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ADDITION OF EVAPORATED MILK ALTERS ANTIOXDANT PROPERTIES OF MOST COMMONLY CONSUMED COFFEE IN SAUDI ARABIA

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ABSTRACT

Coffee is one of the most commonly consumed beverages worldwide. The aim of current study is to evaluate the effect of adding evaporated milk (full fat and low fat) on the antioxidant properties of coffee and to assess the possible alterations of polyphenols bioavailability when evaporated milk is added. To determine the effects of adding milk on the antioxidant activities of instant coffee, total polyphenols and flavonoids contents, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, hydrogen peroxide scavenging activity, ferric reducing power and ferrous ion chelating effect were determined. Total polyphenol and flavonoid contents were significantly higher ($p<0.0001$) in both coffee milk samples, coffee with full fat evaporated milk (CFEM) and coffee with low fat evaporated milk (CLEM), in comparison to pure coffee. Moreover, DPPH scavenging activity was significantly increased ($p<0.001$) in CFEM and CLEM whereas, hydrogen peroxide radical scavenging activity and ferric reducing power were significantly decreased ($p<0.001$) in CFEM and CLEM samples. In conclusion, our study showed that adding different concentrations of evaporated milk to instant coffee influences the bioavailability and efficacy of coffee antioxidants. This might be due to the presence of proteins and fats in milk.

Key words: antioxidant activity, instant coffee, evaporated milk, phenolic contents, radical scavenging

INTRODUCTION

Coffee is one of the most often consumed and most prevalent beverages in the world due to its stimulant effect on the central nervous system and for its desirable sensory flavour (Camargo *et al.,* 1999). Epidemiological and experimental studies have shown several positive effects of regular consumption of coffee on human health including psychoactive responses such as alertness and mood change, neurological conditions, metabolic disorders, gonad and liver functions (Dórea & Da Costa, 2005). These positive effects are usually referred to the existence of bioactive compounds with strong antioxidant and antiradical activities (Cämmerer & Kroh, 2006; Parras *et al.,* 2007), especially polyphenols (Pojjana *et al*., 2002). Antioxidants are chemical compounds meant to protect tissues against the damage caused by oxygen free radicals and lipid peroxidation (Ryan & Petit, 2010).

In the Kingdom of Saudi Arabia, coffee brews are usually prepared in different ways, and they are often consumed with the addition of milk. In general, the major components of milk are water, fat, casein, whey proteins, lactose and some minerals (Walstra & Jenness, 1984). Yuksel *et al.* (2010) studied the ability of polyphenols to interact with dietary proteins, including caseins and whey proteins, indicating that these compounds may form polyphenol–protein complexes which affect the bioactive composition of coffee brews. Furthermore, some authors have proposed that the effect on the total antioxidant activity may be related to the fat content of milk (Ryan & Petit, 2010). Based on this, the aim of this study is to evaluate the effect of adding different concentrations of evaporated milk on the antioxidant properties of coffee and to assess the possible alterations of polyphenols bioavailability when consumed with food matrix containing proteins and fat such as milk.

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MATERIALS AND METHODS

Chemicals and reagents

DPPH (2,2-diphenyl-1-picrylhydrazyl), catechin, ethanol, gallic acid, iron(II) chloride, methanol, ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p2-disulfonic acid monosodium salt hydrate), sodium nitrite and TCA (trichloroacetic acid) were obtained from Sigma Aldrich, United States. Di-sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate and sodium carbonate were purchased from CDH, India. Folin-Ciocalteu's reagent and iron (III) chloride were obtained from BDH limited Poole, England. Hydrogen peroxide was supplied by Asaggaf Pharma Holyland, KSA. Potassium ferricyanide was purchased from Koch-light laboratories, England. Aluminium chloride was supplied from Labo Chemie PVT Ltd, India and sodium hydroxide pellets were purchased from AppliChem Paureac, USA. Phosphate buffer saline pellets were supplied by Oxoid, England.

Coffee beverages preparation

Instant coffee (Nescafé red mug) and both types of evaporated milk (full fat and low fat milk) were purchased from Saudi local market. Coffee-milk samples were prepared as commonly drunk in Saudi Arabia. Firstly, coffee stock was prepared by dissolving 2 g of instant coffee in 200 mL of boiling water. Coffee with full fat evaporated milk (CFEM) beverage was prepared by mixing 20 mL of full fat evaporated milk with 80 mL of coffee stock whereas coffee with low fat evaporated milk (CLEM) beverage was prepared by mixing 20 mL of low fat evaporated milk with 80 mL of coffee stock.

Estimation of total phenolic contents

Total phenolic content was determined by Folin-Ciocalteu's reagent method with minor modification (Singelton *et al.,* 1999). Each coffee sample (0.5 mL) was diluted with 5 mL distilled water and mixed with 0.5 mL of Folin-Ciocalteu's reagent. After 5 min, 1 mL of Na_2CO3 (2%) was added to the mixture and was kept in dark place for an hour. Then the absorbance was measured spectrophotometrically at 750 nm and a calibration curve was constructed with different concentrations of gallic acid as standard (0-1000 µg/mL in 50% methanol). The results were expressed as µg of gallic equivalents/2g of coffee.

Determination of total flavonoid contents

Aluminium chloride colorimetric method was used to estimate the total flavonoids (Ayuko *et al.,* 2009). In this method, catechin 0-500 µg/ml was used as standard and flavonoid content was expressed in terms of µg of catechin/2g of coffee. The absorbance was recorded at 510 nm using spectrophotometer.

DPPH scavenging activity assay

Determination of the free-radical scavenging activity of the stable DPPH was done according to Blois (1958) method with slight modification. 250 µl of coffee samples were diluted in 99.5% ethanol. After that, 62.5 µL of 0.02% DPPH was added to the mixture and then shaken vigorously and incubated in dark place for an hour. The absorbance was monitored at 517 nm and the percentage of DPPH scavenging was calculated by the equation percentage of DPPH scavenging = [(Ac – As) / Ac] x 100, where Ac is the absorbance of control and As is the absorbance of sample.

Hydrogen peroxide radical scavenging assay

The ability of coffee beverages to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.* (1989). 40 mM of H_2O_2 solution was prepared in phosphate buffer saline, pH 7.4. After that, 0.6 mL of H_2O_2 solution was added to 1 mL of each sample. All tubes were incubated for 10 min before the absorbance was measured at 230 nm using UV-VIS double beam spectrophotometer. The scavenging activity of hydrogen peroxide was calculated by the equation:

% scavenged H_2O_2 = [(Ac – As) / Ac] x 100, where Ac is the absorbance of control and As is the absorbance of sample.

Reducing power assay

The reducing power of coffee samples was assessed following the protocol of Oyaizu (1986). First, 1.25 mL of phosphate buffer (0.2 M, pH 6.6) was mixed with 1.25 mL of 1% potassium ferricyanide and 0.5 ml of sample. The mixture was incubated for 30 min at 50ºC before 1.25 mL of 10% TCA was added to the mixture to stop the reaction. Then the samples were centrifuged at 6000 rpm for 10 min and the supernatant was diluted with distilled water. Finally, 1% ferric chloride was added and absorbance was measured at 700 nm.

Metal chelation activity

Ferrous ion chelation was estimated according to the method mentioned by Dinis *et al.* (1994). A mixture of 5 µL of each coffee sample and 50 µL of 2 mM ferrous chloride was prepared and each sample was diluted with 1.5 mL distilled water followed by 30s incubation. After that, 100 µL of 5 mM ferrozine was added and allowed to incubate for 10 min and the absorbance was immediately noted at 562 nm. Chelation activity was calculated by the following equation:

% Chelation activity = [(Ac – As) / Ac] x 100, where Ac is the absorbance of control and As is the absorbance of sample.

Statistical analysis

The data obtained from three parallel determinations for each experiment were expressed as Mean±SD. The statistical analysis was carried out using GraphPad Prism 6 by one-way ANOVA test. Results were considered statistically significant at $p<0.05$.

RESULTS

Determination of total phenolic and flavonoid contents

Total phenolic content was expressed as µg of gallic acid/2g of coffee whereas flavonoid content was expressed as µg of catechin/2g of coffee. As shown in Table 1, the concentrations of total phenolic and flavonoid contents have significantly increased ($p<0.0001$) in milk-containing beverages compared with pure coffee.

DPPH scavenging activity

As shown in Figure 1, coffee-milk samples (20% CFEM and 20% CLEM) showed highly significant ($p<0.01$) increases in DPPH scavenging activity (91.4% and 84.9%, respectively) when compared to pure coffee (45.7%).

Hydrogen peroxide radical scavenging

Coffee-milk samples showed lower scavenging ability in comparison to pure coffee as shown in (Figure 2). The presented data were significantly different ($p<0.001$) compared with the control instant coffee. There is a clear trend of decreasing in scavenging activity, pure coffee, 20% CFEM and

Table 1. Total phenolic (µg of gallic /2g of coffee) and flavonoid (µg of catechin /2g of coffee) contents of pure coffee, CFEM and CLEM

	Pure coffee	20% CFEM	20% CLEM	p value
Total phenolic contents	648±70	2534±253	2241±153	p<0.0001 (a,b)
Total flavonoid contents	746±74	2677±158	1937±76	p<0.0001 (a,b)

Values were represented as mean ± SD (a: Pure coffee vs. 20% CFEM, b: Pure coffee vs. 20% CLEM).
p- value ≤ 0.0001 was used as a criterion of highly significance.

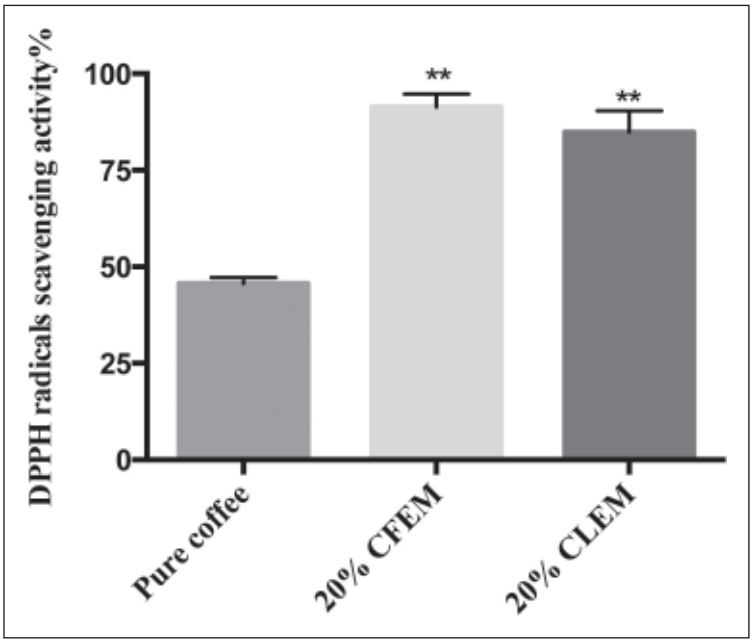

Fig. 1. DPPH free radical scavenging activity of pure coffee, 20% CFEM, and 20% CLEM. Three independent experiments were performed and the data were represented as Mean±SD. The *p*-values were calculated by one-way ANOVA followed by Bonferroni's test correction (**p≤0.01).

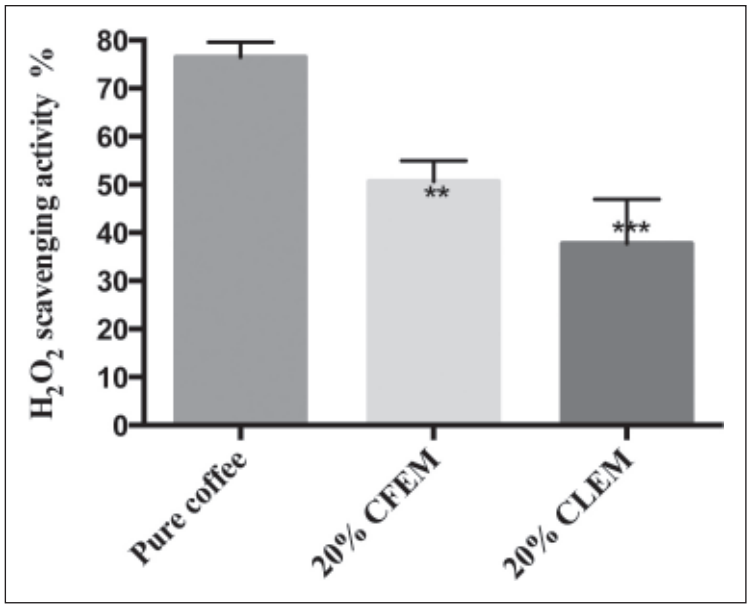

Fig. 2. Hydrogen peroxide radical scavenging activity of pure coffee, 20% CFEM and 20% CLEM. Three independent experiments were performed and the data were represented as Mean±SD. The p-values were calculated by one-way ANOVA followed by Bonferroni's test correction (**p≤0.01, ***p≤0.001).

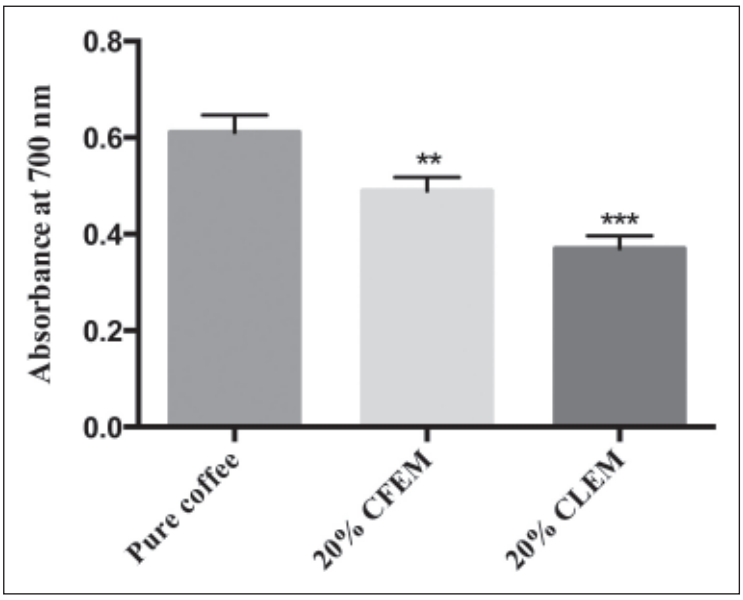

Fig. 3. Reducing power activity of pure coffee, 20% CFEM and 20% CLEM. Three independent experiments were performed and the data were represented as Mean±SD. The p-values were calculated by one-way ANOVA followed by Bonferroni's test correction (**p≤0.01, ***p≤0.001).

20% CLEM showed 76.5%, 50.7% and 37.7% of scavenging activity, respectively.

Reducing power

The absorbance of reducing power of coffee-milk samples was 0.49 and 0.37 nm for 20% CFEM and 20% CLEM, respectively. The values were significantly decreased in both coffee milk samples when compared to pure coffee, the absorbance of pure coffee was 0.61 nm as shown in Figure 3. In addition, there was a significant difference ($p<0.01$ -$p<0.001$) between the two types of milk in comparison to pure coffee.

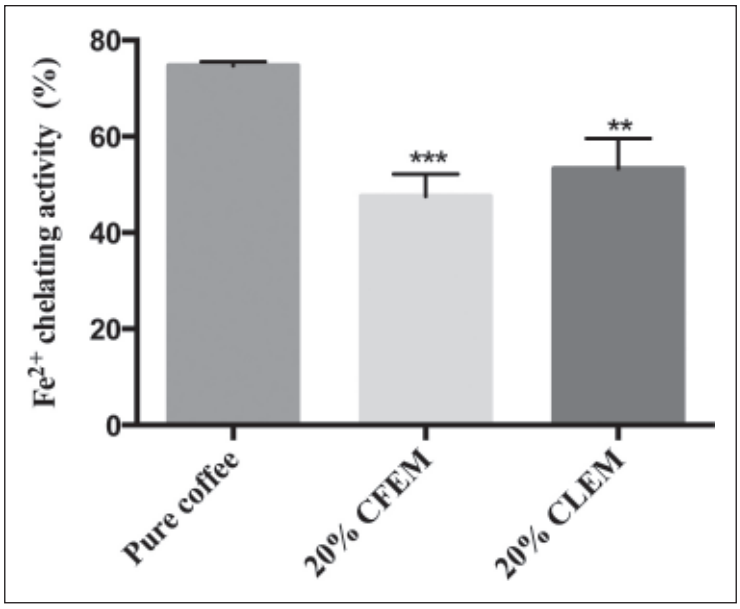

Fig. 4. Ferrous-ion chelation activity of pure coffee, 20% CFEM and 20% CLEM. Three independent experiments were performed and the data were represented as Mean±SD. The p-values were calculated by one-way ANOVA followed by Bonferroni's test correction. (**p≤0.01, ***p≤0.001).

Metal chelation activity

The formation of ferrous-ferrozine complex will not be complete in the presence of metal chelating agent. According to the results, coffee-milk samples were not strong enough as pure coffee in chelation ferrous ion. Figure 4, shows that there were highly significant decreases in the percentage of ferrous ion chelating activity ($p<0.01$ -$p<0.001$) in the two types of coffee-milk samples when compared to pure coffee. However, 20% CFEM and 20% CLEM beverages (47.58% and 53.28%, respectively) showed lower metal chelation activity in comparison to pure coffee (74.69%).

DISCUSSION

Coffee associated with milk is one of the most consumable and favourable beverages in Saudi Arabia. Evaporated milk is also considered as one of the most desirable additives to coffee or tea due to its unsweetened-freshness texture. Although it is produced by removing water under vacuum by heating, it does not lose its nutritional value. Full-fat evaporated milk contains 6.9 g protein and 6.2 g of fats per 100 g in average, while low-fat evaporated milk contains 6.6 g protein and 4.2 g of total fats per 100 g. The amount of proteins is decreased by the process of removing fats from milk. In this study, estimation of antioxidant activity of coffee and coffee-milk beverages was done using four different assays. Total polyphenols and flavonoid content were also determined.

Total phenolic content determination relays based on that Folin-Ciocalteu's reagent reacts with phenolic compounds under basic conditions (Huang *et al*., 2005) and these conditions are adjusted by sodium carbonate. Phenolic compounds, as well-known, can donate H+ because they have one or more aromatic ring with one or more -OH groups (Brewer, 2011). However, since the reagent can interfere with nonphenolic compounds like tyrosine residues in proteins, vitamin C and copper, therefore, reagent is not perfectly specific for phenolic compounds. This explains the highly significant increases in the concentrations of total polyphenols and flavonoid contents in CFEM and CLEM samples in comparison to pure coffee samples and as well known, evaporated milk contains high level of proteins. Our results were in agreement with Niseteo *et al.* (2012) results, which studied the effect of the addition of milk on the bioactive composition of coffee and found that total polyphenols contents have increased by the addition of milk. Flavonoids, generally, play an important role in medical industries due to their antioxidant activities. They can scavenge the free radicals or chelate metals such as iron. The total flavonoid content was estimated using aluminium chloride reagent which reacts with C-4 keto groups and either C-3 or C-5 hydroxyl group of flavones and flavanols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Bhaigyabati *et al.,* 2014). Addition of milk greatly enhanced the flavonoid content which may be due to the interactions

between $AlCl_3$ reagent and milk content (either proteins or fats). Some molecules can work synergistically with flavonoid antioxidants as amino acids and citric acid. In a basic medium, the -OH groups can give a H+, while in a more neutral medium they give H• (Brewer, 2011). The results of this study were in agreement with (Poonia *et al.,* 2011; Mohan *et al.,* 2012) indicating that the extracts had high flavonoid and polyphenol contents.

Antioxidants can act as free radical scavengers or metal ions chelators. Reducing power assay is a general indicator of antioxidant activity, either by scavenging free radicals or metal ions chelation. To determine if the antioxidants present in the samples could act as radical scavengers or metal ions chelators, the H_2O_2 assay, DPPH and Fe(II) chelation methods were applied. Fe(II) chelation method determines if these antioxidants could act as metal ion chelators, while H_2O_2 and DPPH assays are free-radical scavenging assays. H_2O_2 assay measures hydrogen atom transfer, while DPPH assay depends on single electron transfer.

DPPH radical scavenging assay is based on the reduction of DPPH in the presence of hydrogen-donating substance in methanol solution. DPPH accepts the donated electron (H•), thus reducing it into DPPH-H decolorizing the solution from purple to yellow. The reaction is based on delocalization of the spare electron over the molecule (Alam *et al*., 2013; Poonia *et al.,* 2011). Milk contains many lipophilic antioxidants, which play an important role in the prevention of lipid peroxidation, so the greatest antioxidant potential was noted in full-fat milk, which is known to contain number of fat-soluble antioxidants. Thus, decreasing the fat content may eliminate a number of fat-soluble antioxidants, therefore decreasing antioxidant potential (Ryan & Petit, 2010). DPPH free radical is considered as a lipophilic radical, this may explain the increase of DPPH inhibition activity by the addition of milk and the decrease of the activities by the decrease of fat content. Sharma *et al.* (2008) studied the effect of the addition of different concentrations of milk to tea on the antioxidant potential and found that the addition of milk has increased the antioxidant activity, so these results were in agreement with our results.

Hydrogen peroxide itself is not very reactive, but it can penetrate cell membranes rapidly and cause cytotoxicity. Thus, it is very important to remove hydrogen peroxide throughout food systems (Nabavi *et al.,* 2008). The decrease in absorbance of hydrogen peroxide determines the scavenging activity of samples by donating electrons. Phenolic groups are capable to donate electrons thus neutralizing hydrogen peroxide into water (Ebrahimzadeh *et al.,* 2009). Scavenging percentage was significantly highest in pure coffee and significantly decreased by the addition of milk opposing the results of DPPH assay. The decrease of H_2O_2 scavenging activity compared with DPPH assay is may be due to that DPPH molecule has a quite long-life time comparing to H_2O_2. Interactions between milks' fat and proteins may mask the radical scavenging activity thus preventing the scavenger to reach its optimum capacity due to the presence of proteins (Arts *et al.,* 2002). These results suggested that the interactions may create a hydrophobic environment, due to the presence of fats, which form hydrogen bonds between proteins, fats and polyphenols.

Reducing power assay is based on the basis of increasing the absorbance of the reaction mixture which indicates the increase of reducing power of the sample. Potassium ferricyanide in the presence of substances with reduction potential will be converted to potassium ferrocyanide, which then reacts with ferric chloride forming ferric-ferrous complex. This method gives a good reflection for antioxidant activity. Compounds with reducing power are electron donors and can reduce oxidized intermediates of lipid peroxidation processes (Jayanthi & Lalitha, 2011). Several previous studies showed that the addition of milk has decreased the reducing power of the sample (Ryan & Petit, 2010; Niseteo *et al.,* 2012). Interference between milk and coffee components could cause masking of polyphenols and flavonoids by milk proteins and fats, so milk-derived compounds have decreased analytical accuracy.

In the presence of metal chelator, the formation of ferrozine-ferrous complex will be disrupted and the red colour of the complex decreases. Thus, measurement of the decrease in the colour determines the chelation activity of samples that compete with ferrozine to chelate ferrous (Chanda & Dave, 2009). According to the results, coffee-milk samples were not strong as pure coffee, but the coffee could be considered as metal chelator. Chelation of iron after the addition of both types of milk varying in fat content wasn't different. This maybe referred to that metal ion chelators present in coffee were saturated by milk minerals, so they lost their ability to chelate the ferrous added to the solution.

The antioxidant activity of coffee beverage has enhanced, by the addition of 20% evaporated milk. Milk-coffee beverages have worked as free-radical scavengers but not as metal ion chelators. These beverages present a very complex matrix where strong polyphenol-protein interactions occur (Siebert *et al*., 1996). Polyphenol-protein interactions can be formed either covalently or non-covalently by multisite interactions (many phenolic compounds bound to a protein molecule) or

multidentate interactions (phenolic molecule bounds to several proteins). In proline-rich proteins, polyphenol-protein complex formation is favourable (Siebert *et al*., 1996). The binding occurs between the -OH groups of polyphenols and protein chains. These interactions are mainly hydrophobic due to the presence of high molecular weight fats. Since milk proteins are mostly associated with fats, these associations interact with coffee polyphenols producing larger aggregates. Hydrogen bonding and other non-covalent forces stabilize these aggregates. Phenolic compounds found often surrounded by lipids and proteins, protecting these nutrients from early oxidation during their passage inside the gastrointestinal tract. Moreover, these aggregates protect the polyphenols and help delivering them to lower parts in the digestive system, where they can be metabolized by the effect of digestive enzymes and the action of microflora (Jakobek, 2015).

CONCLUSION

The results of the study showed that adding different concentrations of evaporated milk to instant coffee has altered the antioxidant properties of coffee and this might be due to the presence of proteins and fats. Continued researches are needed to assess the effects produced by these popular beverages on human health using different food matrix.

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