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Effects of Aqueous Extracts of Propolis on Total Polyphenol Content and Antioxidant Activity of Raw Milk

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Abstract

In recent years, the use of propolis in food products has received attention owing to its functional role. This study was conducted to investigate the effect of different concentrations of propolis extract on the total polyphenol content and antioxidant activity of raw milk. For this purpose, an aqueous extract of dry propolis was prepared and stored in dark-colored bottles at 4 °C until the day of experiments. The propolis extract was added to raw milk in concentrations of 0, 4.7, 9.1, 16.6, and 28.5%. Total phenolic content and antioxidant activity were measured using the colorimetric Folin-Ciocalteu method and DPPH assay, respectively. Measurements were performed on the first day at zero hour and after 6 and 24 h of treatment, and the storage temperature was maintained at 5°C until analyzed. The amount of total polyphenol increased with the increase in the concentration of propolis extract in the treated milk in 0 hour; accordingly, the lowest and the highest amounts of total polyphenol were related to control milk and milk containing 28.5% propolis extract, respectively. A decreasing trend was observed in total polyphenol in the control sample and raw milk containing 4.7% propolis extract during 24 hours. There was an increase in total polyphenol content in raw milk containing 9.1% and 16.6% propolis extracts, the trend of changes in raw milk containing 28.5% extract was insignificant. The addition of propolis extract caused an increase in the antioxidant activity and total phenolic content in raw milk. According to the results, it is recommended to carry out more studies to clarify the functions of propolis's total polyphenol content and its interaction with milk proteins.

Keywords: Antioxidant activity, Aqueous extract, Propolis, Raw milk, Total polyphenol



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Introduction

Milk and dairy products are important suppliers of a wide range of essential nutrients, some especially important at certain stages of life (Givens, 2020). Recently, various studies have been implemented to enhance the nutritional value of this food or its products (Givens & Kliem, 2009). The complex biochemical composition, high water activity, and nutrient content of milk serve as an ideal medium for microorganisms that cause milk deterioration (Fusco *et al.*, 2020), and the application of heat treatment in milk affects the flavor, and the milk proteins (Bezie, 2019). Orcho *et al.* examined the shelf life extension ability and antioxidant activity of the ethanolic extracts of *Moringa stenopetale*, *Artemisia anua*, and *Mentha spicata* to preserve milk. All the plant extracts showed suitable antioxidant activities, and *Moringa stenopetale* had the most effect on extending shelf life of raw milk samples (Orcho *et al.*, 2023). The vitamins, such as E and C, beta-carotene, and the enzyme system in the milk provide a possible complex set of pro-oxidative and anti-oxidative reactions (Zulueta *et al.*, 2007). Currently, we are faced with increasing controversial research on the antioxidant capacity of milk (Gülcin, 2012).

The antioxidant activity of propolis has been reported in various studies (Kasiotis *et al.*, 2017; Özkök *et al.*, 2021). More than 300 compounds, including different flavonoids, polyphenolic esters, terpenoids, steroids, amino acids, and caffeic acids and their esters, as well as mineral compounds, have been reported in propolis (Mouhoubi-Tafinine *et al.*, 2016). Today, considerable attention is conferred on the activity and content of the bioactive compounds of propolis (Andrade *et al.*, 2017). It has attained wide acceptance across numerous countries as a diet supplement that enhances health (Azemin *et al.*, 2017). The antimicrobial activity of propolis against food spoilage microorganisms, such as *Bacillus cereus* (Kim & Chung, 2011) and *Escherichia coli* (Tosi *et al.*, 2007), is confirmed, and this feature can contribute to the maintenance of greater nutritional value.

The different concentrations of propolis have improved the milk production, fatty acid composition, and antioxidant capacity of milk in dairy cows (Santos *et al.*, 2019). Using Brazilian red propolis, as a substitute for potassium sorbate preservative, its antioxidant activity was significantly increased during yogurt storage (Aguar *et al.*, 2014). The addition of aqueous extract of propolis in concentrations of 1%, 2%, and 3% was accompanied by an increase in phenolic and flavonoid compounds, and antioxidant activity in raw milk (El-Deeb, 2017). Also, the adding 0.5 and 1% of propolis extracts to milk increased the shelf life of raw and pasteurized milk (Shaban *et al.*, 2021). The propolis can be used as an antioxidant, where lipid autoxidation may reduce the sensory quality and the nutritional value of food (Pobiega *et al.*, 2019).

In recent years, consumer demand has grown for raw milk and dairy products manufactured from unpasteurized milk (McLauchlin *et al.*, 2020). During pasteurization, approximately 5-15% of whey protein is denatured (Deeth & Lewis, 2017). Superoxide dismutase activity and glutathione level as antioxidant parameters are affected by the heat treatments (Martysiak-Żurowska *et al.*, 2019). The current research aimed to investigate the effect of extract of propolis on the total polyphenol content and antioxidant activity of raw milk.

Materials and Methods

The milk was obtained by hand milking on a livestock farm after washing the cow's udders. Samples were transported to the laboratory in sterile bottles away from light and heat. Folin-Ciocalteu reagent, 2, 2-diphenyl-1-picrylhydrazyl free radical (DPPH), gallic acid, and methanol were from Merck Company (Darmstadt, Germany). Other chemicals were of the highest commercial grade and used without further purification.

Although the alcoholic extraction of propolis contains high phenolic compounds, this method has disadvantages, such as strong residual flavor and intolerance of some consumers to alcohol (Pobiega *et al.*, 2019). In this study, the

aqueous extract was chosen. For this aim, dry propolis (brown with green veins) purchased from a beekeeper in the Otaghvar district of Langarud County, northern Iran, in the summer. It was transferred to the laboratory in a completely sterile condition. Then, 5 g, 10 g, 20 g, and 40 g propolis were weighed in separate Erlenmeyer flasks, and 100 ml of deionized water (65°C) was added to the flasks. After shaking for two hours, centrifugation was performed at 1500 rpm for 5 min. The supernatant was separated and stored in dark-colored bottles (Said *et al.*, 2006). Extract was added to raw milk with concentrations of 4.7%, 9.1%, 16.6%, and 28.5%. The sampling of raw milk and propolis extract was performed by previously sterilized instruments in an autoclave at 121°C for 15 min.

Measurement of total polyphenols content

Polyphenol was measured according to the colorimetric Folin-Ciocalteu method. For this purpose, 1 ml of the control sample and milk samples containing propolis extracts were pipetted into a 100 ml volumetric flask and diluted with distilled water, and mixed well. In control samples, 1 ml of water was added separately to two test tubes. Moreover, 1 ml of the diluted extract was poured into two separate tubes, and 5 ml of Folin-Ciocalteu reagent solution was added to all the tubes and mixed well. After 3-8 min, 4 ml of a sodium carbonate solution was added to all the tubes and then capped. The tubes were placed at ambient temperature for 60 min; then, the optical absorption was measured by a 2100 UV/VIS spectrophotometer (UNICO, USA) at 765 nm (Singleton & Rossi, 1965).

Measurement of antioxidant activity

Antioxidant activity was measured using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay. This method is one of the most popular and frequently employed methods to evaluate the antioxidant capacity of foods (Pyrzynska & Pękal, 2013). After preparing different concentrations of propolis extract, 1 ml of the sample was mixed with 3 ml of methanolic

DPPH radical solution, and stirred vigorously. The reaction mixture was kept at room temperature (23°C) for one hour in the darkness. Absorbance was measured at 517 nm by a 2100 UV/VIS spectrophotometer (UNICO, USA). Methanolic DPPH solution and methanol were used as standard and to zero the spectrophotometer, respectively. Finally, the antioxidant activity was obtained using the following formula:

$$\% \text{ DPPH}^{\cdot} \text{ scavenging activity} = (1 - [A_s / A_c]) \times 100,$$

Where A_s and A_c are the absorption of the sample solution and methanolic DPPH solution, respectively (Li *et al.*, 2009). It should be noted that the measurements were taken on the first day at the zero hour and after 6 and 24 h of treatment, and the storage temperature was maintained at 5°C until analyzed.

Data Analyses

This study was conducted based on a random design, and the collected data were analyzed in SPSS software (version 16). Measurements with three repetitions were performed. Data were reported as mean \pm standard deviation. A p -value < 0.05 was considered a significant level.

Results and Discussion

In this study, the total phenolic content of the extracts was determined by comparison with a calibration curve of gallic acid as a standard (the standard curve equation: $y = 0.0118x + 0.0434$, $r^2 = 0.9992$). The comparison of the mean and standard deviation of total polyphenol and antioxidant activity of different concentrations of propolis extract is presented in Table 1. There was a significant difference in total polyphenol and antioxidant activity of the studied treatments ($p < 0.001$).

The comparison of the total polyphenol content and antioxidant activity ($\mu\text{g/ml}$) of the studied samples during 24 hours is tabulated in Tables 2 and 3, respectively. The total polyphenol content and antioxidant activity of the propolis extract-treated milk sample was higher than the control in all three times of examinations ($p = 0.001$).

Table 1- Mean and standard deviation of total polyphenol content and antioxidant activity of different concentrations of propolis extract

Group	Total polyphenol content(mg/g)	Antioxidant activity (µg/ml)
4.7% extract	2.512±0.047 ^a	8.501±0.100 ^a
9.1% extract	4.203±0.551 ^b	11.926±0.490 ^b
16.6% extract	7.106±0.010 ^c	17.671±0.068 ^c
28.5% extract	11.670±0.005 ^d	27.302±0.152 ^d

Table 2- Mean and standard deviation of total polyphenol content (mg/g) in the propolis extract-treated milk during the storage period

Treatment	Storage Period			p-value ₁
	0 hour	6 hour	24 hour	
Control sample	0.693±0.001 ^{aA}	0.504±0.010 ^{aB}	0.422±0.005 ^{aC}	0.010
4.7% extract	1.491±0.003 ^{bA}	1.453±0.010 ^{bB}	1.421±0.001 ^{bC}	0.010
9.1% extract	1.582±0.021 ^{cA}	1.621±0.010 ^{cB}	2.094±0.001 ^{cC}	0.001
16.6% extract	2.216±0.003 ^{dA}	2.092±0.021 ^{dB}	2.251±0.001 ^{dC}	0.030
28.8% extract	2.257±0.002 ^{eA}	2.206±0.020 ^{eA}	2.173±0.001 ^{eA}	0.150
p-value ₂	0.001	0.001	0.001	

p-value₁: Significant difference in one treatment over timep-value₂: Significant difference in different treatments at a time**Table 3- Mean and standard deviation of antioxidant activity (µg/ml) in the propolis extract-treated milk during the storage period**

Treatment	Storage Period			p-value ₁
	0 hour	6 hour	24 hour	
Control sample	16.063±0.003 ^{aA}	15.608±0.010 ^{aB}	14.923±0.008 ^{aC}	0.020
4.7% extract	34.680±0.002 ^{bA}	33.863±0.011 ^{bB}	32.495±0.001 ^{bC}	0.010
9.1% extract	36.662±0.010 ^{cA}	37.391±0.015 ^{cB}	37.094±0.001 ^{cC}	0.001
16.6% extract	41.216±0.003 ^{dA}	41.078±0.001 ^{dB}	40.865±0.001 ^{dC}	0.040
28.8% extract	42.278±0.001 ^{eA}	42.216±0.020 ^{eA}	42.193±0.002 ^{eA}	0.189
p-value ₂	0.001	0.001	0.001	

p-value₁: Significant difference in one treatment over timep-value₂: Significant difference in different treatments at a time

In the present study, the total polyphenol content and the antioxidant activity of selected concentrations of propolis aqueous extract were evaluated. High TPC is generally regarded as an indication of high total antioxidant capacity (Li *et al.*, 2009). The results of the present study were in line with those of previous studies reporting the antioxidant activity of propolis extract (Devequi-Nunes *et al.*, 2018; Mohammadzadeh *et al.*, 2007).

Propolis is rich in phytochemicals (Abdullah *et al.*, 2020), and flavonoids, polyphenols, carboxylic acids, quercetins, fatty acids, cinnamic acid, esters, and terpenoids are its most important bioactive compounds (Sawicka *et al.*, 2012). It contains various compounds with biological activities, such as antioxidant, antibacterial, and anti-inflammatory. These

properties would make it an ideal candidate which could be used as a beneficial ingredient (Irigoit *et al.*, 2021).

The effect of collection time (Isla *et al.*, 2009), the geographical region, and plant species (Alvear *et al.*, 2021) on propolis polyphenol has been confirmed. The extraction method affects the antioxidant activity of propolis extract; accordingly, the highest activity was observed in methanolic extract (Esfandiarifard, 2021). Therefore, all the mentioned factors were influential in the results.

The total polyphenol content was obtained at 0.69 mg/g in the control sample at zero hour. These phenolic compounds can be derived from food and/or catabolism products of amino acids (Lopez & Lindsay, 1993). Different

concentrations of aqueous propolis extract led to an increase in the total polyphenol content in treated milk. The interaction between propolis extract polyphenols and milk casein might be the main reason. The results of previous studies have shown that phenolic compounds have a high affinity with milk proteins, especially casein (Arfaoui, 2020; Yildirim-Elikoglu & Erdem, 2018). Since caseins are proteins rich in proline, they have a high affinity for the hydroxyl group of polyphenolic compounds (Yuksel *et al.*, 2010). It has been shown that the formation of the casein-flavonoid complex can increase the absorption of flavonoids by biological membranes (do Nascimento *et al.*, 2022). It seems that adding propolis to milk can enhance its health effects due to its polyphenol content and antioxidant activity.

Based on the results, a significant decrease was observed in the total polyphenol content of the control sample and raw milk containing 4.7% propolis extract, during 24-hour storage. This result agreed with previous studies indicating the decrease of total polyphenols in yogurts enriched with fruits containing high polyphenols during storage (Arfaoui, 2020; Sánchez-Bravo *et al.*, 2018). A slight decrease in the total phenolic content in dragon fruit treated with 0.5% ethanol extracts has been reported (Zahid *et al.*, 2013). A significant increase was found in total polyphenol content in the treatments of 9.1% and 16.6% propolis extracts within 24 hours. It seems that 9.1% and

16.6% propolis extracts are suitable for further use. The results highlight the necessity of further studies to clarify the functions of total polyphenol of propolis and its interaction with milk proteins.

This work has limitations that must be mentioned. In this study, only DPPH assay was used to evaluate antioxidant activity. Furthermore, we measured total polyphenol content and antioxidant activity during a short-term storage period. Unlike several works that have used the effects of ethanol extraction, the current study investigated the effect of aqueous propolis extract. For further studies, it is suggested to investigate the effect of different extraction methods on sensory properties, microbial and physicochemical characteristics, and their potential as a natural preservative.

Conclusion

The present study showed the effect of different concentrations of aqueous propolis extract on the increase of total polyphenol content and antioxidant activity of raw milk. It seems that one of the advantages of using propolis extract relates to its higher antioxidant capacity. Therefore, it is recommended to be used for fortifying milk by conducting more studies.

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اثرات عصاره آبی بره‌موم بر محتوای پلی‌فنل کل و فعالیت آنتی‌اکسیدانی شیر خام

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چکیده

در سال‌های اخیر استفاده از بره‌موم در محصولات غذایی به دلیل نقش عملکردی آن مورد توجه قرار گرفته است. این مطالعه به منظور بررسی اثر غلظت‌های مختلف عصاره بره‌موم بر میزان پلی‌فنل کل و فعالیت آنتی‌اکسیدانی شیر خام انجام شد. برای این منظور عصاره آبی بره‌موم خشک تهیه و تا زمان آزمایش در قوطی‌های تیره رنگ در دمای ۴ درجه سانتی‌گراد نگهداری شد. عصاره بره‌موم در غلظت‌های ۰، ۴/۷، ۹/۱، ۱۶/۶ و ۲۸/۵ درصد به شیر خام اضافه شد. محتوای فنلی کل با استفاده از روش رنگ‌سنجی فولین سیوکالتیو اندازه‌گیری شد. براساس یافته‌ها، میزان پلی‌فنل کل با افزایش غلظت عصاره بره‌موم در شیر تیمار شده در ساعت صفر افزایش یافت. بر این اساس، کمترین و بیشترین مقدار پلی‌فنل کل به ترتیب مربوط به شیر شاهد و شیر حاوی ۲۸/۵ درصد عصاره بره‌موم بود. در طول ۲۴ ساعت، روند کاهش در پلی‌فنل کل در نمونه شاهد و شیر خام حاوی ۴/۷ درصد عصاره بره‌موم مشاهده شد. در حالی که میزان پلی‌فنل کل در شیر خام حاوی ۹/۱ و ۱۶/۶ درصد عصاره بره‌موم افزایش یافت، روند تغییرات شیر خام حاوی ۲۸/۵ درصد عصاره معنی‌دار نبود. افزودن عصاره بره‌موم باعث افزایش فعالیت آنتی‌اکسیدانی و محتوای فنلی کل شیر خام شد. با توجه به نتایج، توصیه می‌شود مطالعات بیشتری برای روشن شدن عملکرد محتوای پلی‌فنل کل بره‌موم و برهمکنش آن با پروتئین‌های شیر انجام شود.

واژه‌های کلیدی: بره‌موم، پلی‌فنل کل، شیر خام، عصاره آبی، فعالیت آنتی‌اکسیدانی

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