Evaluation of interactions between coffee and cardamom, their type, and strength in relation to interactions in a model system

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ABSTRACT
This paper presents a study on determination of antiradical potential, ferric-reducing antioxidant power (FRAP), chelating power (CHEL), OH- scavenging capacity (OH), superoxide dismutase-like activity, lipoxigenase (LOXi), and xanthine oxidase (XOi) inhibitory potential and also interactions between the phytochemicals from coffee and cardamom based on their bioaccessibility in vitro. Evaluation of interactions between coffee and cardamom in a model system showed that phenolic compounds may be responsible for the analyzed activity of the tested extracts. It was observed for FRAP, CHEL, and XOi that raw and digested extracts showed the same interactions as chemical standards. However, the LOX inhibitors present in raw extracts acted synergistically like chemical standards, but due to the changes during the simulated digestion process the kind of interaction between active compounds changed. Correlation between tested extracts and model system, despite the high bioaccessibility of the compounds with this capacity, was not only found for OH-radical neutralization.

RESUMEN
Este trabajo presenta un estudio acerca de la determinación del potencial antiradical (ABTS), el potencial reductor férrico antioxidante (FRAP), el poder quelante (CHEL), la capacidad de barrido de OH- (OH), la actividad de SOD, la lipoxigenasa (LOXi) y el poder inhibidor de xantina oxidasa (XOi), además de las interacciones entre los fitoquímicos del café y el cardamomo en base a su bioaccesibilidad in vitro. La evaluación de las interacciones entre el café y el cardamomo en un sistema modelo mostró que los fenólicos podrían ser responsables de la actividad analizada en los extractos examinados. Se observó en FRAP, CHEL y XOi que los extractos crudos y digeridos mostraron las mismas interacciones que los estándares químicos. Sin embargo, los inhibidores de LOX presentes en los extractos crudos actuaron sinérgicamente como estándares químicos, aunque debido a cambios durante el proceso digestivo simulado, el tipo de interacción entre los compuestos cambió. La correlación entre los extractos examinados y el sistema modelo, a pesar de la alta bioaccesibilidad de los compuestos con esta capacidad, no se encontró únicamente en la neutralización de radicales OH.

Introduction
Nutrition is a fundamental constituent for a healthy lifestyle, and epidemiological evidence links diets rich in food from plants origin with healthy longevity (Bonaccio, Iacoviello, de Gaetano, & Moli-Sani Investigators, 2012). In agreement with the evidence, there is an increasing interest in the so-called functional food. The investigation of the therapeutical effects of coffee has endured the same shortcomings that plague most of the whole plant research paradigm. The standard scientific model has failed to consider coffee as a whole plant complex as it is not divisible into single chemical isolates. Just as studying the benefits of beta-carotene is not the same as studying the benefits of eating a carrot, studying the properties of caffeine is not the same as understanding what makes coffee a useful medicinal plant. Divergent thinking, as opposed to the convergent analysis of medicinal plants, provides the foundation for the discovery of new and synergistic constituent blends that may affect the physiology of human health (Meletis, 2006). Similar to other plant-derived products, coffee is rich in polyphenols. These compounds are classified into flavonoids, phenolic acids, lignans, and stilbenes with a considerable antioxidant potential (Wang & Ho, 2009). The most common polyphenols in coffee are phenolic acids, especially hydroxycinnamic acids, mainly consisting of caffeic acid, ferulic acid, and quinic acid. The resulting forms include caffeoylquinic acids (CQA) and feruloylquinic acids that may be found in several isomeric forms based on the position of the ester link. The most common form is 5-O-CQA, which is often called chlorogenic acid (CGA). The particular profile of the compounds depends on the coffee variety, roasting, and processing (Clifford, 1999). There has been an increasing interest in using natural ingredients in foods and beverages. Consumers like to have increasingly flavored food products which contain natural ingredients due to

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concerns over adverse health effects of synthetic raw materials particularly some synthetic antioxidants. Presently, there is an increasing interest both in the industry and in the scientific research for spices and aromatic herbs due to their strong antioxidant properties (Deepa, Ayesha, Nishtha, & Thankamani, 2013). Spices and herbs are rich sources of powerful antioxidants and have been used as whole or ground spice/herb, extracts, encapsulated, or emulsions (Emboscado, 2015).

In this work, we have proposed cardamom as a functional additive to coffee beverage. Fruit of *Elettaria cardamomum* is used as a spice throughout the world. Also its wide use for culinary purpose, cardamom has folkloric repute as carminative, stomachic, diuretic, abortifacent, antibacterial, antiviral, and antifungal, and also it is considered useful in treatment of constipation, colic, diarrhea, dyspepsia, vomiting, headache, epilepsy, and cardiovascular diseases (Duke, Bogenschutz-Godwin, DuCelliar, & Duke, 2002). Its seed powder (3.5–7 g) is frequently prescribed in the treatment of gastrointestinal disorders and is used as stomachic, resolvent, retentive, digestive, antiemetic, and carminative. It has also been used in the treatment of acid peptic disorders and gastritis. Literature survey revealed that the fruit of *E. cardamomum* contains essential oil (EO), sterol, phenolic acids, and lipids (Jamal, Javed, Aslama, & Jafri, 2006). The main phenolic compounds present in cardamom are vanillic acid, caffeic acid, p-hydroxybenzoic acid, gentisic acid, protocatechuic acid, and p-coumaric acid (Varyjar, Bandyopadhyay, & Thomas, 1998).

Many studies on understanding the antioxidant potential of phenolic compounds in fruits, vegetables, and different beverages have concluded that it is impossible to predict the antioxidant power of a given product by just studying one type of compound contained in the product. In some cases, the possible existence of synergic or antagonistic effects between the various antioxidants in the plant foods and derived products has been postulated (Garcia-Alonso, Rimbach, Rivas-Gonzalo, & de Pascual-Teresa, 2004; Vinson, Su, Zubik, & Bose, 2001). In general, all the results for pure chemical compounds from the group of phenolics suggest high antioxidant activity. Although the *in vitro* antioxidant properties of isolated polyphenols have already been well documented (Rivero-Pérez, Muñiz, & González-Sanjose, 2008), in this paper, we will extend this knowledge to analyze the antioxidant properties produced by the interaction of coffee and cardamom water extracts for synergistic or antagonistic effects. Furthermore, the *in vitro* digestion process evaluates the bioaccessibility of the active compounds and provides information on whether and how the digestion processes may modulate the biological activity of phenolic compounds and the type of interaction between them. A comprehensive assessment of the interaction between the main active ingredients of the tested materials will provide input about their effect on developing physiological activity of the supplemented coffee.

**Materials and methods**

**Chemicals**

Ferrozine (3-(2-pyridyl)-5,6-bis-(4-phenyl-sulfonic acid)-1,2,4-triazine), ABTS (2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), pancreatin, pepsin (EC 3.4.23.1), bile extract, CGA, vanillic acid, xanthine oxidase (XO), lipoygenase, linoleic acid, xanthine were purchased from Sigma-Aldrich Company (Pozań, Poland). Acetonitrile and methanol gradient HPLC grade and formic acid LC–MS grade for LC–UV–MS separations were purchased from J. T. Baker (Phillipsburg, NJ). Water was purified in-house with a Milli-Q water purification system Simplicity-185 (Millipore Co.). All other chemicals were of analytical grade.

**Materials**

Roasted coffee (*Coffea arabica*) and cardamom in a form of ground spice were bought in the local market (Lublin, Poland).

**Extraction procedures**

For extraction of water-soluble antioxidants and phenolics 8 mL of boiling water were poured over 0.5 g of coffee and cardamom, the samples were shaken for 30 min at 37°C. After centrifugation (15 min, 8000g), the supernatants were decanted from the precipitate, and the final volume was brought to 10 mL with distilled water. The final extract concentration was 50 mg dry weight (DW)/mL. The coffee brew and the cardamom brew – the aromatic supplement – were analyzed separately and in appropriate combinations (4:1; 3:2; 1:1; 2:3; 1:4 v/v).

For bioaccessibility studies, simulated gastrointestinal digestion *in vitro* was performed according to the procedures described by Gawlik-Dziki (2012). The water extracts of coffee and cardamom and their mixtures (10 mL) were adjusted to pH 1.2 using 5 mol/L HCl, and subsequently, 0.75 mL of simulated gastric fluid (300 U/mL of pepsin in 0.03 mol/L NaCl, pH 1.2) was added. The samples were shaken for 120 min at 37°C. After that, the samples were adjusted to pH 6 with 0.1 mol/L of NaHCO₃ and then 3.75 mL of the simulated intestinal juice (prepared by dissolving 0.05 g of pancreatin (activity equivalent 4 × USP) and 0.3 g of bile extract in 35 mL 0.1 mol/L NaHCO₃ was added). The extracts were adjusted to pH 7 with 1 mol/L NaOH and finally 5 mL of 120 mmol/L NaCl and 5 mL of mmol/L KCl were added. The prepared samples were submitted for *in vitro* digestion for 60 min, at 37°C in the darkness. After that, the samples were centrifuged and supernatants (extracts after simulated digestion) were used for further analysis.

Model compound solutions were prepared in 50% ethanol and the final concentration was 50 µg/mL. The CGA solution and the solution of vanillic acid were analyzed separately and in volumetric combinations (4:1; 3:2; 1:1; 2:3; 1:4 v/v) CGA/vanillic acid.

**Analysis of bioactive compounds**

**Total phenolic compounds**

The amount of total polyphenols in the extracts was determined by the use of Folin–Ciocalteu reagent (Singleton & Rossi, 1965). A 0.1-mL of the extract was mixed with 0.1 mL of H₂O₂, 0.4 mL of Folin reagent (1:5 H₂O), and after 3 min with 2 mL of 10% Na₂CO₃. After 30 min the absorbance was measured using a UV–vis spectrophotometer at the wavelength of 725 nm. Gallic acid was employed as a calibration standard and TPC was expressed in terms of mg gallic acid equivalents (GAE)/g dry matter (DM). The linearity range for this assay was determined as 100–500 µg GA/mL ($R^2 = 0.995$), giving an absorbance range of 0.350–1.722 AU.
Ultra-performance liquid chromatography

Ultra-performance liquid chromatography (UPLC)/MS methods were used to identification of main phenolic compounds present in water extracts of coffee and cardamom. The brew was concentrated using a vacuum evaporator. However, for qualitative analysis of cardamom, 100 mg both fresh and digested sample were subjected to alkaline hydrolysis (5 mL of 0.2 M NaOH + 0.05% ascorbic acid, 2 h), carried out at room temperature in the dark. The samples were subsequently acidified with 2-M HCl to achieve a pH value of about 2, and laberated phenolic acids were extracted with ethyl acetate (3 x 5 mL). The organic extracts were dried with an evaporator under reduced pressure, dissolved in 50% methanol and analyzed to identify phenolic acids.

UPLC–DAD–ESI–MS analyses were performed using a Waters ACQUITY UPLC® BEH C18 column (100 × 2.1 mm, 1.7 µm), maintained at 40°C. The following gradient of solvent A (water with 0.1% FA, v/v) and solvent B (in acetonitrile with 0.1% FA, v/v) was used to elute analytes: 0–1.00 min, 5% B; 1.00–24.00 min, 5–50% B; 24.00–25.00 min, 50–95% B; 25.00–27.00 min, 95% B; 27.00–27.10 min, 95–5% B; 27.10–30.00 min, 5% B; the flow rate was 0.4 mL/min. ESI–MS analyses were performed using negative ionization mode and the identity of compounds was based on their UV and MS spectra.

Analyzes of antioxidant activities

The potential protective activities of coffee and cardamom extracts against the reactive oxygen species (ROS) were determined by using several methods. The antiradical activity was analyzed according to Re et al. (1999) using an improved ABTS decolorization assay, whereas ferric-reducing power was determined based on the approach proposed by Oyaizu (1986) and chelating power (CHEL) was determined using the method of Guo, Lee, Chiang, Lin, and Chang (2011). OH–scavenging assay was performed according to the procedure described by Su, Wang, and Liu (2009). Hydroxyl radicals were generated using Fenton reaction in the system of FeSO₄ and H₂O₂. In turn, assay for superoxide dismutase-like activity (superoxide dismutase; SOD–like) was based on the method described by Marklund and Marklund (1974). The inhibitory effect on lipooxygenase (LOX) activity was assessed (spectrophotometrically at 20°C by measuring the increase in absorbance at 234 nm over a 3-min period) according to Axelroad, Cheesborough, and Laakso (1981), while the inhibition of xanthine oxidase (XOI) with xanthine as a substrate was measured spectrophotometrically based on the procedure reported by Sweeney, Wyllie, Shalliker, and Markhan (2001) with the following modification: the assay mixture consisted of 0.5 mL of test solution, 1.3 mL of 1/15 mol/L phosphate buffer (pH 7.5), and 0.2 mL of enzyme solution (0.01 U/mL in M/15 phosphate buffer). After preincubation of the mixture at 30°C for 10 min, the reaction was initiated by adding 1.5 mL of 0.15 mmol/L xanthine solution. The assay mixture was incubated at 30°C, and the absorbance (295 nm) was measured over a 3-min period.

Antioxidant activities (except reducing power) were determined as EC50 – the extract concentration (mg DW/mL) which provided 50% of the activity based on a dose-dependent mode of action. Reducing power determined as EC50 is the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis.

Calculations

The following factors were determined for better understanding of the relationships between biologically active compounds in the light of their bioaccessibility:

- Phenolics bioaccessibility factor (ACP), which is an indication of the bioaccessibility of phenolic compounds

\[ ACP = \frac{C_D}{C_R} \]

where \( C_D \) = concentration of compounds after simulated gastrointestinal digestion, and \( C_R \) = concentration of compounds after water extraction (raw extract).

- The antioxidant bioaccessibility index (BAC), which is an indication of the bioaccessibility of antioxidative compounds

\[ BAC = \frac{A_R}{A_D} \]

where \( A_D \) = EC50 of extract after simulated gastrointestinal digestion and \( A_R \) = EC50 of raw extract (after water extraction).

- The interaction factor (IF), which provides an explanation for the mode of interaction

\[ IF = \frac{A_M}{A_I} \]

where \( A_M \) = measured activity of a mixture of samples and \( A_I \) = theoretically calculated mixture activity (based on the dose response of single components at various concentrations).

IF value <1 indicates synergistic interaction; IF > 1 indicates antagonism; IF = 1 indicates additional interactions.

Consumer acceptance test of coffee enriched with cardamom

A total of 50 g of coffee brews (control and coffee with different cardamom doses) were brewed with 1200 mL of water. The samples were served at a temperature range from 70 to 60°C after cooling at room temperature. The consumer acceptance test on the coffee beverages was conducted with 50 coffee drinkers (23 males and 27 females, ages 22–30). Consumers rated smell, bitter taste, acid taste, spicy taste, and the overall quality. The 9-point hedonic scale test (1 was the worst and 9 was the best) was used to evaluate all coffee samples.

The isobolographic analysis of interactions

The dose-normalized isobologram described by Chou (2006) were used in order to determine interactions between bioactive compounds of coffee and cardamom (raw and digested extracts) in respect to pure chemical standards identified as main phenolic extracts present in the analyzed samples.

Statistical analysis

The experimental results were mean ± SD of three parallel experiments (n = 9). Statistical analysis was performed using STATISTICA 7.0 for mean comparison using Tukey’s test at the significance level α = 0.05.
Results and discussion

Qualitative–quantitative analysis of phenolics

Classically, CGAs are a family of esters formed between certain trans-cinnamic acids and quinic acid (1L-1OH,3,4/5-tetrahydroxycyclohexane carboxylic acid), which has axial hydroxyls on carbons 1 and 3 and equatorial hydroxyls on carbons 4 and 5. During the process, trans-isomers may be partially converted into cis (Clifford, 1999). Coffee beans are one of the richest dietary sources of CGA, and for many consumers, it seems to be the major dietary source. The CGA in coffee and coffee products have been extensively reviewed (Clifford, 1998). In this study, isomer 5-CQA was determined to be the highest content, followed by 4-CQA, and finally the 3-CQA (Table 1). These results are similar to the results obtained by Fuijoka and Shibamoto (2008), who studied the content of CGA in various commercial coffee brews and detected CGA isomers in the same decreasing order (5-CQA > 4-CQA > 3-CQA). Also Oliveira-Neto et al. (2016) studied the main phenolic compounds in selected coffee samples: soluble granules, power for infusion, capsules for espresso which were subclassified into ‘lungo’ or ‘lunghí’ (L), regular (R), and decaffeinated (d). The highest content of CGA was identified in capsules for espresso (89.03 ± 0.85 mg/g). The coffee variety used in our study contained 34.93 ± 2.36 mg/g of CGA in water extract, and this value is similar to those obtained for soluble granules in the study by the above-cited authors. In turn, Belguidoum, Amira-Guebailla, Boulmokh, and Houache (2014) showed in their results that CGA level varied from 2815.0 ± 54.0 to 1010.0 ± 9.0 mg/100 g DW with the highest values found for green coffee samples. CGA concentration in unpackaged roasted Arabica coffee was 1189.0 ± 40.6 mg/100 g DW, which was lower than the value found for Robusta variety 1517.2 ± 24.6 mg/100 g DW, and much lower value was found for Arabica coffee tested in our study. The CGA content in brewed coffee may be affected by the kind of coffee beans used as Arabica beans contain less CGA than Robusta beans (Ký et al., 2001). The roasting method used might also play an important role in the CGA content of the final coffee product. For example, the light medium roasting condition resulted in the highest amount of transformation from CGA to the corresponding lactones, suggesting that this process reduced the amount of CGA in coffee (Farah, De Paulis, Trugo, & Martin, 2005). We have also observed a change in the quantity and quality of the phenolic compounds after the in vitro digestion process. The concentrations of all phenolic compounds were higher in water extract than in digested extract. Furthermore, extracts of coffee after digestion did not contain six compounds that were identified in water extract. Coffee extracts contained substantial amounts of caffeine: 41.72 ± 2.67 mg/g DM before and 27.44 ± 0.94 mg/g DM after digestion in vitro (Table 1). The CGA (5-CQA) was adopted as a model compound for coffee because it was the dominant compound from the hydroxycinnamic acid family in coffee extracts. These observations are consistent with those published in the work of Budryn, Zaczýriska, and Rachwal-Rosiak (2016).

Natural spices and their derivative products, EOs and extracts, are known to have a wide spectrum of biological activity; moreover, these products are the only natural flavorings available (Charles, 2013). The addition of spices to food is common and makes it possible to significantly improve the organoleptic characteristics of the products, diversify their assortment, taste and flavor, and enrich them with the natural preservatives and antioxidants. The use of spices increases the shelf life of products due to the decrease in microbiological activity and intensity of lipid oxidation. Synthetic antioxidants (AOs), butylated hydroxytoluene or butylated hydroxyanisole, are commonly used in the food industry. However, these compounds are found to be dangerous for health, and the search for harmless and effective natural AOs is a promising challenge. Misharina (2016) studied the antiradical properties of EOs and extract from cardamom. This author noticed that the total content of compounds with high antiradical activity in cardamom EO was twice higher than that in the cardamom extract; the antiradical efficiency value for the oil was 3.2 times higher than that for the extract. Phenols and flavonoids were found in cardamom extract; thus, the treatment of the compound with cardamom extract resulted in increased activity of glutathione S-transferase and reduced the oxidation level of lipids in liver and plasma of mice (Suganthi, Rajamani, Ravichandran, & Anuradha, 2007; Bhattacharjee, Rana, & Sengupta, 2007). In our study, we have identified four phenolic compounds in the water extract of cardamom: protocatechuic acid, vanillic acid, p-coumaric acid, and ferulic acid. Our findings are in accordance with Vairiyar et al. (1998), who identified p-coumaric acid, protocatechuic acid, and caffeic acid in cardamom samples. The vanillic acid was adopted as a dominant hydroxycinnameric acid, since its concentration was the highest and this value increased after in vitro digestion.

Furthermore, the total phenol content (TPC) shown in Table 1 was necessary to evaluate the ACP. This factor was calculated in foothold of results obtained for water extracts, and thus, it may show the extraction efficiency of the

### Table 1

| Table 1. Analysis qualitative-quantitative of the compounds fenólicos del café y el cardamomo. |
|--------------------------------------------------|
| Water extract | Digested extract |
| Coffee | | |
| Compound concentration (mg/g DM) (n = 3) | | |
| 3-Caffeoylquinic acid | 18.82 ± 1.37 | 6.03 ± 0.20 |
| 5-Caffeoylquinic acid | 34.91 ± 2.36 | 6.00 ± 0.29 |
| 4-Caffeoylquinic acid | 20.77 ± 1.50 | 5.25 ± 0.20 |
| Caffeine | 41.72 ± 2.67 | 27.44 ± 0.94 |
| 3-Feruloylquinic acid | 6.82 ± 0.65 | 4.20 ± 0.28 |
| 5-Feruloylquinic acid | 19.94 ± 1.37 | 8.27 ± 0.37 |
| 3-Caffeoylquinic-1,5-lactone | 6.45 ± 0.47 | – |
| 4-Caffeoylquinic-1,5-lactone | 1.78 ± 0.26 | – |
| 3-Feruloylquinic-1,5-lactone | 1.59 ± 0.33 | – |
| 3,4-Dicaffeoylquinic acid | 3.20 ± 0.35 | – |
| 3,5-Dicaffeoylquinic acid | 1.18 ± 0.17 | – |
| 4,5-Dicaffeoylquinic acid | 1.67 ± 0.11 | – |
| Total phenolic content (mg GAE/g DM) (n = 6) | 47.16 ± 2.36 | 41.92 ± 2.09 |
| ACP = 0.88 | | |
| Cardamom | | |
| Protocatechuic acid | 0.213 ± 0.003 | 0.117 ± 0.019 |
| Vanillic acid | 0.834 ± 0.018 | 1.140 ± 0.052 |
| p-Coumaric acid | 0.389 ± 0.025 | 0.120 ± 0.026 |
| Ferulic acid | 0.685 ± 0.048 | 0.405 ± 0.062 |
| Total phenolic content (mg GAE/g DM) (n = 6) | 0.61 ± 0.03 | 1.74 ± 0.08 |
| ACP = 2.85 | | |

*Phenolics bioaccessibility factor (ACP), which is an indication of the bioaccessibility of phenolic compounds.*

*Factor de bioaccesibilidad fenólica (ACP), el cual es una indicación de la bioaccesibilidad de los compuestos fenólicos.*
simulated gastrointestinal tract. The value obtained for cardamom exceeds 1, which indicates a high potential bioaccessibility of phenolic compounds. Although Hinneburg, Dorman, and Hiltunen (2006) determined the total amount of phenolic compounds as 24.2 ± 0.29 mg GAE/g DM in cardamom extract, Przygoda, Zieleńska, Ciesarów, Kukurová, and Zieleński (2014) characterized it as a poor source of phenolics compared to other tested spices, and these findings are in agreement with our studies. In case of coffee, after in vitro digestion TPC decreased from 47.16 ± 2.36 to 41.92 ± 2.09 mg GAE/g DM and ACP was below 1, which showed the low bioaccessibility of the active compounds. It does not change the fact that the tested coffee contained high concentration of total phenolic compounds compared to the results obtained for Arabica roasted coffee samples by Belguidoum, Amira-Guebailla, Boulmokh and Houache (2014) (15.97 ± 0.93 mg/g DM).

**Antioxidant activity of potentially bioaccessible compounds**

The multidirectional antioxidant activity of coffee has been proved by many investigators (Budryn, Nebesny, Rachwal-Rosiak, & Oracz, 2013; Sato et al., 2011). Administration of antioxidants may therefore help to remove the ROS and improve the clinical outcome. It is identified that dietary antioxidants can enhance the cellular defense and help to prevent the oxidation damage to cellular components (Sato et al., 2011). Brewed coffee has been consumed for many centuries due to its stimulating properties and other beneficial health activities. As presented in this study, coffee and cardamom as aromatic supplement are excellent source of antioxidative compounds with multidirectional activity.

The antioxidant capacity of the tested samples determined against ABTS+ radical is shown in Table 2. The coffee extract was found as a better source of antiradical compounds compared to cardamom infusion. In both samples, it was noted that simulated gastrointestinal digestion caused an increase in the antiradical power. The highest activity was observed in coffee extract after digestion in vitro (EC50 = 1.49 ± 0.07 mg/mL). However, the BAC index indicates that compounds in cardamom with antiradical activity are more bioaccessible than the compounds from coffee extracts. Bioaccessibility determines the biological activity of phytochemicals defined as the amount of a food constituent released from the solid food matrix inside the gut which might be able to pass through the intestinal barrier (Manach et al., 2005). Most polyphenols cannot be absorbed in their native form as they are in the form of esters, glycosides, or polymers in food (D’Archivio et al., 2005). Before absorption, these compounds must be hydrolyzed either by intestinal enzymes or by colonic microflora. In general, only digestively stable aglycones are bioavailable, i.e. they can be absorbed in the small intestine. The effect of gastrointestinal digestion on the bioaccessibility of the compounds can be mimicked in vitro by extraction procedures reconstituting the conditions characteristic of the gastrointestinal tract. Such an in vitro approach enabled us to show that the conditions in the gastrointestinal tract may affect the amount and biological activity of the phenolic compounds from tested samples.

The CGA alone, as a pure chemical compound, showed ABTS+ radical scavenging activity and the EC50 value was 42.75 ± 2.14 µg/mL. On the other hand, vanillic acid, as a model compound for cardamom extract, had a lower antioxidant capacity and the EC50 value was 83.22 ± 4.16 µg/mL (Figure 1). In complex systems, such as food and food preparations, various different mechanisms may contribute to the oxidative processes, such as in Fenton reactions, where transition metal ions play a vital role, different ROS might be generated, and various target structures such as lipids, proteins, and carbohydrates can be affected (Halliwell, 1997). The ferric-reducing antioxidant power (FRAP), CHEL, and ability of coffee and cardamom to scavange hydroxyl radicals under the given experimental conditions is listed in Table 2. While determining the FRAP, we obtained a very low EC50 value for coffee extract before digestion indicating a very high activity. However, after in vitro digestion EC50 value increased, which unfortunately indicates the low bioaccessibility of the compound characterized by the FRAP (BAC = 0.48). For cardamom, simulated gastrointestinal digestion caused an increase in FRAP, which indicates higher antioxidant bioaccessibility (BAC = 1.35) compared to coffee extract. Model compounds for coffee and cardamom also possessed FRAP: the EC50 value for CGA was 41.81 ± 2.09 µg/mL and for vanillic acid was 200.00 ± 0.99 µg/mL (Figure 1). Thus, a similar correlation was observed in this study for pure chemical standards as for tested extracts (Table 2).

### Table 2. Comparison of the antioxidant bioaccessibility index (BAC) for coffee and cardamom.

| Activity | Coffee | Cardamom |
|----------|--------|---------|
|          | A<sub>a</sub> | A<sub>b</sub> | A<sub>a</sub> | A<sub>b</sub> |
| ABTS     | 1.79 ± 0.09a | 1.49 ± 0.07b | 1.20 | 6.38 ± 0.34c |
| FRAP     | 0.58 ± 0.03a | 1.21 ± 0.06b | 0.48 | 5.07 ± 0.25d |
| CHEL     | 0.37 ± 0.02a | 1.37 ± 0.07b | 0.27 | 1.13 ± 0.07b |
| OH       | 0.77 ± 0.04a | 0.26 ± 0.04b | 2.96 | 0.31 ± 0.02b |
| SOD-like activity | 10.30 ± 0.52a | 14.96 ± 0.75b | 0.69 | 48.57 ± 2.43d |
| LOXI     | 4.58 ± 0.23a | 1.37 ± 0.07b | 3.34 | 1.67 ± 0.08d |
| XOI      | 2.74 ± 0.14a | 2.44 ± 0.12b | 1.12 | 2.48 ± 0.12b |

BAC: Bioaccessibility index; ABTS: antiradical potential; FRAP: ferric-reducing antioxidant power; CHEL: chelating power; OH: OH• scavenging assay; SOD: superoxide dismutase; LOXI: inhibition of lipoxygenase; XOI: inhibition of xanthine oxidase.

*<sub>a</sub> = EC50 (mg/mL) of raw extract (after water extraction).

*<sub>b</sub> = EC50 (mg/mL) of extract after simulated gastrointestinal digestion.

Means with different letter within a same row are significantly different (α < 0.05).
Table 2

|                | EC50 [µg/mL] |
|----------------|-------------|
| ABTS          | 22.66 ± 1.13 µg/mL for CGA and 22.28 ± 1.11 µg/mL for vanillic acid. |
| FRAP           | 48.57        |
| CHEL           | 0.31         |
| OH             | 0.77         |
| SOD-like       | 1.67 ± 0.08  |
| LOX           | 2.53 ± 0.13  |
| XO             | 1.45         |

Figure 1. Comparison of EC50 values for antiradical and anti-inflammatory activity of pure chemical standards: antiradical potential (ABTS), ferric reducing antioxidant power (FRAP), chelating power (CHEL), OH-scavenging assay (OH), SOD-like activity, inhibition of lipooxygenase (LOX), inhibition of xanthine oxidase (XO).

The ability of the tested extracts to chelate iron ions (Fe^{2+}) was also determined. Table 2 shows that both tested trials showed the ability to chelate iron ions. The highest activity, and thus the lowest EC50, was observed for coffee infusion before digestion. In both cases, in vitro digestion process caused a decrease in the ability to chelate iron ions (Fe^{2+}) and the antioxidant BACs were low: 0.27 for coffee and 0.38 for cardamom (Table 2). As shown in Figure 1, for pure chemical standards ability to chelate iron ions (Fe^{2+}) was almost the same: EC50 = 22.66 ± 1.13 µg/mL for CGA and EC50 = 22.28 ± 1.11 µg/mL for vanillic acid.

Then, the potential for OH radical neutralization was determined. Experience has shown that where mixtures are characterized by the highest activity (Table 2), EC50 values are the lowest (0.77 mg/mL for coffee, 0.26 mg/mL for coffee after digestion, and 0.31 mg/mL for digested cardamom); so, cardamom extract showed the highest EC50 value (1.45 mg/mL) and this sample least efficiently neutralized the hydroxyl radicals. The antioxidant BAC in OH-scavenging assay was almost two times higher in case of cardamom as compared to coffee. However, for both model compounds, values obtained for EC50 were similar (Figure 1).

Considering the raw extracts, SOD-like activity was much higher for coffee water extract EC50 = 10.30 mg/mL compared to cardamom extract EC50 = 62.82 mg/mL (Table 2). A similar correlation was observed in this study for pure chemical standards: CGA possessed higher SOD-like activity compared to vanillic acid (Figure 1). After digestion, coffee capacity decreased to an EC50 value of 14.96 mg/mL, but we have observed an increase in the activity of cardamom extract (EC50 = 48.57). Nevertheless, bioaccessibility of cardamom compounds with SOD-like activity was almost two times higher than that for coffee extract. SOD is an enzyme that acts as a catalyst for the process of dismutation of superoxide into non-ROS and hydrogen peroxide. Therefore, it is a critical antioxidant defense that is present in nearly all cells which are exposed to oxygen (Monk, Fagerstedt, & Crawford, 1989). SOD helps in neutralizing the toxic effects of free radicals (Gralla & Kosman, 1992), and therefore the discussed activity was presented as a way to neutralize superoxide radical.

Different studies have showed the existence of a close relationship between inflammation and synthesis of oxidative species; furthermore, plant species that show antioxidant properties tend to have anti-inflammatory properties (Radmark, Werz, Steinhilber, & Samuelsson, 2007). Therefore, we analyzed the effect of coffee and cardamom extracts on pro-inflammatory enzyme activity such as LOX and XO. Both extracts showed an inhibitory capacity of LOX activity, with EC50 values of 4.58 ± 0.23 mg/mL for water coffee extract and 2.53 ± 0.13 mg/mL for cardamom extract. This potency increased after digestion in both trials (EC50 of 1.37 ± 0.07 and 1.67 ± 0.08 mg/mL for coffee and cardamom, respectively), but the antioxidant BAC noted for coffee was higher than that for cardamom sample (Table 2). This enzyme has been related to chronic diseases such as rheumatic arthritis, bronchial asthma, and psoriasis; and studies have showed that it is overexpressed in malignant tumor tissues. Moreover, the regulation of LOX activity is associated with cyclic redox reaction of a non-heme iron in its active site. When the enzyme is at rest, the iron is in the reduced form (Fe^{2+}), but is quickly oxidized to its ferric state (Fe^{3+}) by hydroperoxides, which allows LOX to enter into a catalytic cycle in which the iron acts as electron acceptor and donor (Radmark et al., 2007). Most of the LOX inhibitors exert their action on the enzyme’s active site by chelating the iron or reducing it into its ferrous form or by scavenging electrons participating in the redox cycle of iron. Therefore, LOX inhibitors can be classified as redox active compounds, iron ligand inhibitors, and non-redox inhibitors (Werz & Steinhilber, 2005). Coffee and cardamom extracts exerted a strong iron CHEL (Table 2) with EC50 values lower than their corresponding EC50 for LOX enzyme. Therefore, we could suggest that the tested trials exert its action on LOX activity mostly by chelating the iron. Model compounds for coffee and cardamom were also LOX inhibitors: the EC50 value for CGA was 63.68 µg/mL and for vanillic acid was 83.32 µg/mL (Figure 1). Thus, a reverse correlation was observed as for raw extracts, but similar to digested extracts. The CGA was a more potent inhibitor of LOX than vanillic acid.

XO is a molybdenum (Mo)-containing enzyme which belongs to the Mo hydroxylase family of protein. XO controls the sequential hydroxylolation of hypoxanthine to xanthine then to uric acid, along with the generation of ROS. The over-activity of XO within the living system is because of the excess levels of blood uric acid (hyperuricemia), which is the underlying cause of gout. Thus, a specific inhibitor for XO may be a significant therapy for hyperuricemia, gout, and some other related diseases induced by the concomitant-free radicals including DNA damage, ischemia–reperfusion injury, heart attacks, stroke, and renal hypoxia (Lin, Zhang, Pan, & Gon, 2015). Phenolics and flavonoids may act as potent inhibitors against the metabolic enzymes such as XO and lipooxygenase (Hoorn et al., 2002). Thus, the phenolic constituents may play an essential role in the inhibition of XO, and these XO inhibitors and uricosuric agents are commonly used for treating and curing inflammatory diseases. The Allopurinol is the drug of choice against XO activity; however, it has serious side effects (Nile, Kumar, & Park, 2013). Thus, new alternatives with increased therapeutic activity and lesser side effects are desired. This study suggested that coffee and cardamom impart XOi and significantly reduce the risk of related inflammatory disorders.
As Table 2 shows, coffee water extract was noted as a better XO inhibitor; however, after digestion in vitro both trials, coffee and cardamom, showed a similar level of inhibitory activity (EC50 = 2.44 ± 0.12 and 2.48 ± 0.12 for coffee and cardamom, respectively). Considering the BAC index, it was much higher for bioactive compounds of cardamom. In their work, Nile, Ko, Kim, and Keum (2016) showed that the ferulic acid-related compounds showed a good to excellent activity profile toward XO inhibitory activity and formation of hydroperoxide. All of the 10 ferulic acid-related compounds showed XO-inhibiting activity among which ferulic acid, gallic acid, caffeic acid, p-coumaric acid, alkyl gallate, and methyl gallate showed an inhibition >50% at 100 μg/mL. In our study, CGA and vanillic acid were found as good XO inhibitors, EC50 values: 40.66 ± 2.03 and 49.39 ± 2.47 μg/mL for vanillic and CGA, respectively (Figure 1).

Bioaccessibility is a major factor that should be considered when assessing the potential health benefit of functional foods. As our results show the changes occurring during the simulated digestion process (variable pH conditions and/or action of digestive enzymes) affected the levels of the tested activities.

**Analysis of interactions**

The estimation of interactions between active compounds was carried out in two ways: isobolographic analysis and IF determination. As per Chou (2006), we used normalized isobolograms to suggest the interactions between tested samples with respect to interactions in the model system containing pure chemical compounds identified as main phenolics in the tested extracts of coffee and cardamom. When two compounds are combined and if compound A has an effect and compound B has no effect and if in combination they have an effect that is greater than that of compound A, then it is enhancement or potentiation. We can describe the effect simply as percent enhancement or fold of potentiation. If A and B alone each has an effect, then in combination they may produce a synergistic, an additive, or an antagonistic effect. By definition, synergism is an effect that is more than additive (isobole is concave), whereas the definition for antagonism (convex isobole) is an effect that is less than additive (isobole is a straight line).

The isobolographic analysis clearly showed that the water-extractable LOX inhibitors from coffee and cinnamon acted synergistically, whereas changes during the simulated digestion caused antagonism between them, and antagonism was observed for pure chemical standards (CGA and cinnamic acid) as well. These findings are in accordance with those obtained in this study. For coffee and cardamom raw extracts and for pure chemical standards, we have observed synergistic interactions, whereas extracts digested in vitro acted antagonistically (Figure 3(f)). Also IF values presented in Table 3 confirm these data. The reverse situation occurred for LOX inhibitors derived from coffee and cinnamon (Durak et al., 2015), where antagonism was noted for digested extracts and in the model system, but raw extracts acted antagonistically.

Another tested activity was the capacity to XOi. As Figure 3(g) shows, for coffee and cardamom raw and digested extracts, isoboles took a concave shape compared to pure chemical standards (chlorogenic and vanillic acid), which indicates a synergistic interaction. As noted in Table 3, the strongest synergism was noted for raw extracts (IF = 0.28) and the weakest for digested extracts (IF = 0.89).

This study showed that the IF proposed by Gawlik-Dziki (2012) enables studying interactions between any number of components and it is less complicated than isobolographic methods. Moreover, the ‘strength’ of interaction may be estimated approximately based on the IF value. In this study, we have shown that the type of interaction
determined from isobolographic analysis (Figure 2(a–g)) is confirmed by IF (Table 3). Therefore, as mentioned earlier, this index can be used in the preliminary studies of the interaction between two active ingredients.

Consumer acceptance test of coffee enriched with cardamom presented in Table 4 showed that the addition of cardamom at a level from 1% to 2.5% to coffee had no significant effect on its sensory attributes and consequently on consumer’s acceptance. However, cardamom at 0.5% level is the most acceptable for consumers.

**Conclusion**

In conclusion, our findings have shown that coffee and cardamom provide an excellent source of potentially bioaccessible compounds (especially those from hydroxycinnamic acids family) with multidirectional antioxidant activity. Particular attention should be paid to the results concerning the analysis of the interaction between the phenolic compounds contained in the coffee and cardamom raw and digested extracts compared to pure chemical standards (chlorogenic and vanillic acid), within the antioxidant and anti-inflammatory activities. It was observed in case of FRAP, CHEL, and XOi, where raw and digested extract showed the same kind of interactions as pure chemical standards. For antiradical capacity (ABTS) and SOD-like activity for digested extracts of coffee and cardamom, the kind of interaction was the same as in the model system. However, LOX inhibitors present in raw extracts acted synergistically like chemical standards, but the changes occurring during simulated digestion process affected on the kind of interaction between active compounds. Only for OH· radical neutralization there is no correlation between tested extracts and model system, though the high bioaccessibility of compound was seen with this capacity. The sensory characteristics linking results showed that addition of 0.5% of cardamom gives satisfactory overall consumer acceptability. To conclude, cardamom is an aromatic additive that could affect not only the flavor and aroma of coffee, but also its biological activities.
Table 3. Comparison of IF of mixtures of coffee with cardamom and pure chemical compounds identified in experimental material.

| Sample | Extract | Activity | Activity | Activity | Activity | IF |
|--------|---------|----------|----------|----------|----------|-----|
| Coffee/Cardamom mixture (1:1 v/v) | Raw | ABTS | 2.33 | 2.74 | 0.85 |
| | | FRAP | 1.05 | 3.71 | 0.28 |
| | | CHEL | 0.36 | 0.39 | 0.92 |
| | | OH | 2.35 | 1.11 | 2.12 |
| | | SOD-like activity | 16.69 | 36.56 | 0.46 |
| | | LOXI | 3.29 | 3.55 | 0.92 |
| | | XOI | 3.62 | 5.14 | 0.71 |
| | Digested | ABTS | 2.73 | 1.86 | 1.47 |
| | | FRAP | 1.83 | 3.14 | 0.58 |
| | | CHEL | 1.23 | 1.25 | 0.98 |
| | | OH | 0.29 | 0.29 | 1.00 |
| | | SOD-like activity | 34.45 | 20.77 | 1.66 |
| | | LOXI | 2.15 | 1.52 | 1.42 |
| | | XOI | 2.18 | 2.46 | 0.89 |

Chlorogenic acid/Vanillic acid mixture (1:1)^1

| Sample | Activity | Activity | Activity | Activity | Activity |
|--------|----------|----------|----------|----------|----------|
| ABTS | 97.45 | 62.98 | 1.55 |
| FRAP | 64.77 | 120.90 | 0.34 |
| CHEL | 21.97 | 22.37 | 0.98 |
| OH | 75.14 | 151.21 | 0.50 |
| Assay for SOD-like activity | 611.36 | 370.25 | 1.65 |
| LOXI | 62.72 | 73.50 | 0.85 |
| XOI | 36.03 | 45.03 | 0.80 |

IF: Interaction factors; ABTS: antiradical potential; FRAP: ferric-reducing antioxidant power; CHEL: chelating power; OH: OH scavenging assay; SOD: superoxide dismutase; LOXI: inhibition of lipoxygenase; XOI: inhibition of xanthine oxidase.

^1The measured activity of a mixture of samples (expressed as EC50 [mg/mL]).

^2The theoretically calculated mixture activity (based on the dose response of single components at various concentrations, expressed as EC50 [mg/mL]).

^3For pure chemical standards EC50 is expressed in μg/mL.

^4Actividad calculada de una mezcla de muestras (expresada como EC50 [mg/mL]).

^5La actividad de la mezcla calculada teóricamente (en base a la respuesta a la dosis de los componentes individuales a diferentes concentraciones – expresada como EC50 [mg/mL]).

^6Para los estándares químicos puros EC50 se expresa en [μg/mL].

Table 4. Consumer acceptance test of coffee enriched with cardamom.

| Sample | Smell | Acid taste | Bitter taste | Spicy taste | Overall |
|--------|-------|------------|-------------|-------------|---------|
| | C | 6.21 ± 0.99a | 5.13 ± 1.27a | 6.93 ± 0.81a | 4.77 ± 1.42a | 6.32 ± 0.57a |
| | C 0.5% | 6.39 ± 1.15b | 5.40 ± 1.10b | 6.57 ± 0.93b | 5.04 ± 1.20b | 6.75 ± 0.72b |
| | C 1% | 6.3 ± 1.2b | 5.22 ± 0.97c | 6.3 ± 1.05a | 5.41 ± 1.26c | 5.58 ± 1.02c |
| | C 1.5% | 6.21 ± 1.19a | 5.66 ± 1.14d | 6.39 ± 1.10c | 5.76 ± 1.28d | 5.54 ± 1.10c |
| | C 2% | 6.3 ± 1.05a | 5.58 ± 1.29e | 6.12 ± 0.99d | 5.76 ± 1.24e | 5.57 ± 0.83c |
| | C 2.5% | 6.66 ± 1.13c | 5.77 ± 1.35b | 6.12 ± 0.94c | 5.82 ± 1.33c | 5.38 ± 0.85c |
| | C 3% | 6.75 ± 1.16 | 6.03 ± 1.33g | 6.19 ± 1.15d | 6.93 ± 1.46e | 5.41 ± 1.15d |

C – control brew (coffee without cardamom), C 1%–C 5% – coffee with cardamom addition (from 1% to 5%, respectively)

Means with different letter (a–g) superscript within a same column are significantly different (p < 0.05).

The point scale was from 1 (the worst) to 9 (the best).

C – infusión control (café sin cardamomo), C 1%–C 5% – café con adición de cardamomo (de 1% a 5%, respectivamente)

Los promedios con distintas letras (a–g) en los superíndices en una misma columna son significativamente distintos (p < 0.05).

La escala de puntuación fue de 1 (la peor) a 9 (la mejor).

Moreover, main phenolic compounds identified in tested material in most cases showed the same kind of interaction as tested (raw and/or digested) samples. Therefore, these phe-nolic compounds possessed a very high effect on the kind of interaction observed between tested samples.

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