometer. Absorbance measurements were compared to a
138 standard curve of gallic acid (purity ≥ 98%; Sigma-Aldrich Quimica) and expressed as
139 mg of gallic acid equivalents (GAE) per gram of product. Flavonoid content was
140 measured with the colorimetric method of aluminum chloride (Luximon-Ramma et al.
141 2002). Determinations were conducted on aqueous solutions of the samples. 1.5 mL of
142 each solution were vigorously mixed with 1.5 mL of aluminum chloride solution (2% w/v
143 in methanol) and allowed to react for 15 min. Apigenin (purity ≥ 98%; Sigma-Aldrich)
144 was chosen as a standard due to apigenin being one of the most common flavonoids in
145 sugarcane juice (Duarte-Almeida et al. 2006, 2011) and having a maximum absorbance
146 after reaction with $AlCl_3$ close to tricin, the other major flavone in sugarcane. Apigenin
147 equivalents (mg AE) per gram of product were obtained from absorbance at 337 nm.

148 ***Antiradical capacity (DPPH and ABTS methods)***

149 Radical scavenging ability of non-refined sugars against 1,1-diphenyl-2-picryl hydrazyl
150 (DPPH·) and 2,20-azobis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) radicals was
151 assayed. DPPH antiradical capacity was determined as proposed by Brand-Williams et
152 al. (1995). 2 mL of a DPPH solution in methanol (0.1 mM) were mixed with different
153 amounts (10, 30, 50 and 70 mL) of sample consisting of solutions of the different sugars
154 (1:10 w/v sugar solution in bidistilled water). Scavenging capacity was then monitored
155 spectrophotometrically by measuring the decrease in the absorbance at 517 nm during 3
156 h and percentage inhibition of DPPH (% I) was calculated as relative reduction in the
157 absorbance with respect to the blank. The amount of sample needed to scavenge 50% of
158 the DPPH (IC50) was also calculated. The ABTS or TEAC (Trolox Equivalent
159 Antioxidant capacity), which measures the ability of an antioxidant to scavenge the
160 preformed radical cation ABTS+ relative to that of the standard antioxidant Trolox, was
161 determined according to Re et al. (1999). ABTS (7 mM) was made to react with potassium
162 persulfate (2.45 mM) during 16 h at room temperature in order to obtain the ABTS+
163 radical. Then, the solution was diluted in phosphate buffer (pH 7) to an absorbance of
164 0.70 ± 0.02 at 734 nm. 90 mL of the sample or blank were then added to 2.910 mL of the
165 ABTS+ in phosphate buffer and absorbance at 734 nm was read at 1, 2, 3 and 6 min of
166 reaction. In controls, deionized water was used. TEAC values were expressed in mmol
167 Trolox per gram of sample. Reagents used in AO determinations, DPPH, ABTS (purity
168 ≥ 98%) and Trolox (purity ≥ 97%), were from Sigma-Aldrich Quimica, Spain.

169 **Identification and quantification of antioxidant constituents by HPLC**

170 ***Extraction of phenolic constituents***

171 Determination of phenolic constituents in sugarcane products by HPLC (High-
172 Performance Liquid Chromatography) was based in the protocol developed by Duarte-
173 Almeida et al. (2006, 2011). Sugarcane products were dissolved in bidistilled water (1:3

174 w/v) and further centrifuged at 3,500 rpm during 10 min. Supernatant was collected for
175 further analysis. Solid phase extraction was performed using polyamide columns
176 (CHROMABOND® PA. 6 mL/500 mg; Macherey-Nagel GmbH & Co.) previously
177 conditioned with 10 mL of methanol and 30 mL of bidistilled water. 5 mL aliquots of the
178 extracts were fractionated in the polyamide columns and further washed with 10 mL of
179 bidistilled water and eluted with 25 mL of methanol and 25 mL of methanol:ammonia
180 (99.5:0.5 v/v). The volume extracted (50 mL) was then evaporated to dryness at 40 ºC
181 under vacuum conditions in a Rotavapor (Heidolph, Germany). Concentrated extracts
182 were dissolved in 1 mL methanol and filtered to a chromatography vial through a 0.45
183 mm PTFE filter. All reagents used in the present protocol were of HPLC grade.

184 ***Analytical HPLC***

185 Identification and quantification of phenolic substances in the eluates were carried out
186 using analytical reversed phase HPLC on an Agilent 1100 system with autosampler and
187 quaternary pump coupled to a diode array detector, and filled with a C18 reversed-phase
188 column (250 x 4.6 mm and 5 μm; Luna II Phenomenex). The following elution solvents
189 were used: A. water:tetrahydrofuran:trifluoroacetic acid (98:2:0.1) and B, acetonitrile.
190 Solvent gradient was similar to Duarte-Almeida et al. (2007). Each phase was filtered
191 through 0.2 μm nylon mesh. Determinations were performed in triplicates, 20 μL being
192 the volume injected. Identification followed comparison of UV spectra (200 to 400 nm)
193 and retention times with standards, and quantification was based on external calibration.
194 Standards used for hydroxycinnamic acids were: caffeic, coumaric, ferulic, chlorogenic
195 and sinapic acids; as for flavones: tricin, luteolin and apigenin were chosen. Results are
196 given as mg/100 g. All standards were of HPLC grade and purchased from Sigma-
197 Aldrich, except for tricin (synthetized by ©Syncom, The Netherlands). Fortified samples
198 were also prepared in order to take into account components recovery factor.

199 Chromatograms were examined by means of Empower Pro (Waters) so as to identify and
200 quantify phenolic constituents.

201 **Antimicrobial activity against cariogenic bacteria**

202 *Streptococcus mutans* (CECT 479 T) and *Streptococcus sobrinus* (CECT 4034)
203 (Colección Española de Cultivos Tipo, Burjassot, Valencia) were used as cariogenic
204 bacteria for antimicrobial assays. Lyophilized microorganisms were reconstituted in
205 Brain Heart Infusion (BHI) broth and agar (Scharlau) with further incubation (PSelecta
206 Incudigit) at 37 ºC during 48 h, following the CECT recommendations. Solid and liquid
207 media inhibition assays were based on the available literature (Chitnis et al. 2007;
208 Mosquera and Veloz 2011; Takara et al. 2007).

209 ***Inhibition assay in solid medium: paper disk-agar diffusion assay***

210 Extracts of the different sugarcane products were obtained as for the HPLC analysis and
211 bring to 1 mL. Paper disks were submerged in the prepared extracts and introduced in
212 Petri dishes prepared with BHI agar and further inoculated with the corresponding
213 microorganism (150 or 300 μL of a suspension of *S. mutans* or *S. sobrinus*, obtained by
214 incubating at 37 ºC during 48 h in BHI broth). Chlorhexidine (Sigma-Aldrich, Spain) was
215 used as a positive control.

216 ***In vitro inhibition assay in liquid medium (extracts)***

217 Inhibition was also evaluated spectrophotometrically (Mosquera and Veloz 2011). Solid
218 phase extraction was slightly modified so that the phenolic content of 10 mL (2 x 5 mL)
219 of the sugar solution was concentrated, and finally dissolved in 1 mL of bidistilled water.
220 Serial dilution of the obtained extracts were prepared by mixing the extract (1 mL) with
221 1 mL of BHI broth. After homogenization in vortex, 1 mL of the mixture was introduced
222 in the subsequent tube, up to 5 tubes, so that final amount of extract in the tubes was: 0.5,
223 0.25, 0.125, 0.0625, 0.0125 mL. In order to estimate the amount of phenolic compounds

able to inhibit or reduce bacterial growth, phenolic content in the dilution tubes was determined by the Folin-Ciocalteau method. The inoculum was prepared by seeding the microorganisms in 5 mL of BHI broth and growing during 48 h at 37 ºC (PSelecta Incugidit). After incubation, microorganisms were collected by centrifugation (miniSpin, Eppendorf®) at 3,500 rpm during 20 min. Supernatant was removed and pellet resuspended in 4 mL of a 0.9% NaCl sterile solution. Initial inoculum was brought to an optic density (O.D.) of 0.1 (λ = 665 nm) by addition of the 0.9% NaCl solution (~10^4 CFU/mL). Each tube was inoculated (1 mL) and further incubated at 37 ºC during 48 h. Absorbance at 665 nm was measured before (A_0) and after incubation (A_1), and absorbance increments were registered.

In vitro inhibition assay in liquid medium (sugarcane derivatives)

Anticariogenic effect of sugarcane products was also tested by liquid inhibition assay. In this case, anticariogenic properties of solutions of the non-refined products was directly evaluated. For this purpose, BS, JB, GJ and CH were selected and compared to white sugar (WS). Serial dilutions were prepared from a solution of each sugarcane product in bidistilled water in order to obtain the following concentrations: 60, 45, 30, 15 g non-refined sugar/100 mL. The initial inoculum was standardized by adjusting the optical density at 665 nm with a 0.9% NaCl solution (10^4 CFU/mL). Tubes containing 2 mL of BHI broth and 4 mL of the corresponding solution were inoculated with 10^4 CFU (1 mL inoculum), and absorbance at 665 nm was measured before (A_0) and after (A1) incubation (A_1) at 37 ºC during 48 h.

Statistical significance of the results

Analytical determinations were performed at least in triplicate. Statgraphics Centurion XVI was used to calculate One-Way ANOVAs and determinate statistically significant differences with a 95% confidence interval and multiple range tests were used to

249 determine the significance of the difference among samples (p-value < 0.05). Results are
250 given as the mean ± standard deviation

251 **RESULTS AND DISCUSSION**

252 **Physicochemical properties of non-refined sugars**

253 Physicochemical properties of the sugarcane products analyzed are given in Table 1.
254 Water content was significantly different among the products analyzed: crystal sugar (BS)
255 presenting the lowest value (0.12%), and cane honey (CH) the highest one (16.9%).
256 Jaggeries presented an intermediate moisture content, block jaggeries containing more
257 water (4.0-6.4%) than the granulated ones (1.70-1.83%). Results are consistent with sugar
258 processing: crystal sugars (either white or brown) are dried after the crystallization stage,
259 thus decreasing their moisture content. On the other hand, jaggeries are solidified by
260 cooling after evaporation, for which final product retains more water. As for cane honey,
261 this is obtained as the mother liquor of the crystallization process, for which they contain
262 a significant amount of water with respect to the other sugarcane products. Then, values
263 are in agreement with the literature, and the differences found are a result of particular
264 manufacturing processes (Jaffe 2012). Moisture content is related to sugar shelf life
265 (Guerra and Mujica 2010), but it is water availability that indicates the availability of
266 water to participate in reactions. The latter was rather homogeneous among samples, as
267 in Seguí et al. 2015, which could be related to hygroscopic properties of inverted sugar.
268 In fact, samples containing higher contents of glucose and fructose (CH, RJB) showed
269 significantly higher moisture contents for not such a significant increase in a_w. As for total
270 soluble solids, values were close to 100% except for CH, this suggesting the presence of
271 other compounds different from sugars or either other sugars different from sucrose
272 interfering in the refractometric index. CH contained significantly higher amounts of
273 glucose and fructose than the other products analyzed. These sugars, which may be of

plant material origin or be presents as a result of sucrose inversion during processing or storage, were identified in all samples. Brown sugar and jaggery blocks presented the lowest IU values for colour, which implied lighter solutions, whereas cane honey solution was the darkest one. The refining process applied to crystal sugars eliminates most of the phenolic constituents of sugarcane responsible for colour, while, on the other hand, molasses concentration and Maillard reactions would be responsible for CH high IU values. Physicochemical attributes of the products analyzed were in the expected range (Seguí et al. 2015; Lee et al. 2018; Mujica et al. 2008; Saska et al 2010; Wojtczak, et al. 2013).

Antioxidant properties of non-refined sugars

Antioxidant properties of non-refined sugars are summarized in Table 2. Results reveal that degree of refining determine the antioxidant properties of sugarcane derivatives. In particular, phenol and flavonoid contents were significantly lower for brown sugar, in which the refining process would have eliminated most of the antioxidant compounds originally present in the sugarcane juice. Among the other products, granulated jaggeries and cane honey (GJ, MS and CH) exhibited the highest contents. Values were in the range of the published for similar products taking into account that differences in processing as well as origin and sugarcane cultivar may influence the results (Harish Nayaka et al. 2009; Payet et al. 2005; Seguí et al. 2015).

All the products analyzed showed certain *in-vitro* antioxidant capacity as measured by the ABTS-TEAC and the DPPH methods, results being in line with the values registered for phenol and flavonoid contents. In particular, antioxidant capacity was significantly higher for the GJ, followed by CH and MS, and slightly lower for both jaggery blocks. In contrast, BS exhibited very low ABTS-TEAC and DPPH antioxidant abilities, significantly far from the rest of products.

Phenolic profile of non-refined sugarcane products

HPLC chromatograms obtained for the phenolic fraction of the six sugarcane products analyzed, at 323 and 348 nm are presented in Figure 2. Complex chromatograms were obtained in all cases, with a significant amount of picks, in line with the presented by other authors for sugarcane parts, juice or derivatives (Colombo et al. 2006; Duarte-Almeida et al. 2006; Vila et al. 2008). Spectroscopic characteristics of the standards were considered to select the wave lengths to identify and quantify phenolic constituents (323 nm for hydroxycinnamic acids and 348 nm for flavones). Spectroscopic characteristics of the standards were used to select wave lengths (323 nm for hydroxycinnamic acids and 348 nm for flavones). Identification was achieved by comparing the UV-visible spectra with those of the standards. As in (Duarte-Almeida et al. 2011) compounds with similar spectra but different retention times were considered derivatives and flavonoids identified by their corresponding aglycone. Figure 3 shows an example of pick identification (GJ), and details of the UV-spectra for caffeic acid and apigenin.

To date, flavones and hydroxycinnamic acids had been identified in sugarcane (leaves, culms, juice) and some derivatives such as molasses or very high polarization (VHP) sugar (Colombo et al. 2006; Duarte-Almeida et al. 2006 2011; Vila et al. 2008). Other authors (Payet et al. 2005, 2006) found phenolic acids but no flavones in brown sugar samples. The present work reveals that both hydroxycinnamic acids and flavones are present in non-refined commercial cane sugars (Table 3). Except for sinapic acid, not detected in any sample, and luteolin, not identified in BS and CH, the rest of phenolics evaluated were present in all the products analyzed. The amount of flavones and cinnamic acids obtained is in agreement with the antioxidant capacity registered for the products. In fact, the lowest concentration of phenolics was found in brown sugar, which presented poor antioxidant properties; whereas granulated jaggeries (GJ and MS) were the richest

in hydroxycinnamic acids and flavones, followed by both jaggery blocks and cane honey. Brown and other non-refined sugars have been said to exhibit antioxidant activity also due to the presence of Maillard reaction products such as melanoidins (Payet et al. 2005). In this paper, however, it is confirmed that the antioxidant capacity of sugarcane derivatives is strongly related to the natural phenolic constituents present in sugarcane, which are preserved during processing in the case of non-refined sugars.

Hydroxycinnamic acids are very common in nature, and are present in many plant foods such as fruits, usually in their bound form (Murkovic 2003). The cane sugars analyzed contained less cinnamic acids than flavones, chlorogenic acid being most abundant in granulated jaggery and muscovado sugar. Duarte-Almeida et al. (2011) also reported a higher amount of this phenylpropanoid in sugar molasses. The same authors also found chlorogenic acid in brown sugar, as in the present study, and in contrast Payet et al. (2005). Contrarily to Duarte-Almeida et al. (2011), caffeic acid was also identified in all sugarcane products, and was mostly present in granulated jaggeries. Ferulic acid was more abundant in jaggery blocks and CH.

As for flavones, apigenin was the most abundant flavone in all cases, followed by tricin. In contrast, luteolin was only identified in very small amounts and not present in brown sugar and cane honey. These results agree with the obtained by Duarte-Almeida et al. (2011) who found higher amounts of apigenin followed by tricin and finally luteolin in sugarcane juice, molasses and sugar. In a previous study, however, Duarte-Almeida et al. (2006) found tricin to be the most abundant flavone in sugarcane juice. Tricin and its derivatives (glucosides, esters) are present in rice bran and other grass species (Verschoyle et al. 2006), and have also been identified in different parts of sugarcane (leaves, bagasse, juice). Biological potential of tricin has been reported by several authors (Alves et al. 2016; Duarte-Almeida et al. 2007). In particular, this natural occurring

flavone has shown antiproliferative potential again breast and colon cancer cells (Cai et al. 2004; Cai et al. 2007; Hudson et al. 2000; Verschoyle et al. 2006), as well as a chemopreventive potential (Al-Fayez et al. 2006). Tricin have also exhibited antiviral activity against influenza and human cytomegalovirus (Akuzawa et al. 2011; Yazawa et al. 2011). In 2010, Zhou and Ibrahim presented tricin as a potential multifunctional nutraceutical in a review paper. Apigenin has also been reported to have health benefits, including anti-inflammatory and antiproliferative properties. Among other flavones, it is believed to possess therapeutic potential against cancer and has evolved as a promising pharmacological agent in cancer treatment (Chiang et al. 2006; Jaganathan and Mandal 2009; Shuckla and Gupta 2010). In addition, some studies show that flavones such as apigenin and luteolin may potentiate the effect of chemotherapeutic drugs (Johnson and Gonzalez de Mejia 2013).

Antibacterial properties against cariogenic bacteria

Antimicrobial activity of the extracts against *S. mutans* and *S. sobrinus* was confirmed as deduced from the inhibition halos observed in the plates (Figure 4). Inhibition halos were small as compared to chlorhexidine and resulted evident only in the plates seeded with *S. mutans*. This could be explained taking into account microorganism population, since *S. sobrinus* grew to a higher extent. Halos observed for the GJ and MS extracts were bigger than the observed for the jaggery blocks, while no halos were observed in the case of CH and BS.

Results of the disk-diffusion had some limitations since they depend on the antimicrobial properties of the compounds being analyzed but also on their diffusion properties and other factors such as microorganism population. Thus, in order to complete the antimicrobial study, a liquid assay was performed. In this case, microorganisms were grown in tubes containing the liquid medium (BHI-broth) enriched with the extracts.

374 Results are summarized in Table 4. As deduced from the absorbance increments, the
375 amount of extract present in the assay tube significantly affected microorganism growth
376 for both *S. mutans* and *S. sobrinus*. With regard to the origin of the extract, significant
377 differences BS and both jaggery blocks, in the case of *S. mutans*. In most cases, an
378 inflection point is observed in tube 2 (0.25 mL of extract), in which the amount of phenols
379 present estimated by the Folin-Ciocalteau method were: 28.8 mg GAE/mL (GJ), 27.3 mg
380 GAE/mL (MS), 26.6 mg GAE/mL (LJB), 25.5 mg GAE/mL (CH). For some sugars such
381 as GJ, CH and MS, the amount of extract needed to inhibit microorganism growth was
382 lower since transition was observed between tubes 2 and 3 (0.25-0.125 mL). Most
383 probably, not only phenol concentration but also specific phenolic constituents are
384 responsible for the antibacterial activity of non-refined sugars. Flavonoids, including
385 flavones, have been claimed to possess antibacterial activity (Cushnie and Lamb 2011).
386 Tricin, apigenin and luteolin are especially mentioned in the literature as being present in
387 materials exhibiting antibacterial properties (Moniruzaman et al. 2015; Tanaka et al 2011;
388 Sato et al. 2000). Flavonoid aglycones are said to be effective glucosyl-transferase
389 inhibitors, which may contribute to their anticariogenic properties (Takara et al. 2007).
390 The decreased absorbance increment observed in the last tubes could be attributed to
391 carbon source depletion.
392 Results of the present work confirm the hypothesized for other researches who stated that
393 in sugarcane sucrose is accompanied by specific compounds that inhibit microorganisms
394 responsible for dental caries development (Jaffé 2012; Osborn et al. 1937a; Singh 2006;
395 Takara et al. 2007). This result is considered of sufficient interest, but anticariogenic
396 properties of non-refined sugars themselves is still to be confirmed, for which
397 microorganism growth in the presence of sugarcane products was also studied. In this
398 case, inhibition of *Streptococcus mutans* in the presence of BS, GJ, JB and CH were

399 analyzed using WS as a control. Results of this assay (Table 5) suggest that sugarcane
400 derivatives may exhibit certain antibacterial activity, which was more evident in case of
401 jaggeries. In contrast, WS and BS showed no inhibition of microbial growth.
402 Antimicrobial properties of sugarcane products and their extracts are consistent among
403 assays (liquid and solid media), but also with respect to the antioxidant properties and
404 phenolic profile of the non-refined sugars. In general, granulated jaggeries (GJ, MS) and
405 cane honey have exhibited higher antimicrobial activity than jaggery blocks, which is in
406 line with the antioxidant properties of these products and their flavone content. In
407 contrast, no antibacterial properties have been attributed to brown sugar, which had
408 exhibited poor antioxidant properties and scarce phenolic content.

409 **CONCLUSION**

410 Results of the present work reveal the presence of naturally occurring bioactive
411 compounds in six selected commercial non-refined sugarcane products, which are
412 available in common supermarkets to be used as sugar substitutive. Various
413 hydroxycinnamic acids and the flavones apigenin, tricin and luteolin have been identified
414 as constituents of non-refined cane sugars. Physicochemical and antioxidant properties of
415 non-refined cane sugars have been related to degree of refining, and phenolic constituents
416 present in the different sugarcane samples have been found to be consistent with the
417 antioxidant and antibacterial properties of the sugars analyzed.
418 Several health benefits, including antiproliferative, chemopreventive, radio-protective,
419 anticariogenic and immunoregulating properties had been attributed to sugarcane extracts
420 obtained from leaves, culms or juice. Confirmation of particular phenolic constituents in
421 non-refined sugars, especially the flavones tricin and apigenin, suggest that non-refined
422 sugars could provide similar health beneficial effects. Consumption of unrefined sugars
423 may contribute towards the prevention of certain diseases and promote well-being

maintenance, for which the use of non-refined sugarcane alternatives to white sugar at home and industry is encouraged. Among the non-refined sugars analyzed, granulated jaggeries (including muscovado sugar) provide the best nutritional benefits, giving their phenolic profile and antioxidant properties. Physicochemical properties of granulated jaggeries can be classified as intermediate among the products analyzed, and have exhibited safe levels of moisture content and water activity. Besides, granulated jaggery is presented in an appropriate format for dosing which facilitates formulation at home and industrial uses. For all the previous, granulated jaggeries postulate as the best alternative to white sugar, taking into account not only sweetening but also preservative properties of sugar.

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