

## The Effect of Adding Different Concentrations of Cows' Milk on the Antioxidant Properties of Coffee

Ayat B. Al-Ghafari\*, Rahaf H. Alharbi, Manal M. Al-Jehani, Shoroq A. Bujeir, Huda A. Al Doghaither, and Ulfat M. Omar

Department of Biochemistry, Faculty of Science,  
King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia.

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**Polyphenols have been widely studied and considered as a health promoting and disease preventive agents in humans. Several studies investigated the antioxidant properties of polyphenols and their abilities to eliminate free radicals. In this study, the antioxidant activity of coffee in the presence of different types of cows' milk at different concentrations, 10% or 20%, was investigated. Our results showed that milk could either enhance the scavenging of DPPH or decrease the metal chelating and metal reducing activity of polyphenol. However, more investigations are required to evaluate the mechanisms by which fats in milk can alter the antioxidant activity of coffee.**

**Keywords:** Whole milk; Semi-skimmed milk; Skimmed milk;  
Scavenging activity; reducing power; metal chelating.

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Oxygen is an essential element for the survival of aerobic organisms. In many chemical and biochemical reactions, oxygen-derived free radicals are produced from oxidation-reduction processes<sup>1</sup>. These free radicals are responsible for oxidative stress that leads to variety of diseases and disorders such as cancer<sup>2</sup>, cardiovascular diseases<sup>3</sup>, neural disorders<sup>4</sup>, and Alzheimer's disease<sup>5</sup>. They are produced under normal physiological conditions and are generated by exogenous chemicals<sup>6</sup>. Reactive oxygen species (ROS) can present in different forms of activated oxygen such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) or as singlet oxygen<sup>7</sup>. The redox homeostasis of cell is maintained by the antioxidant protection mechanism of the human body. If this mechanism

has been unbalanced, the reducing of oxidative damage may be occurred by using antioxidant supplement<sup>6</sup>.

Coffee is an exogenous antioxidant that is widely consumed due to its desirable sensory properties<sup>8</sup>. The proper consumption of coffee is important to health as many negative and positive effects can occur according to the dose. Negative effects of coffee consumption include hypertension, heart diseases, psychiatric disorders and bone diseases. These side effects are mainly attributed to increased caffeine consumption of coffee<sup>9,10</sup>. On the other hand, a regular intake of coffee has shown many positive effects on metabolic, psychoactive and neurological diseases<sup>11</sup>. The positive effects depend on the phenolic and flavonoid compounds such as chlorogenic acids (quiny esters of hydroxycinnamic acid)<sup>12</sup>, caffeic, ferulic, p-coumaric acids<sup>13</sup> and proanthocyanidins<sup>13,14</sup>. These

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\* To whom all correspondence should be addressed.  
Tel.:00966504686549;  
E-mail: abalghafari@kau.edu.sa

biological compounds are secondary metabolites from plants<sup>6</sup> and have potent strong antioxidant and radical scavenging activities<sup>15,16</sup>, which help the body to reduce the oxidative stress<sup>17</sup>.

Most of people prefer to drink coffee with additives. Milk is one of the preferable choices of additives because it contains various compounds such as water, fats, sugars, minerals, vitamins, and proteins<sup>18</sup>. When milk is added to coffee, the milk proteins (caseins and whey proteins) can interact with the coffee polyphenols and form a complex, which might affect many properties of coffee particularly the structural, functional, and nutritional ones<sup>19-21</sup>. Many studies have investigated the antioxidant capacity of tea after adding different types of milk to it. However, the obtained results are contradictory. Some of them have reported an inhibitory effect, while others have shown no significant effect of adding milk<sup>22-24</sup>. Milk fat exists in emulsified form coated by thin layer known as a globule membrane<sup>25</sup>, and its effect on antioxidant activity and bioavailability of coffee polyphenols is unclear until now. Therefore, the objective of this study was to assess the *in vitro* effect of adding different concentrations of whole, semi-skimmed, and skimmed cow's milk on the antioxidant activity of coffee.

## MATERIALS AND METHODS

### Preparation of samples

Red mug coffee (Nescafé) and different types of long life (Almarai) cow's milk (whole, semi-skimmed, and skimmed), the most popular consumed milk brand according to the survey, were purchased from a local supermarket. The types of milk and the percentages used were selected according to a survey performed on 900 participants in Jeddah, Western region of Kingdom of Saudi Arabia.

Coffee brews have been prepared as people in the survey commonly drink it. Each sample was prepared by adding 2g of instant coffee to 200 ml boiling water and then either 20 ml or 40 ml of different types of cow milk was added to have a final concentration of 10% or 20%, respectively.

### Determination of total phenolic and flavonoid contents

In a clean test tube, 0.5ml of each coffee

sample (with or without milk) was incubated at room temperature for 5 minutes with 5ml deionized water and 5ml Folin-Ciocalteu's reagent (BDH, Poole, England). Then, in a dark place, all tubes were incubated with 1ml of anhydrous sodium carbonate (CDH, New Delhi, India) for 1 hour before the absorbance was measured at 750 nm. A standard curve was prepared from a serial concentration (0 to 1000µg/ml) of Gallic acid (Sigma-Aldrich, Poole, UK) which was prepared in methanol: water (50: 50 v/v)<sup>26,27</sup>.

On the other hand, the total flavonoid content was determined by incubating 250µl of each coffee sample (with or without milk) with 1.25 ml deionized water and 75µl sodium nitrite (Sigma-Aldrich, Poole, UK) for 5 minutes. Then, 150µl of (10%) aluminum chloride (loba chemie PVT.LTD., Mumbai, India), 0.5 ml of (1M) sodium hydroxide (AppliChem Panreac, Missouri, USA) and 275µl of deionized water were added to the previous mixture. After that, the absorbance was read at 510 nm and a standard curve was prepared from a serial dilution (0 to 500µg/ml) of β-Catechin (Sigma-Aldrich, Poole, UK)<sup>28</sup>.

### Determination of radical scavenging activity with H<sub>2</sub>O<sub>2</sub> and DPPH assays

In test tubes, 1 ml of sample was incubated for 10 minutes at room temperature with 0.6 ml of (40 mM) hydrogen peroxide (Sigma-Aldrich, Poole, UK). Then, the absorbance was recorded at 230 nm and the radical scavenging activity for H<sub>2</sub>O<sub>2</sub> was calculated by the following equation: inhibition % = [(Absorbance of blank – Absorbance of sample) / Absorbance of blank] × 100<sup>29,30</sup>.

In the DPPH-radical scavenging assay, ethanol (Sigma-Aldrich, Poole, UK) and DPPH reagent (Sigma-Aldrich, Poole, UK) were added to water extract solutions of samples and were incubated in dark place for 1 hour at room temperature. The absorbance was measured at 517 nm and the DPPH radical scavenging activity was calculated using the same equation used to calculate the inhibition percentage of H<sub>2</sub>O<sub>2</sub><sup>31</sup>.

### Determination of metal chelating and reducing power activity

To estimate the ferrous ion chelating activity of samples<sup>32</sup>, 5µl of each sample (with or without milk) was incubated for 30 seconds with 50µl of (2mM) ferrous chloride (Sigma-Aldrich,



significantly the activity of coffee to scavenge any radicals (as shown by DPPH assay) at both concentration of milk (10% or 20%).

#### Estimation of metal chelating and reducing power activity

Two assays were performed to estimate the ability of antioxidants, present in the samples, to act as metal chelating agents and as reducing agents. In ferrous chelating activity assay, the dark color which is formed by the interaction of ferrozine with  $Fe^{2+}$  is changed into a lighter color in the presence of a chelating agent in the sample<sup>37</sup>. On

the other hand, ferric reducing power assay used the  $Fe^{3+}$  reduction as an indicator of electron-donating activity which is an important mechanism of any phenolic antioxidant<sup>38</sup>.

Table 3 represents the results from metal chelating and reducing power assays. Interestingly, the results in Fig 2 showed that adding any type of milk (whole, semi-skimmed, and skimmed) at any concentration, either 10% or 20%, reduces significantly the metal chelating and the metal reducing power activities of the coffee compared to the coffee alone (with no milk added).

**Table 1.** Total phenolic and flavonoid contents

Samples	10% Milk		20% Milk	
	Total phenolic content ( $\mu$ g Gallic acid/2g coffee)*	Total flavonoid content ( $\mu$ g of Chatecin/2g coffee)*	Total phenolic content ( $\mu$ g of Gallic acid/2g coffee)*	Total flavonoid content ( $\mu$ g of Chatecin/2g coffee)*
Coffee (no milk added)	695 $\pm$ 2.5	506 $\pm$ 1.5	647 $\pm$ 3.4	506 $\pm$ 4.2
Coffee with whole milk	1181 $\pm$ 3.0	977 $\pm$ 6.0	1678 $\pm$ 7.0	1353 $\pm$ 5.0
Coffee with semi-skimmed milk	836 $\pm$ 5.0	822 $\pm$ 7.0	1061 $\pm$ 3.0	907 $\pm$ 2.5
Coffee with skimmed milk	732 $\pm$ 7.0	694 $\pm$ 5.0	897 $\pm$ 6.0	639 $\pm$ 5.0

\* Data were represented as Mean $\pm$ SD of three independent experiments

**Table 2.** Estimation of the radical scavenging activity

Samples	10% Milk			
	$H_2O_2$ radical scavenging activity		DPPH radical scavenging activity	
	Mean $\pm$ SD	P-value	Mean $\pm$ SD	P-value
Coffee (no milk added)	77% $\pm$ 3.82	REFERENCE	45% $\pm$ 1.54	REFERENCE
Coffee with whole milk	80% $\pm$ 4.96	P>0.05 (NS)	94% $\pm$ 3.0	*** $\leq$ 0.001
Coffee with semi-skimmed milk	88% $\pm$ 5.0	P>0.05 (NS)	84% $\pm$ 1.0	** $\leq$ 0.01
Coffee with skimmed milk	85% $\pm$ 6.5	P>0.05 (NS)	65% $\pm$ 7.0	* $\leq$ 0.05
Samples	20% Milk			
	$H_2O_2$ radical scavenging activity		DPPH radical scavenging activity	
	Mean $\pm$ SD	P-value	Mean $\pm$ SD	P-value
Coffee (no milk added)	76% $\pm$ 3.0	REFERENCE	45% $\pm$ 1.5	REFERENCE
Coffee with whole milk	72% $\pm$ 2.0	P>0.05 (NS)	55% $\pm$ 7.0	P>0.05 (NS)
Coffee with semi-skimmed milk	62% $\pm$ 1.6	**P $\leq$ 0.01	80% $\pm$ 7.0	**P $\leq$ 0.01
Coffee with skimmed milk	38% $\pm$ 5.0	***P $\leq$ 0.001	83% $\pm$ 5.0	**P $\leq$ 0.01

-All data were represented as Mean  $\pm$ SD calculated by one-way ANOVA test followed by Bonferroni's test correction.  
-P-values were calculated by a comparison made versus the (coffee only) value, which was referring to it as reference.

NS: Not Significant

## DISCUSSION

Coffee is one of the most popular beverages in the world. Many of the studies indicated that adding some additives to coffee can alter the bioactive compounds present in it, and therefore, may affect the biological activity of coffee as an important antioxidant. In many cases, coffee is consumed with milk. Milk protein may form interaction with coffee polyphenols, therefore, the present study aimed to investigate the total phenol and flavonoid contents and the antioxidant activity of bioactive components when different types of milk (whole, semi-skimmed, and skimmed) at both concentration (10% and 20%) were added to coffee. Our results indicated that adding milk to coffee increased the total phenol and flavonoid contents. This might be explained by the presence of other compounds in milk that can act as an antioxidant such as lactoferrin, ascorbic acid, tocopherols and tocotrienols<sup>39</sup>.

Hydrophobic interaction and hydrogen bond are the major reactions that derive polyphenol-protein complex formation. Several factors can influence the extent of these binding forces such as structure of polyphenols and proteins as well as some physical conditions particularly (temperature, pH, and ionic strength)<sup>40</sup>. In our study, our results revealed that at 10% milk

concentration, there was no significant difference in hydrogen peroxide scavenging activity between the coffee with and without milk. Interestingly, adding milk at higher concentration (20%) decreased the H<sub>2</sub>O<sub>2</sub> scavenging activity for coffee especially with skimmed milk. Some flavonoids have a high affinity to bind to proteins<sup>40</sup>. This affinity makes many phenol groups of flavonoids occupied and thus H<sub>2</sub>O<sub>2</sub> scavenging do not occur properly. The formation of polyphenol-protein complex can result in protein unfolding. In the presence of lipids, which are classified as Amphiphilic molecules, hydrophobic lipid phase interacts with hydrophobic part of the protein, which has been exposed to the outer phase due to denaturation. This prevents the association between hydrophobic sites of polyphenols with the aromatic groups and aliphatic side chains of hydrophobic amino acids<sup>41</sup>. However, those polyphenols when bind with the polar head groups of lipid can probably make phenol groups somehow in a position that allows phenols to scavenge H<sub>2</sub>O<sub>2</sub>, while polyphenol-protein complex without lipid presence require the involvement of many hydrogen and hydrophobic bonds in addition to the high affinity of flavonoids toward protein binding<sup>40</sup>. These findings may explain why whole milk sample has H<sub>2</sub>O<sub>2</sub> scavenging activity higher than semi-skimmed and skimmed milks.

**Table 3.** Estimation of metal chelation and reducing power activity

Samples	10% Milk			
	Ferrous chelating activity		Ferric reducing power activity	
	Mean ± SD	p-value	Mean ± SD	P-value
Coffee (no milk added)	37% ± 5.50	REFERENCE	0.62 ± 0.05	REFERENCE
Coffee with whole milk	23% ± 1.89	**P≤0.01	0.38 ± 0.03	**P≤0.01
Coffee with semi-skimmed Milk	18% ± 1.42	**P≤0.01	0.43 ± 0.09	*P<0.05
Coffee with skimmed milk	17% ± 2.84	***P≤0.001	0.42 ± 0.06	*P<0.05
Samples	20% Milk			
	Ferrous chelating activity		Ferric reducing power activity	
	Mean ± SD	P-value	Mean ± SD	P-value
Coffee (no milk added)	38% ± 3.40	REFERENCE	0.61 ± 0.04	REFERENCE
Coffee with whole milk	22% ± 2.80	**P≤0.01	0.35 ± 0.02	***P≤0.001
Coffee with semi-skimmed milk	23% ± 5.40	**P≤0.01	0.37 ± 0.02	***P≤0.001
Coffee with skimmed milk	27% ± 0.86	*P<0.05	0.33 ± 0.04	***P≤0.001

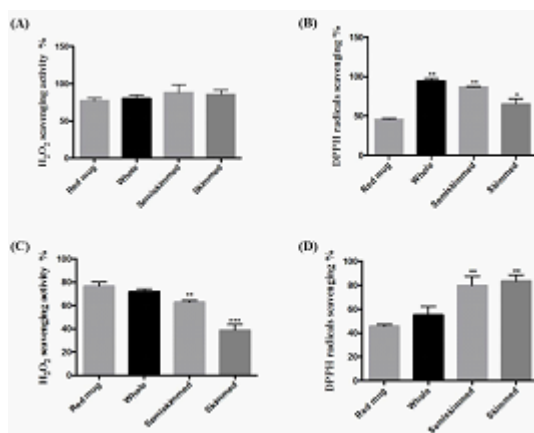
-All data were represented as Mean ±SD calculated by one-way ANOVA test followed by Bonferroni's test correction.  
-P-values were calculated by a comparison made versus the (coffee only) value, which was referring to it as reference.

On other hand, in the present study, results from DPPH radical scavenging activity experiments are in contrast with that of  $H_2O_2$  for all tested samples. In addition to the probabilities that mentioned above about the difference of phenols, some amino acids with aromatic and bulky side groups are considered as effective radical scavengers because they can act as H-donors due to the presence of special groups in their side chains. For example, the phenolic group in tyrosine, indolic group in tryptophan, imidazole group in histidine, and sulfur hydrogen<sup>42</sup> in methionine and cysteine. Moreover, several studies refer to the correlation between the high concentration of flavonoids and polyphenols with the high DPPH scavenging activity<sup>43,44</sup>. In our study, the polyphenol and flavonoid contents were higher in all coffee with milk samples compared to the plain coffee sample.

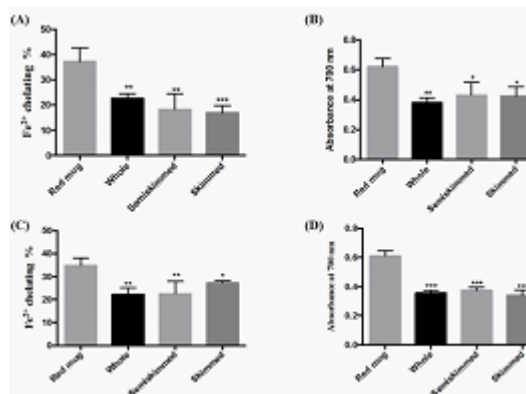
In general, many studies, reported that

the polyphenols interaction with proteins, not only affects the scavenging activity but also affects the chelating and metal reducing activity of coffee<sup>18,45</sup>. The hydrophobic stacking of the aromatic groups of the protein and polyphenols, or the interaction of the -OH groups of the polyphenols with the protein chain can result in the formation of aggregates<sup>40</sup>. Our results showed that the ferrous chelating activity increased when coffee is not mixed with milk. This might be due to the presence of free -OH groups of polyphenols or might be related to the binding of polyphenols to the milk caseins by covalent and non-covalent interactions either in a multi-site or in a multi-dentate interaction<sup>24</sup>. Our results are in agreement with other similar studies<sup>22,46</sup> that used FRAP assay to assess the ferrous chelating activity.

In conclusion, the results of the current study revealed that milk might either enhance the scavenging of DPPH or decrease the chelating and metal reducing activity of polyphenol. However,



**Fig. 1.** Radical scavenging activity of red mug coffee with and without various milk types and concentrations. Three different types of milk were tested (whole, semi-skimmed, and skimmed) at two concentrations (10% and 20%). A & B) represents the  $H_2O_2$  and DPPH radical scavenging activity of samples at 10% milk concentration, whereas; C & D) represents the  $H_2O_2$  and DPPH radical scavenging activity of samples at 20% milk concentration. Results revealed that the addition of milk at any concentration or type decreases the  $H_2O_2$  radical scavenging activity of the coffee, whereas; increases the DPPH radical scavenging activity. The values expressed as mean $\pm$ SD (n=3). Comparisons of means were made using one-way ANOVA followed by a correction with Bonferroni's test (\*P<0.05, \*\* P $\le$ 0.01, and \*\*\* P $\le$ 0.001).



**Fig. 2.** Metal chelating and reducing power activity of red mug coffee with and without various milk types and concentrations. Three different types of milk were tested (whole, semi-skimmed, and skimmed) at two concentrations (10% and 20%). A & B) represents the metal chelating and reducing power activity of samples at 10% milk concentration, whereas; C & D) represents the metal chelating and reducing power activity of samples at 20% milk concentration. Results revealed that the addition of milk at any concentration or type decreases the metal chelating and reducing power activity of the coffee. The values expressed as mean $\pm$ SD (n=3). Comparisons of means were made using one-way ANOVA followed by a correction with Bonferroni's test (\*P<0.05, \*\* P $\le$ 0.01, and \*\*\* P $\le$ 0.001).

more investigations are required to evaluate the mechanisms by which fats in milk can alter the antioxidant properties of coffee.

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