Antioxidant Activity of Sugar Molasses, Including Protective Effect Against DNA Oxidative Damage

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ABSTRACT: Extracts were obtained from molasses, a byproduct of the sugar industry, via a number of chromatographic steps. Their antioxidant capacity was studied, including the inhibitory effect upon DNA oxidative damage; the phenolic compound profile thereof was ascertained as well. Two extracts exhibited significant antioxidant features, expressed by their capacity to decolorize ABTS radical cation and to scavenge hydroxyl free radicals (via deoxyribose assay). Those 2 extracts also brought about protection against induced DNA oxidative damage (via decreasing DNA scission, as assessed by electrophoresis). The phenolic compounds syringic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, and ferulic acid were positively identified and quantified.

Keywords: ABTS assay, deoxyribose assay, DNA protection, natural antioxidants, sugar cane

Introduction

Antioxidants are compounds which, even when present at much lower concentrations than those of oxidizable substrates, significantly delay (or even prevent) oxidation thereof (Halliwell 1990). This capacity has been largely taken advantage of in the food industry for preservation purposes as well as in the cosmetic, pharmaceutical, and plastic industries to avoid deterioration of labile feedstocks. In addition, several biological properties, anticarcinogenic, antimutagenic, antiallergenic, and antiaging activities, have been associated with the presence of specific antioxidants.

A few synthetic antioxidants may unfortunately exhibit a detrimental impact upon health, although not toxic at the levels normally used in the food industry, they actually have been implicated in some diseases (for example, cancer) (Ito and others 1983). On the other hand, natural product demand has in general been on the rise. Both these realizations account for an impetus toward identification of alternative sources of antioxidants—preferably natural, and thus probably safer. Therefore, research on plant sources has been fostered worldwide, including comprehensive screening of raw materials for novel antioxidants. Many compounds have accordingly been isolated, most of which belong to the phenolic compound family (Rice-Evans and others 1997). Special attention has in particular been paid to antioxidants extracted from inexpensive sources such as leftovers of agricultural industries; these include sugar cane molasses, potato peel waste, olive mill waste water, grape seeds and peels, citrus seeds and peels, green-vegetable byproducts, and cocoa byproducts (Moure and others 2001; Llorach and others 2004).

Cane molasses is a thick syrup, obtained as a byproduct of the manufacture or refining of sucrose from sugar cane. More than 35 million metric tons of molasses are produced annually on a world basis; they are used mainly as a supplement for livestock feed or as a substrate for ethanol production. In addition to sugars, which account for approximately 46% (w/w), molasses contains several nonsugar organic materials—compounds originated from the cane plant (for example, phenolic compounds) or generated during the production process (for example, colored products of Maillard reactions—melanoidins, and products of thermal or alkaline degradation of sugars) (Curtin 1983; Clarke 1985). Nagai and others (2001) have reported that sugar cane extracts possess significant antioxidant activity; such extracts also exhibit interesting physiological functions, enhancement of resistance to infections as well as vaccine adjuvant and anti-inflammatory features. Despite said functional properties of some sugar cane components, they are often disposed of in molasses form without any recovery downstream.

The aim of this research effort was thus to characterize the antioxidant capacity of molasses, encompassing also its protective effect on induced DNA oxidative damage. Such antioxidant features were measured via 2 alternative methods: ABTS radical cation decolorization assay and deoxyribose assay; their phenolic compound composition was studied as well, in attempts to rationalize said antioxidant performance.

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Materials and Methods

Chemicals and reagents

Cane molasses was kindly supplied by a major sugar refinery, RAR–Refinarias de Açúcar Reunidas, S.A. (Porto, Portugal). All HPLC standards, as well as ascorbic acid, ABTS (2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)) diammonium salt, EDTA (ethylenediaminetetracetic acid), 2-deoxy-D-ribose, calf thymus DNA, and agarose were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium persulfate, TBA (2-thiobarbituric acid), and formic acid were obtained from Merck (Darmstadt, Germany). Iron(III) chloride, hydrogen peroxide (33%), TCA (trichloroacetic acid) and Cu(II) sulfate were purchased from Panreac (Barcelona, Spain). Methanol SpS™ was obtained from Romil (Cambridge, U.K.).

Molasses extraction

A modification of the chromatographic method previously developed by Bento (2002) was chosen for our study. A stock solution was

prepared by diluting molasses with deionized water to 20 g/L and adjusting pH to 8.5 with concentrated NaOH. Said stock solution (2 L) was then loaded onto a column previously packed with 150 mL of anion exchange resin (Lewatit 56368, Bayer, Pittsburgh, Pa., U.S.A.), and washed with plain water. Elution was brought about by a 17.5 g/L NaCl solution (1.2 L), followed by 41.0 g/L NaCl solution (1.2 L) at a flow rate of 5 mL/min. Fractions of eluate (50 mL) were collected and assayed for antioxidant activity (ABTS assay, as described below), sugar colorants (absorbance at 420 nm, using a Heλios α spectrophotometer from Unicam, Cambridge, U.K.) (Clarke and others 1985), and sodium chloride concentration (Mohr titration, Vogel 1961).

Antioxidant activity

This type of activity was assessed via 2 alternative methods, ABTS and deoxyribose assays, as follows.

ABTS assay (radical cation decolorization). This method is based on decolorization of radical cation $ABTS^{\bullet+}$, measured as percent reduction of absorbance at 734 nm (Re and others 1999; Gião and others 2005). The radical $ABTS^{\bullet+}$ was initially produced by mixing a 7 mM solution of ABTS with a 2.45 mM solution of potassium persulfate at a 1:1 (v/v) ratio, and then allowing the mixture stand in the dark at room temperature for 16 h prior to use. The $ABTS^{\bullet+}$ solution was then diluted in ultrapure water so as to obtain an absorbance of 0.700 ± 0.020 at 734 nm (using a UV-1203 spectrophotometer, Shimadzu, Kyoto, Japan). Each experimental sample was finally added to 1 mL $ABTS^{\bullet+}$ solution, using a volume such that the percent reduction in absorbance, by 6 min of reaction, would lie in the range 20% to 80%. Calibration curves were produced in parallel, using solutions of ascorbic acid as reference compound, at various concentrations and under the same experimental conditions as the samples. Results were expressed in milligrams of equivalent ascorbic acid per liter of sample ($mg_{equiv\,AA}/L$).

Deoxyribose assay (hydroxyl free radical scavenging activity). Deoxyribose is oxidized when exposed to hydroxyl radicals; such a degradation can be detected by heating the products in the presence of thiobarbituric acid under acidic conditions, which leads to development of a pink chromogen, and measuring absorbance at 532 nm against appropriate blanks; this method was initially proposed by Halliwell and others (1987). The assay mixtures, containing the sample, used a final volume of 1 mL, that is, 1 mM in deoxyribose, 24 mM in sodium phosphate (containing 15 mM NaCl, pH 7.4), 0.1 mM in $FeCl_3$, 0.1 mM in EDTA, 1 mM in H_2O_2, and 0.1 mM in ascorbic acid. After incubation at 37 °C for 1 h, color development was promoted via addition of 1.5 mL of 28% (w/v) TCA and 1.0 mL of 1% (w/v) TBA in 0.05 M NaOH, followed by heating at 100 °C for 15 min. Inhibition of deoxyribose degradation was expressed as percent decrease in absorbance, when compared to the control (assay without sample).

DNA oxidative damage inhibition

This effect was assessed according to the methodology described by Rivero and others (2005), with slight modifications—as described below.

Incubation. The assay mixtures, containing the sample, used a final volume of 1 mL—that is, 200 μg/mL in DNA, 100 mM in sodium phosphate (pH 7.4), 10 mM in ascorbic acid, and 100 μM in Cu(II). Two controls were run in parallel: one containing only DNA and the other containing DNA, ascorbic acid, and Cu(II). The mixtures were incubated for 1 h in a shaking water bath at 37 °C. The maximum volume of sample allowed in the assay was 400 μL—so the maximum concentration that could be tested would be 40% (v/v), expressed as extract volume/reaction mixture volume. In order to test the effect of higher concentrations, extracts were previously concentrated (say 2.5-fold) under vacuum at 38 °C, and adequate volumes of sample were then assayed.

Electrophoresis. The testing solutions were, after incubation, subject to electrophoresis in 0.7% (w/v) agarose gels, prepared with 100 mM sodium phosphate buffer (pH 7.8) containing 5 mM EDTA. Gels were run in the above buffer, at 400 mA, and the DNA bands were visualized under UV light after ethidium bromide staining.

Phenolic compound analysis

These analyses were carried out according to Pintado and others (1999) with the modifications described next.

Resolution. Separation of compounds was via HPLC using an Alliance 2690 (Waters, Milford, Mass., U.S.A.), with a 996 Photodiode Array Detector, and a Waters Symmetry C18 column (250 mm × 4.6 mm, 5 μm); the flow rate was 1 mL/min, the column temperature was 25 °C, and the injection volume was 40 μL. Two mobile phases were used: A—methanol:formic acid:water (5:2.5:92.5, v/v/v); and B—methanol:formic acid:water (92.5:2.5:5, v/v/v); elution was performed under the following gradients: from 0% to 30% B over 30 min; from 30% to 70% B over 35 min; from 70% to 80% B over 10 min; from 80% to 0% B over 5 min; and finally, equilibration with A for 11 min.

Identification and quantification. Peak identification was putatively achieved by comparing both the retention time and the UV-Vis absorption spectrum of each peak, with those produced by known reference standards. These reference standards were *p*-hydroxybenzoic acid, vanillic acid, syringic acid, gallic acid, protocatechuic acid, gentisic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, chlorogenic acid, umbelliferone, aesculin, kaempferol, quercetin, rutin, luteolin, apigenin, catechin, and *p*-hydroxybenzaldehyde. The internal standard used was 4-hydroxy-3-methoxycinnamaldehyde at 0.526 mg/L. Peak quantification was achieved via calibration curves, obtained for each reference standard, which was prepared in a way similar to the samples.

Statistical analysis

The influence of the type of molasses extract on its antioxidant activity, measured by the ABTS assay, was ascertained using one-way analysis of variance (ANOVA). The influence of the type and concentration of extract on its antioxidant activity measured by inhibition of deoxyribose degradation was analyzed using two-way ANOVA. Furthermore, Duncan's post hoc test was applied, so as to determine the statistically significant different values. Due to failure of our data to satisfy the homoshcedasticity assumption of ANOVA, unequal variance *t*-tests were applied. All statistical handling was performed using SPSS software, v. 14.0 (SPSS, Chicago, Ill., U.S.A.).

Results and Discussion

Molasses extraction

Ion exchange resins have met with success in cane sugar decolorization for more than three decades now because of their capacity to remove organic, nonsugar compounds, thus producing low colored syrups that are concomitantly rich in sucrose. Recently, Bento (2002) has shown that it is possible to fractionate the aforementioned nonsugar compounds by fixing them in a strong base styrenic resin and eluting them afterwards with increasing concentrations of sodium chloride.

In this work, a similar technique was used to obtain the extracts from crude molasses. In order to characterize the elution profile of molasses via the ion exchange resin using NaCl solutions as eluant, fractions of eluate were collected and assayed for their antioxidant

activity using the ABTS assay, their sugar colorant content using absorbance at 420 nm, and their sodium chloride concentration using the Mohr method. Two main peaks were produced during elution, which exhibit both high antioxidant activity and high absorbance at 420 nm: the 1st one was obtained during elution at low salt concentration, so it is accounted for by compounds with low affinity to the resin; the 2nd one, on the other hand, comprises compounds with high affinity to the resin, as it appears during elution at high salt concentration. A small shoulder was observed in between, after the 1st peak, which corresponds to compounds with intermediate affinity to the resin.

The sequential 50 mL fractions resolved by the aforementioned ion exchange column were pooled together, so as to obtain 3 distinct extracts: Extract I (fractions 2 to 9), Extract II (fractions 10 to 24), and Extract III (fractions 25 to 33).

Antioxidant activity

The antioxidant activities of sugar molasses extracts, using the ABTS assay, were statistically different from each other ($P < 0.05$): Extract I exhibited the highest activity (120 ± 2 $mg_{equivAA}/L$), followed by Extract III (86 ± 1 $mg_{equivAA}/L$) and finally Extract II (50 ± 1 $mg_{equivAA}/L$).

In order to ensure a deeper characterization of the antioxidant activity of the aforementioned extracts, their hydroxyl radical scavenging capacity was evaluated via the deoxyribose assay. All extracts exhibited a positive effect on protection of deoxyribose from degradation, as apparent from inspection of Figure 1. For a volume of 50 μL, the inhibition percentage observed was 63%, 19%, and 59%, for Extracts I, II, and III, respectively. However, when the volume was increased to 200 μL, the inhibition effect increased to 82%, 31%, and 80%, respectively. On the other hand, when the extracts were incubated with deoxyribose only, no degradation was observed, even at the highest volumes (400 μL), indicating that the extracts possess no pro-oxidant activity. Extract II yielded the lowest protective effect at all concentrations tested, whereas Extracts I and III exhibited no statistically significant difference between them ($P < 0.05$).

The 2 assays referred to above are based on different principles, so they represent not fully overlapping aspects of the antioxidant capacity; this may explain why Extract I showed a significantly higher activity than Extract III via the ABTS method, but an essentially similar activity to Extract III via the deoxyribose method. However, irrespective of the method chosen, Extract II exhibited the lowest activity.

DNA oxidative damage inhibition

Some authors (Muñiz and others 2001; Rivero and others 2005) have shown the usefulness of using DNA scission as an effective method to assess the antioxidant activity against active oxygen species *in vitro*. Both ascorbic acid and copper may induce damage of DNA, because they produce highly reactive oxygen species (viz. hydroxyl radicals) which have been implicated in degradation of DNA (Halliwell and Aruoma 1991), thus producing DNA strand breaks that can be adequately visualized by electrophoresis. The effects of concentration of each of our 3 extracts on DNA degradation are depicted in Figure 2, which conveys electrophoretic separation of its fragments. The results obtained pertaining to Extract I (lanes 3, 4, and 5) indicate that the protective effect took place only at 100% (v/v) concentration. At the lower concentrations tested, Extract I was not able to protect DNA, which was consequently degraded, with fragments presenting a pattern similar to that of the control with Cu(II) and ascorbic acid (lane 2). Likewise, almost no protection of DNA was observed in the case of Extract II (lanes 6, 7, and 8), even at the highest concentration tested. In what concerns Extract III (lanes 9, 10, and 11), a protective effect was already observed at 60% (v/v) concentration, which became obviously stronger at 100% (v/v).

The fact that Extracts I and III were able to protect against DNA oxidative damage is consistent with their high overall antioxidant

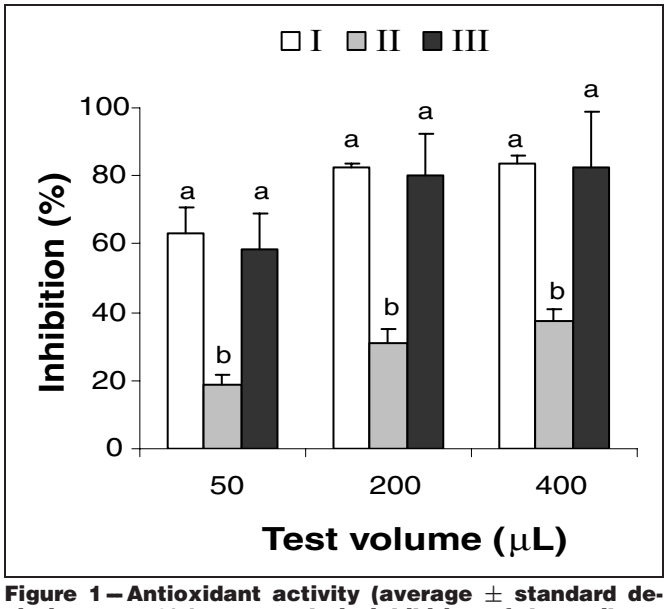

Figure 1—Antioxidant activity (average ± standard deviation, $n = 3/4$) assessed via inhibition of deoxyribose degradation (percent) for distinct assay volumes of each of the 3 extracts obtained from sugar molasses via ion exchange chromatography: I (fractions 2 to 9), II (fractions 10 to 24), and III (fractions 25 to 33). Within the same test volume, values followed by different letters are significantly different ($P < 0.05$).

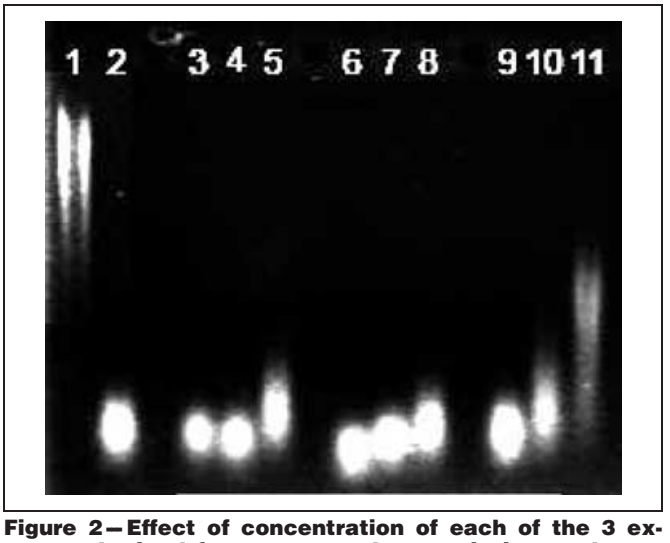

Figure 2—Effect of concentration of each of the 3 extracts obtained from sugar molasses via ion exchange chromatography (I— fractions 2 to 9; II— fractions 10 to 24, and III— fractions 25 to 33), on DNA oxidative damage [induced by Cu(II)-ascorbic acid], assessed by agarose gel electrophoresis. DNA alone (lane 1); DNA + Cu(II)-ascorbic acid (lane 2); DNA + Cu(II)-ascorbic acid + Extract I, at 40% (v/v) (lane 3), or 60% (v/v) (lane 4), or 100% (v/v) (lane 5); DNA + Cu(II)-ascorbic acid + Extract II, at 40% (v/v) (lane 6), or 60% (v/v) (lane 7), or 100% (v/v) (lane 8); and DNA + Cu(II)-ascorbic acid + Extract III, at 40% (v/v) (lane 9), or 60% (v/v) (lane 10), or 100% (v/v) (lane 11).

activity and in full agreement with their free radical scavenger activity as well.

Phenolic compound analysis

Phenolic compounds are known to possess antioxidant activity in general—hence, the phenolic composition of our extracts was actively sought. The resulting profiles obtained by HPLC are shown in Figure 3. Those compounds that underwent positive identification were then quantified in the 3 extracts.

The major phenolic compound in Extract I was syringic acid (peak 4, Figure 3. I), at a concentration of 2.86 mg/L. Syringic acid was also present in Extract II (Figure 3. II), but at an even higher concentration (4.72 mg/L), together with *p*-hydroxybenzoic acid (peak 1) and vanillic acid (peak 3) at 1.84 and 2.84 mg/L, respectively. The phenolic compounds detected in Extract III (Figure 3. III) were *p*-hydroxybenzoic acid (peak 1), *p*-hydroxybenzaldehyde (peak 2), and ferulic acid (peak 5) at concentrations of 0.82, 0.36, and 0.19 mg/L, respectively. Although several other peaks appeared in the HPLC chromatogram of Extract III, they could not be identified beyond doubt; however, the peak eluted at 42.09 min exhibited a UV-Vis spectrum which resembled that of ferulic acid.

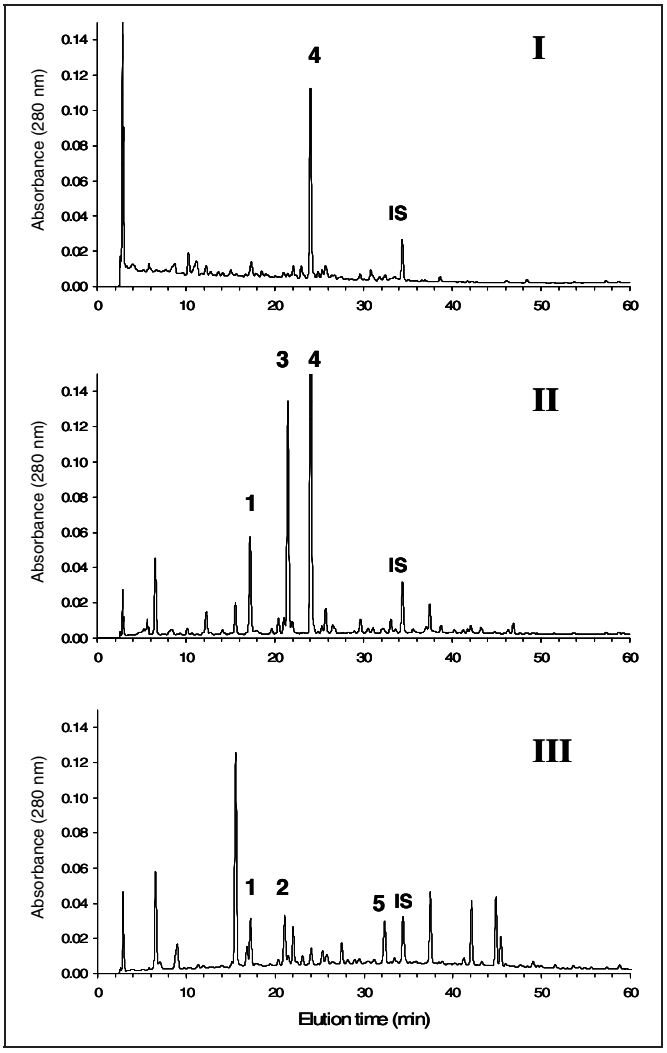

Figure 3—Elution profile, via HPLC, of the 3 extracts obtained from sugar molasses via ion exchange chromatography (I— fractions 2 to 9; II— fractions 10 to 24; and III— fractions 25 to 33), using methanol/formic acid/water as eluant and detection by absorbance (at 280 nm). Phenolic compounds identified are as follows: (1) *p*-hydroxybenzoic acid; (2) *p*-hydroxybenzaldehyde; (3) vanillic acid; (4) syringic acid; and (5) ferulic acid. IS—internal standard.

The aforementioned compounds have already been reported in sugar cane extracts obtained in the 1st step of sugar processing (Kort 1979); they are indeed released when cane is crushed, and can consequently be found in a wide range of cane sugar products. Ferulic acid, *p*-hydroxybenzoic acid, syringic acid, and vanillic acid were also found in oat and wheat bran extracts (Martínez-Tomé and others 2004), and they apparently contribute, at least in part, to the overall antioxidant capacity of said extracts.

Recall that Extract I contains mainly syringic acid, but at a lower concentration than in Extract II, which also presents other phenolic compounds to relevant levels. However, Extract I showed higher antioxidant activity than Extract II. Guimarães and others (1996) claimed that, among the sugar colorants in molasses, melanoidins are eluted by ion exchange chromatography at lower NaCl concentrations; therefore, Extract I would probably contain also melanoidins, which agrees with the elution profile recorded by absorbance during extraction of molasses via ion exchange chromatography (data not shown). Several authors (Borreli and others 2002; Wagner and others 2002) have suggested that melanoidins account for the strong antioxidant properties of some foods and beverages, which may provide a further rationale for the different features of Extracts I and II stated above. In addition, melanoidins may improve the defense system of living organisms (Faist and Erbersdobler 2001). In what concerns Extract III, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, and ferulic acid may account (as a set) for its high antioxidant activity. However, the possible contribution of other phenolic compounds not identified in this study cannot be ruled out. Hence, further studies are in order to clarify which components are actually responsible for the observed antioxidant features of the extracts.

Positive contributions to many physiological functions have been claimed for the phenolic compounds identified in our experimental extracts: ferulic, syringic, and vanilic acids have been described to possess antioxidant, antimicrobial, anti-inflammatory, and anticancer activities; and antioxidant and antimicrobial activities were associated as well with *p*-hydroxybenzoic acid (Aziz and others 1998; Losso 2002; Kampa and others 2004; Ou and Kwok 2004). As such, the composition of our molasses extracts in terms of those compounds would already support potential upgrade of such a byproduct for eventual consideration as functional ingredient.

Knowledge on the bioavailability of the aforementioned phenolic acids is also necessary to assess their actual biological effect and hence provide a rationale for the significance of our research effort. At present, there is a lack of comprehensive information on absorption, pharmacokinetics, metabolism, and excretion of polyphenols in general and phenolic acids in particular in the human body (Manach and others 2004). Yet, some authors suggest that phenolic acids, including ferulic, syringic, vanillic, and *p*-hydroxybenzoic acids, can be bioavailable in selected food matrices. Bourne and Rice-Evans (1998) provided evidence for absorption and bioavailability of ferulic acid in humans, following consumption of tomato via monitoring the pharmacokinetics of excretion; the peak time for maximum urinary excretion was approximately 7 h and recovery of ferulic acid in urine was in the range 11% to 25% of that ingested. Furthermore, using Wistar rats fed with a diet enriched in ferulic acid, Adam and others (2002) reported that 56% of the perfused ferulic acid was absorbed through the small intestine. On the other hand, the bioavailability of phenolic acids from oat was examined in hamsters by Chen and others (2004); the oral dose administered was 0.03, 0.10, 0.13, and 0.50 μmol for *p*-hydroxybenzoic,

syringic, vanillic, and ferulic acid, respectively. Those authors found that such phenolics were indeed absorbed to considerable degrees and that peak concentrations appeared in plasma by 40 min and ranged from 0.10 to 1.20 μmol/L. Likewise, Germano and others (2006) reported high levels of phenolic acids (ferulic, syringic, and vanillic acids) in plasma of rats by 15 min following oral administration of extracts rich in said compounds; this study also confirmed greater bioavailability of free than of bound forms of phenolic acids.

As far as our bibliographic search could go, this is the 1st report of a substantial antioxidant activity and a specific protective effect against DNA oxidative damage that is recorded in sugar molasses, which adds to the potential commercial interest of upgrading this byproduct. However, commercial incorporation of such extracts in dietary supplements or trade as natural antioxidant ingredients still requires further studies on their actual bioavailability in the specific (formulated) matrices in question, as well as in vivo activity, toxicity, and stability tests.

Conclusions

Molasses constitutes an interesting and nonexpensive source of antioxidants that is available in large amounts as a byproduct of the cane sugar industry. Functional features thereof include protective effect against DNA oxidative damage—besides radical scavenger capacity, which may be accounted at least in part by syringic acid, *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, and ferulic acid.

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