Journal of Animal & Plant Sciences, 34(1): 2024, Page: 138-144
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2024.1.0702

BIOCHEMICAL CHARACTERIZATION AND ANTIMICROBIAL PROPERTIES OF ICE CREAM ENRICHED WITH ANTIOXIDANT ENCAPSULATED PROPOLIS

M. E. Güneş[1], Ş. Keskin[2], P. E. Alkan[3], M. Keskin[2*] and S. Kolayli[4]

[1]Vocational School of Technical Science, Department of Food Processing, Uludag University, Bursa, Türkiye
[2]Vocational School of Health Services, Bilecik Şeyh Edebali University, Bilecik, Türkiye
[3]Vocational School of Health Services, Uludağ University, Bursa, Türkiye
[4]Department of Chemistry, Faculty of Science, Karadeniz Technical University, Trabzon, Türkiye
Corresponding author's email: merveozdemirkeskin@gmail.com

ABSTRACT

Microorganism food poisoning is one of the foodborne illnesses in the world. The interest in natural preservatives has increased since the lack of acceptability in using synthetic preservatives. Propolis, a resinous mixture, is a natural product produced by honey bees to protect their hives against microorganisms. It is considered a natural food supplement and preservative owing to its high antimicrobial and antioxidant activities. However, its resinous nature (not readily soluble in water), ethanol solubility, specific strong smell and taste limits its usage as a natural preservative. Encapsulation of propolis ethanol extract with natural polymers like alginate and pectin may overcome these limitations. Main aim of this study was to test powdered form of propolis as a natural preservative in ice cream as model food. For this purpose propolis, after extraction and characterization, was encapsulated by using pectin and converted into powdered form. Antimicrobial activity of prepared ice cream itself against S. *aureus* (ATCC 25923), E. *coli* (ATCC 28712), E. *faecalis* (ATCC 51299), K. *pneumoniae* (ATCC 700603) and B. *cereus* (patient isolate) was tested. Total phenolic content and ferric reducing antioxidant power of propolis were noted as 46.26 ±1.18 mg GAE/mL and as 0.27 ±0.07 µmol $FeSO_4.7H_2O$/mL, respectively. The encapsulation efficiency was found 95%. Encapsulation of propolis ethanol extract improved the homogenization of propolis active compounds in ice cream. This resulted in obtaining an ice cream with high antimicrobial and antioxidant activities. In conclusion, the use of encapsulated active compounds of propolis ethanol extract improved the both antimicrobial and antioxidant activity of the ice cream produced in a natural way.

Keywords: antioxidant activity, antimicrobial activity, ionic gelation, ice cream, propolis

Published first online November 12, 2023 Published final January 20, 2024

INTRODUCTION

Food preservatives and additives are matters added regularly to foodstuffs to increase product durability and to enhance or modify its properties including appearance, flavor, or structure, provided it does not detract from its nutritional value. Food preservatives and additives that could be of natural or synthetic origin, usually without appreciable nutritional value, are added to food in small amounts during the production procedure (Silva and Lidon, 2016). However, the synthetic origin of food preservatives and additives could have side effects on health. Thus, there is an interest in natural substances to protect foodstuffs against the harmful effects of microorganisms and to extend shelf life (Silva and Lidon, 2016).

Propolis is a resinous mixture collected from different parts of the plants or buds by honey bees. It contains more than 300 compounds including aromatic acids, volatiles, and phenolic compounds (Ahn *et al*., 2007; Li *et al*., 2008; Ozkok *et al*., 2021). Therefore, it has antioxidant, antimicrobial, anti-inflammatory, and antitumor activities. Although propolis is a natural preservative, the usage of propolis in food and other industries is quite limited because of its resinous structure. The best solvent to obtain its active compounds is 70% ethanol-water. In addition, its solubility, specific smell, and taste restrict it wider application in foods. Encapsulation is a process that entraps active compounds into another material that could be biocompatible (Nori *et al*., 2011, Keskin 2020). Encapsulation of its active compounds may contribute to solve above mentioned problems and enhance the availability of propolis for the food industry (Nori *et al*., 2011, Keskin 2020).

Ice cream is one of the most consumed dairy food all over the world. It is produced by mixing mainly milk, sugar, milk powder, emulsifier, and some flavors. The ice cream may be considered an oil-in-water

emulsion based on its structure (Güven *et al*., 2018). In this respect, it is easy to explain why propolis ethanol extract is not suitable for usage in ice cream production. This is the result of the apolar structure of propolis ethanol extract. Encapsulation of propolis ethanol extract with more polar or water-soluble polymers improves the miscibility and solubility in ice cream production.

The main purpose of this study was to test the powdered form of propolis as a preservative in ice cream as a model food. Production of more functional ice cream was also designed by using a powdered form of propolis with a distinct physical and pharmacologic future. Especially, the effect of the antimicrobial feature of whole ice cream obtained by adding powdered form of propolis was tested.

MATERIALS AND METHODS

Raw propolis sample was obtained from bee hives from Koyunköy district of Bilecik city in 2019 by using traps. The pectin, ethanol, gallic acid, Na_2CO_3, Folin- Ciocalteu reagent, and $CaCl_2$ were supplied from Sigma Aldrich USA. All other chemicals used were of analytical grade.

Extraction of Raw Propolis: Raw propolis was crushed into fine powder after keeping it at -18 °C overnight. The fine powder propolis was weighed (10 g) and mixed with 100 mL of 70% of ethanol/water as a solvent. Extraction was carried out on a magnetic stirrer by constant stirring at 150 rpm. After 24 h of this process, the extract was filtered by using Whatman No 1 filter paper. The obtained filtrate was labeled as propolis extract and used in further studies.

Determination of Total Phenolic Contents (TPC), Flavonoid Contents (TFC), and Antioxidant Capacity: Folin- Ciocalteu method was used for the determination of the TPC of propolis extract and filtrate. Gallic acid was used as a standard (Singleton and Rossi 1965; Singleton *et al*., 1999). Results were expressed as mg Gallic acid equivalent (GAE) per mL sample. The total flavonoid content (TFC) of the propolis sample was determined according to Fukumoto and Mazza (2000) and expressed as mg Quercetin equivalent (QUE) per mL sample.

Ferric reducing antioxidant power (FRAP) was determined according to Benzie and Strain (1999). The results were presented as µmol $FeSO_4.7H_2O$ equivalents per mL sample. Trolox was used as a positive control to obtain a reference curve in the range of 62.5 to 1000 µM.

Determination of Chemical Characteristics of Propolis Extract: Active compounds of propolis extract were detected by using GC-MS technique as reported in Bankova *et al*., 2019 with minor modifications. Derivatization of propolis extract was achieved by using bis-(trimethylsilyl)-trifluoro-acetamide (BSTFA). Dried propolis extract was dissolved in 50 µL of dry pyridine. 75 µL of BSTFA was added into this mixture and heated at 80°C for 20 min. Separation and detection of active compounds was carried out by using an Agilent 6890NNetwork GC-MS device equipped with DB-5MS column (30 m × 25 mm and 0.25 µm film thickness) and 5973N Selective Mass Detector. Oven temperature was increased from 75 to 325°C at a rate of 5°C/min increment and kept at 325°C for 15 min. The flow rate of Helium as a carrier gas was set to 0.8 mL/min. Injection of the sample was carried out at 300°C as the injector temperature with a 1:50 split ratio and 70 eV of ionization voltage. Semi-quantification was carried out by internal normalization with the area of each compound (Bankova *et al*. 2019).

Encapsulation of Propolis Extract: Propolis extract was encapsulated by using ionic gelation and solvent-changing methods (Keskin 2020). The pectin was used as an encapsulating agent. Briefly, 5% of the pectin solution (50 mL) was prepared. Propolis extract was poured into a beaker and 0.275 g of $CaCl_2$ was dissolved in this extract. Then, the pectin solution was dropped into three different ratios (v/v) of propolis extract (1:1, 2:1, and 2.5:1). By this way the highest amount of propolis active compounds was loaded into pectin beads separately. Obtained beads were filtered and dried at 50 °C in a vacuum oven. The TPC of both the propolis extract and the obtained filtrate was determined separately. Encapsulation efficiency (EE) was calculated according to formula of EE % = (PE-F/PE)*100.

Where; PE and F represented the TPC of propolis extract and filtrate, respectively.

Preparation of Ice Cream Mix: The addition of each propolis sample (1:1, 2:1, 2.5:1) to the ice cream mix was carried out by dissolving them in the water phase at a concentration of 0.75%, 1%, and 1.5% (m/v). The prepared samples were dissolved in an ultrasonic bath (AC-150H) at 40 ºC for 6-8 hours. Ice cream mix was prepared by mixing cow's milk (13% DM), sahlep (1%), sugar (23%), milk powder (4%), vanilla (0.1%), and emulsifier (1.2%) and pasteurized at 100 ºC (Saltan and Güneş, 1998). After pasteurization, the ice cream mix was cooled to 40 °C, and the propolis samples dissolved in an ultrasonic bath were mixed with the ice cream mixture. The mix was then frozen at -6 ºC and stored at -18 ºC.

Determination of Antibacterial Activity of Ice Cream: In the antimicrobial activity test, S. *aureus* (ATCC 25923), E. *coli* (ATCC 28712), E. *faecalis* (ATCC 51299), K. *pneumoniae* (ATCC 700603), and B. *cereus* (patient isolate) were used. Agar-well diffusion method was used for the determination of antimicrobial activity (Bazerque, Perez and Pauli, 1990). Each of the test

bacteria was incubated in Mueller Hinton broth at 25 ºC. Final concentrations were adjusted to 0.5 Mc-Farland and 0.1 mL of test bacterial solution was spread over the surface of Muller Hinton Agar (Oxoid, CM0337). 6 mm wide wells were made on agar surface with a sterile metal cylinder. Whole ice cream samples obtained by adding 1:1, 2:1, and 2.5:1 ratio of powdered propolis at concentrations of 0.75, 1 and 1.5% were prepared separately. 50 microliters of each sample were added to each well. Plates were incubated at 35 °C for 18–24 hours. All trials were repeated twice. Finally, the diameters of the inhibition zone on the plates were measured. Results were evaluated as; <5.5 mm zone diameter, No inhibition; 5.5– 9 mm, very low inhibition; 9–12 mm, low inhibition; 12–15 mm, average inhibition; and >15 mm high inhibition (Small *et al*., 2007). Ice cream samples were kept at -18 °C and their antibacterial activity was also tested on the 7th and 29th days.

Data analyses: The statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SE. The data were analyzed by one-way analysis of variance (ANOVA) and means were separated by Tukey's range test ($P<0.05$).

RESULTS AND DISCUSSION

In recent years, because of the lack of acceptability of synthetic preservatives, there is an interest in using natural preservatives such as plants, bee products, etc. Propolis with its phytochemicals is a natural bee product. Attempts for the usage of propolis in food applications has increased in the last decades. The antimicrobial and antioxidant effects of propolis provide to the production of food and/or food additives, and are generally recognized as safe (Burdock, 1998), making it an attracting applicant as a natural preservative in new food applications.

In this study, propolis sample was collected and extracted. The TPC and TFC of propolis extract were determined as 46.26 ±1.18 mg GAE/mL and 11.63 ±0.97 mg QUE/ mL, respectively. Ferric reducing antioxidant power (FRAP) was determined as 0.27 ±0.07 µmol $FeSO_4.7H_2O$/mL (Table 1). The total phenolic content of the propolis sample is an important criterion for its quality. In a study, it was reported that the TPC of Turkish propolis samples ranged from 16.13 to 178.34 mg GAE/g (Keskin and Kolaylı, 2018). In another study, TPC of propolis samples obtained from the different localities of Bilecik City was reported in the range of 11 to 76 mg GAE/mL (Keskin *et al*. 2019).

The chemical characteristics of propolis extract was presented in Table 2. It was detected that propolis extract contained certain types of phenolic compounds like caffeic acid, ferulic acid, p-coumaric acid, galangin, chrysin, pinostrobin, and caffeic acid phenethyl ester. In earlier studies, the composition of propolis extract was determined as similar to our result. When compared with the literature, the findings of this study showed that the composition of propolis from Bilecik province stayed somehow the same.

Encapsulation efficiency was determined as 95% (Figure 1). According to the results obtained beads prepared by adding 1:1, 2:1 and 2.5:1 ratio of propolis extract contained 43.95, 87.90, and 109.87 mg GAE/g total phenolic content, respectively.

Antibacterial activity is one of the most important biological activities of propolis (Sforcin *et al*., 2000; Letullier *et al*., 2020; Al-Juhaimi *et al*., 2021). The antibacterial activity of propolis is higher against the Gram (+) bacteria (Lindenfelser 1967, Silici and Kutluca 2005). It has been reported that there is an important relation between the phenolic acid and flavonoid content of propolis and its antibacterial activity (Burdock 1998). These compounds were reported to be varied depending on some factors like the season, botanical source, and honey bee species (Sforcin *et al*., 2000; Przybyłek and Karpiński 2019, Letullier *et al*., 2020). In our study, pectin-encapsulated propolis samples obtained from the Bilecik region were added to ice cream at different concentrations and the antibacterial activity of whole ice cream on *S.aureus* (ATCC 25923), *E. coli* (ATCC 28712), *E. faecalis* (ATCC 51299), *K. pneumoniae* (ATCC 700603) and *B.cereus* (patient isolate) bacteria was evaluated over time. The antibacterial activity of propolis-added ice cream was found to be higher against gram-positive bacteria (Table 3). These results are compatible with the literature reports. As the propolis ratio increased, a partial increase was found in obtained antibacterial effect. However, no significant difference was found between the mean zone diameters of 2:1 and 2.5:1 propolis-added ice cream samples. Unpleasant changes in the color and flavor of ice cream were detected in a 2.5:1 ratio of encapsulated propolis added ice cream due to the highest amount of propolis. A similar finding was reported earlier (Özer *et al*., 2021).

Increasing the propolis concentration from 0.75% to 1.5% in ice cream samples obtained by adding encapsulated propolis for all ratios did not cause a remarkable difference in zone diameter. The 1% concentration of all propolis ratios in ice cream has higher antibacterial activity than 0.75% and a similar effect with 1.5%. While the antibacterial activity of encapsulated propolis added ice creams had insignificant zone diameters on *E. coli*, *B. cereus* and *K. pneumoniae* on the 1st day, effective values were detected on these bacteria on the 7th and 29th days (Table 3). It can be said that the sugar in the ice cream contributes to the antibacterial effect during the storage process. Our results showed that the highest efficiency was on *E. faecalis* and *S. aureus*. The effect on *S. aureus* is similar to other studies (Keskin *et al*., 2001; Marcucci *et al*., 2001; Silici

and Kutluca, 2005; Erkmen and Ozcan, 2008; Vardar *et al*., 2008; Kaya *et al*., 2012; Kolaylı *et al*., 2020). Cushine and Lamb (2005) reported that flavonoids were effective on *S. aureus* and *E. faecalis* causing a decrease in the number of living bacterial cells and inhibiting RNA synthesis of *S. aureus*. The antibacterial activity of the propolis sample obtained from the Bilecik region could be explained by its rich phenolic acids and flavonoid content like caffeic acid, ferulic acid, p-coumaric acid, galangin, chrysin and caffeic acid phenethyl ester. The effectiveness of propolis- added ice creams on *B. cereus* and *K. pneumoniae* appeared on the 7th and 29th days. Erkmen and Özcan (2008) reported that a 0.02% concentration of propolis samples obtained from the Gaziantep region had a bactericidal effect on *B. cereus* and *B. subtilis*. Fahad et al. (2021) reported that all of the propolis samples obtained from Konya, Adana, Osmaniye, and Muğla showed antimicrobial activity on *B. cereus*. Kaya *et al*. (2012) reported that ethanol extract of propolis sample from the Kayseri region was effective on *K. pneumoniae* with a MIC value of 512 (µg/ml). In the present study, the antibacterial activity of all ice cream samples on *E. coli* on the first day was insignificant. However, remarkable values were obtained after 7 and even 29 days of storage. It was determined that the average zone diameters of the ice creams containing 2:1 ratio of propolis were higher than the 1:1 ratio and similar to the values of the 2.5:1 ratio (Figure 2). Kaya *et al*. (2012) reported that a much higher MIC (1024 µg/ml) value was detected for *E. coli* than for other bacteria. Fahad *et al*. (2021) declared that all of the propolis samples obtained from Konya, Adana, Osmaniye, and Muğla were effective on *E. coli*. Propolis samples obtained from the Erzurum region showed the highest effect. Inadequate hygienic conditions can cause food contamination with pathogenic bacteria. Especially in foods with high nutritional value like ice cream, pathogenic bacteria can multiply very quickly and pose a risk to human health. Demir (2021) reported that the addition of propolis extract in 400, 800, and 1600 mg/L concentration caused 100% bacterial reduction on the second day of storage in all of the ice cream samples contaminated with *Listeria monocytogenes*. In another study antimicrobial activity of ethanol extract of propolis added ice cream was reported against enterotoxigenic strain of methicillin-resistant *Staphylococcus aureus* (MRSA) which inoculated into lab prepared ice cream. In that study it was mentioned that after two weeks incubation of ice cream at freezing temperature (-20 °C) methicillin-resistant *Staphylococcus aureus* (MRSA) could not be enumerated. It was also mentioned that anti-MRSA activity was increased by time (El-Bassiony *et al*. 2012). Similarly, increased antimicrobial activity of whole ice cream samples by time was detected in the present study.

Figure 1. Encapsulated propolis extract (Keskin *et al*., 2018)

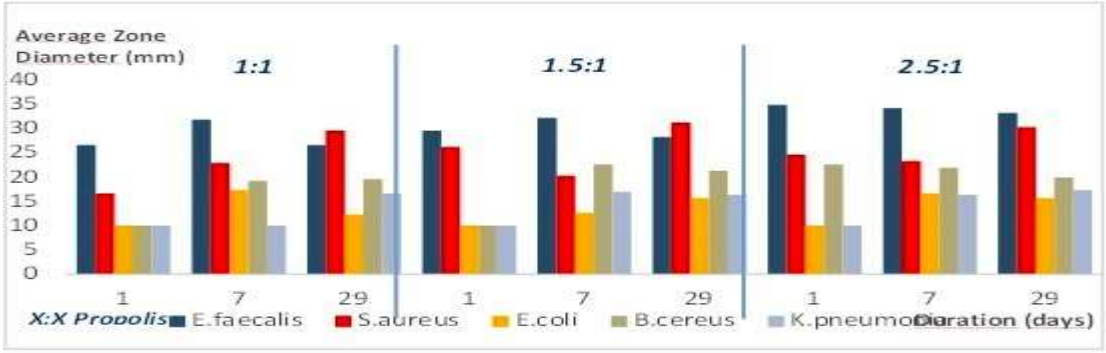

Figure 2. Average antibacterial activity across concentration levels and duration in days

In the light of our findings it could be said that the ice creams produced with the addition of propolis have a protective effect against the contamination risks that may occur during ice cream production. In particular, this effect increases during the storage period. In addition, it can be mentioned that it has a protective effect against bacterial risks that threaten oral health after ice cream consumption.

Table 1. Biochemical properties of propolis extract and encapsulated propolis.

	Total Phenolic Content (mg GAE/ mL)	Total Flavonoid Content (mg QUE/ mL)	Antioxidant Capacity (µmol $FeSO_4.7H_2O$/mL)	Encapsulation Efficiency (%)
Propolis Extract	46.26 ±1.18[a]	11.63 ±0.97[a]	0.27 ±0.07[a]	-
Encapsulated Propolis	43.95 ±1.18[b]	11.05 ±0.81[b]	0.26 ±0.07[a]	95.0 ±0.2

*The values with same letters in a column did not differ significantly (P<0.05)

Table 2. Chemical composition of propolis.

No	Retention Time (minute)	Detected Compound	Area (%)
1	11.68	Malic acid	0.2
2	11.82	Cinnamyl alcohol	0.2
3	11.96	Dihydrocinnamic acid	0.5
4	15.44	Cinnamic acid	1.2
5	19.69	Vanillic acid	0.2
6	22.22	5-Phenyl-2,4-pentadienoic acid	3.2
7	22.66	*p*-Methoxycinnamic acid	0.4
8	22.82	*p*-Coumaric acid	1.0
9	22.92	Palmitic acid	0.2
10	25.61	Caffeic acid	1.5
11	26.09	Ferulic acid	1.0
12	26.34	Isoferulic acid	0.7
13	26.64	Linoleic acid	0.1
14	27.13	Dimethoxycinnamic acid	1.3
15	31.44	Pentenyl caffeate (isomer)	2.8
16	34.33	Pinocembrin chalcone	17.5
17	34.78	Pinobanksin	3.3
18	34.84	Cinnamyl cinnamate	0.3
19	35.02	Pinostrobin chalcone	0.9
20	35.40	Pinocembrin	7.4
21	36.13	Chalcone derivative	5.2
22	36.31	Phenylethyl *p*-coumarate	0.2
23	36.37	Pinostrobin	0.2
24	36.58	Galangin	11.8
25	37.04	Benzyl caffeate	4.9
26	37.61	Benzyl ferulate	0.4
27	37.80	3-Methylpinobanksin	2.6
28	37.89	Pinobanksin-3-acetate	4.9
29	37.99	Chrysin	7.8
30	38.19	Caffeic acid phenethyl ester (CAPE)	2.7
31	39.04	Tectochrysin	0.8
32	39.55	Dihydroxymethoxy flavone	0.5
33	39.73	Pinobanksin-3-pentanoate	0.5
34	39.77	Cinnamyl *p*-coumarate	0.5
35	40.05	Kaempferol	0.5
36	41.41	Cinnamyl caffeate	3.2
37	42.00	Cinnamyl ferulate	2.1
38	42.97	Lupeol	0.5
39	44.36	Quercetin dimethyl ether (isomer)	0.2
40	45.02	Lanosterol	0.3

Table 3. Antimicrobial activities of ice cream samples against a range of microorganisms.

		Minimum inhibition zone diameters (mm)								
Day	**Propolis Rate**	1:1			2:1			2.5:1		
	Propolis concentration (%)	0.75	1.0	1.5	0.75	1.0	1.5	0.75	1.0	1.5
1	***E. faecalis***	20	30	30	25	32	32	35	35	35
	S. aureus	16	16	18	20	28	31	20	25	29
	E. coli	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
	B. cereus	< 10	< 10	< 10	< 10	< 10	< 10	20	20	28
	K. pneumonia	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
7	***E. faecalis***	31	31	34	32	32	33	33	35	35
	S. aureus	16	17	25	17	18	26	19	23	28
	E. coli	12	12	14	16	19	17	16	17	17
	B. cereus	18	20	20	22	23	23	20	22	24
	K. pneumonia	< 10	< 10	< 10	14	18	19	14	15	20
29	***E. faecalis***	19	31	30	25	30	30	31	35	34
	S. aureus	28	29	32	30	32	32	29	29	33
	E. coli	12	12	13	15	16	16	14	16	17
	B. cereus	19	20	20	20	20	24	19	18	23
	K. pneumonia	< 10	15	25	< 10	14	25	< 10	15	27

Conclusion: In this study, propolis ethanol extract was encapsulated by pectin to obtain the powdered form of propolis and it was used as a preservative in ice cream as a model food. Our findings showed that the powdered form of propolis was highly effective against tested pathogenic microorganisms, especially during the storage process of ice cream. Being protected from contamination and multiplying risk is very important for the food sector and this study could offer a solution to solve these problems.

Author contributions: **M.E.G:** Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Validation, Writing – original draft, review & editing **Ş.K:** Data curation, Formal analysis, Methodology, Project administration, Resources, Validation, Writing – original draft, review & editing **P.E.A:** Formal analysis, Validation **M.K:** Project administration, Formal analysis, Validation **S.K:** Formal analysis, Methodology, Validation

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