تأثیر عصاره‌های آبی و اتانولی بره‌موم بر بار میکروبی شیر خام

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چکیده

مقدمه: بره‌موم یکی از مهم‌ترین فراورده‌های فرعی زنبور عسل است که به‌واسطهٔ ویژگی میکروب‌کشی قوی، پتانسیل آن را دارد که به‌شکل افزودنی طبیعی غذایی استفاده شود. مطالعهٔ حاضر به‌منظور بررسی آثار عصاره‌های آبی و اتانولی بره‌موم بر بار میکروبی شیر خام انجام شد.

مواد و روش‌ها: نمونه‌های شیر خام حاوی غلظت‌های ۰/۱، ۰/۵، ۱ و ۲ درصد عصارهٔ اتانولی و ۰/۵، ۱، ۳ و ۵ درصد عصارهٔ آبی تهیه شدند. تیمارها در دماهای ۴ و ۲۵ درجهٔ سانتی‌گراد گرمخانه‌گذاری شدند و در طول زمان نگهداری صفر، ۲، ۲۴، ۴۸ و ۷۲ ساعت، تأثیر غلظت‌های یادشدهٔ بره‌موم بر بار میکروبی شیرخام با کشت پورپلیت در محیط کشت پلیت‌کانت‌آگار و شمارش کلی ریزموجودات بررسی شد. ترکیبات شیمیایی با گازکروماتوگرافی متصل به طیف‌نگار جرمی شناسایی شدند.

نتایج: در دمای ۲۵ درجهٔ سانتی‌گراد، تمام غلظت‌های عصاره‌های آبی و اتانولی بار میکروبی شیر خام را تحت‌تأثیر قرار دادند، ولی این اثر در غلظت‌های کم پس‌از ۲۴ ساعت نمایان شد. در دمای ۲۵ درجهٔ سانتی‌گراد در مقایسه با دمای ۴ درجهٔ سانتی‌گراد، عصاره‌های آبی و اتانولی بره‌موم در غلظت‌های کمتر فعالیت ضدمیکروبی بیشتری را نشان دادند و بره‌موم برای اینکه بتواند فعالیت ضدمیکروبی زیادی داشته باشد، به زمان نیاز داشت؛ ترکیبات شیمیایی بره‌موم باگاز کروماتوگرافی مایع تحلیل و ۴۱ ترکیب با غالبیت ترکیبات فنلی و فلاونوئیدی شناسایی شدند.

بحث و نتیجه‌گیری: خاصیت ضدمیکروبی عصاره‌های بره‌موم مطالعه‌شده می‌تواند با محتوای ترکیبات فنلی و فلاونوئیدی زیاد آنها مرتبط باشد و امکان استفاده از آن به‌شکل افزودنی طبیعی در مواد غذایی مطرح می‌شود.

واژه‌های کلیدی: بره‌موم، عصارهٔ آبی، عصارهٔ اتانولی، بار میکروبی، شیر خام

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Effects of Ethanolic and Aqueous Extracts of Propolis on the Microbial Load of Raw Milk

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Abstract

Introduction: Propolis is one of the most important bee byproducts that, due to its high antimicrobial properties, has the potential to be used as a food additive. This study investigated the effects of aqueous and ethanolic extracts of propolis on the microbial load of raw milk.

Materials and methods: Raw milk samples containing 0.1, 0.5, 1, and 2% ethanolic extracts and 0.5, 1, 3, and 5% aqueous extracts were prepared. The treatments were incubated at 4 °C and 25 °C and were cultivated by pour-plate technique in PCA medium during storage times of 0, 2, 24, 48, and 72 hours. By a total count of microorganisms, the effects of different concentrations of propolis extracts on the microbial load of raw milk were investigated. The chemical compounds of the extracts were identified by gas chromatography–mass spectroscopy (GC-MS) analysis.

Results: At 25 °C, all concentrations of aqueous and ethanolic extracts affected the microbial load of raw milk, but at low concentrations, such effects were observed after 24 hours. Aqueous and ethanolic extracts of propolis at 25 °C compared to 4 °C in lower concentrations showed more antimicrobial activity. It was also found that propolis extracts need time to exhibit high antimicrobial activity. Forty-one compounds were identified with the dominance of phenolic and flavonoid compounds.

Discussion and conclusion: The antimicrobial properties of the tested propolis extracts can be attributed to the high amounts of phenolic and flavonoid compounds and the use of this bee product as an additive is recommended.

Keywords: Propolis, Aqueous Extract, Ethanolic Extract, Microbial Load, Raw Milk.

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Introduction

Propolis is one of the bee byproducts produced by honey bees by combining their own saliva and beeswax with exuded substances they collect from gum trees, sap flows, and other botanical sources. Honey bees produce propolis by ingestion of gum, gastrointestinal activity, and the action of the beta-glucosidase enzyme secreted from the salivary glands. This propolis is used to seal the pores in the hollow and prevent the effects of light, moisture, counteracting external factors, disinfecting the internal environment, and adjusting the internal temperature of the hives. Its color varies depending on its botanical source, the most common being dark brown (1,2).

The chemical composition of propolis depends on botanical, geographical, and meteorological characteristics of the area where the bee feeds (3). In general, raw propolis consists of approximately 45-50% resins, 23-35% waxes, 10% essential oils, 5% pollen, and 5% of various organic compounds and minerals (ketones, calcones, benzoic acid, Vitamins, zinc, iron, ...). Using biochemical analyses, various compounds including flavonoids, alcohols, alpha acids, amino acids, aromatic acids, aromatic esters, calcones, terpenoids, sugars and steroids, and other substances in the propolis have been identified. But, the main compounds are phenolic compounds, flavonoids (flavanons and flavones), phenolic acids, and their esters (4). The biological properties of propolis, such as antibacterial, antifungal, antiviral, antitumor, and antioxidant properties have been proven (3,5,6).

Chemical compounds responsible for the beneficial biological activity of propolis, especially antimicrobial and antioxidant activities, including flavonoids (flavones, flavonools, flavans, dihydroflavonols), and other phenolic compounds are mainly cinnamic acids and other esters. Antimicrobial activity is present in all propolis of different regions, but different geographical origins, areas vegetation, and botany affect the rate of this antimicrobial activity (3,7).

The antimicrobial and antioxidant properties of propolis suggest its use as a preservative in the food industry. Milk is one of the main food groups for supplying many essential nutrients such as calcium and vitamin. This important food is a suitable culture medium for the growth of various microorganisms and thus it quickly gets spoiled. Therefore, it is important to reduce the number of microorganisms in such full food for the food industry and quality control as well as the public health. Antimicrobial preservatives and compounds are one way to control the growth of microorganisms in foods. Adding chemicals and antibiotics is not a suitable method as it could have consequences. Therefore, research is strongly felt on novel food additives with a natural origin. The aim of this study was to evaluate the effects of adding different concentrations of aqueous and ethanolic extracts of propolis on the microbial load of raw milk.

Materials and Methods

Propolis Samples: Crud propolis samples were collected from beehives at Kordkuy township of Golestan province in the north of Iran and until the experiments were kept

at the refrigerator of the Microbiology Laboratory of Islamic Azad University, Azadshahr Branch.

Preparation of Aqueous and Ethanolic Extracts of Propolis: The propolis sample was cut into small pieces and in order to obtain aqueous and ethanolic extracts, distilled water and 80% ethanol (1-10 w/v) were added, respectively, and mixed well on a shaker for 72 hours at the room temperature. Then, the solution of aqueous and ethanolic extracts was filtered with Whatman No. 4 filter paper using the vacuum pump, and condensation and solvent removal were placed in a rotary evaporator under vacuum conditions at 50 °C. These pure extracts were stored in a dark place at 4 °C until the experiments (8-10).

Determination of the Microbial Load of Raw Milk: After the preparation of aqueous and ethanolic extracts, to investigate the effects of different concentrations of these extracts on the microbial load of raw milk, first, the microbial load of raw milk prepared from a milk collection center in Azadshahr city was evaluated.

In this regard, due to the high level of microbial contamination of the raw milk, from the prepared milk samples, serial dilutions and 1 ml of the dilutions were cultivated by the pour-plate technique in a plate count agar medium (PCA; Merck) and after 48 hours incubation at 30 °C, the number of colonies was counted in plates with 30-300 colonies. By multiplying the dilution factor, the total number of microorganisms in each ml of raw milk was obtained and the results were expressed as CFU/ml.

Preparation of Treatments: Certain amounts of aqueous and ethanolic propolis extracts were added to the raw milk whose microbial load was determined. As a result, the raw milk containing concentrations of 0.1, 0.5, 1, and 2% ethanolic extract and concentrations of 0.5, 1, 3, and 5% of aqueous extract of propolis were prepared. The samples were incubated in 4 °C and 25 °C, and after 0, 2, 24, 48, and 72 hours were cultivated by the pour-plate technique in plate count agar medium (PCA; Merck). After the incubation at 30 °C for 48 hours and colonies counting, the total count of microorganisms (CFU/ml) was obtained from each sample of the raw milk containing different concentrations of aqueous and ethanolic extracts of propolis at different temperatures (4 °C and 25 °C) and time periods (0, 2, 24, 48 and 72 hours).

The Chemical Compounds Analysis: Quantitation of composition of propolis was carried out by Gas Chromatography–mass spectrometry (GC-MS). GC-MS was performed using a Hewlett–Packard 5890 Series II gas chromatographer (Palo Alto, CA) equipped with a 60 m × 0.22 mm internal diameter, Supelco 2380 capillary column with 0.25 mm film thickness (Supelco, Inc., Bellefonte, USA). The column oven temperature was programmed from 60 °C (2 min hold) to 250 °C at 3 °C/min. The injector and detector were maintained at 250 °C and 300 °C, respectively. The sample injection volume was employed and the run time was 60 min. Retention times and peak areas were automatically computed by a DP 700 computing integrator Hewlett–Packard 3396 Series II (Palo Alto, CA) identified by the comparison with the absolute and relative retention time of a known standard mixture of 37 components (Supelco, Barcelona, Spain) from our own data bank. The peaks were identified by computer searches in commercial reference libraries.

Statistical Analysis: In order to increase the accuracy and sensitivity, each test was done in triplicate. Statistical analysis was performed using SPSS15.0 (SPSS, Inc., Chicago, IL, USA, 2006), Windows 7. Means were compared with an analysis of variance (ANOVA) followed by the

Duncan test to determine among means at $p \leq 0.05$ level.

Results

The Effect of Different Concentrations of Ethanolic and Aqueous Extracts on Changes of Microbial Load of Raw Milk during Storage at 4 °C: The changes process in the microbial load of raw milk containing different concentrations of aqueous and ethanolic extracts of propolis at 4 and 25°C during storage times of 2, 24, 48, and 72 hours were investigated.

Based on Fig. 1, it is clear that at 4 °C, different concentrations of ethanolic extract of propolis have not made a change in milk microbial load up to 48 hours. Only at a 2% concentration of ethanolic extract, such decrease can be seen from the beginning of the time periods examined (Fig. 1).

In Fig. 2, the results of the aqueous extract show that the effects of 3 and 5% aqueous extracts on the reduction of the logarithm of the number of raw milk microorganisms, such as ethanolic extract, appear to be more pronounced over time (Fig. 2).

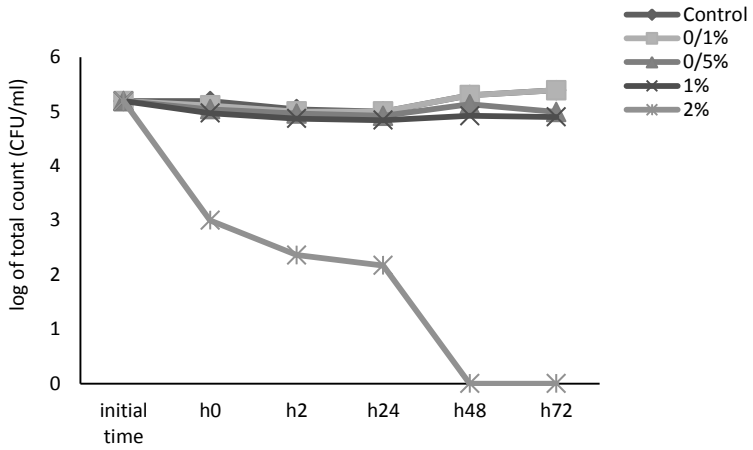

Fig. 1- The effect of different concentrations of ethanolic extract of propolis on the logarithm of microbial total count (log CFU/ml) in raw milk during storage at 4 °C

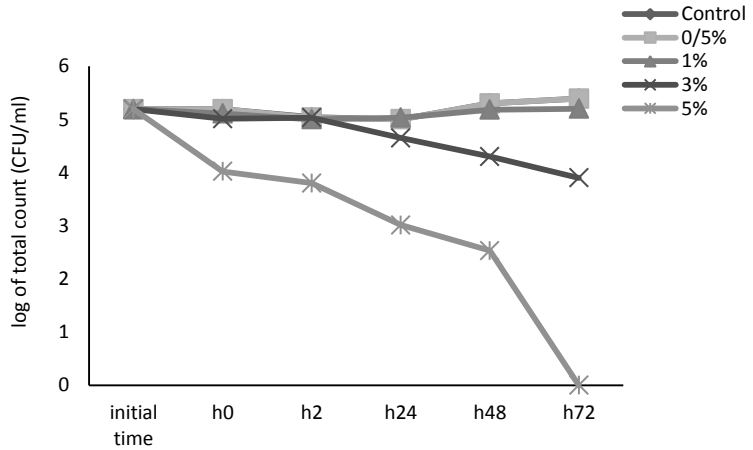

Fig. 2- The effect of different concentrations of aqueous extract of propolis on the logarithm of microbial total count (log CFU/ml) in raw Milk during storage at 4 °C

The Effect of Different Concentrations of ethanolic and Aqueous Extracts on Changes of Microbial Load of Raw Milk during Storage at 25 °C: In Fig. 3, the results of the effect of different concentrations of ethanolic extract of

propolis on the decrease logarithmic microbial count (log CFU/ml) in raw milk during storage at 25 °C are shown. As can be seen, at 25 °C, there is a significant difference between the amount of microbial load in the control treatment and the raw milk containing different concentrations of the ethanolic extract.

Unlike the temperature of 4 °C, the concentration of 0.5% ethanolic extract can also reduce the microbial load of raw milk at 25 °C (Fig. 3). This indicates that propolis at 25 °C has a greater effect on the microbial load than the temperature of 4 °C and this may be because the solubility of propolis is higher at 25 °C. This can affect the penetration and transition from the cellular barrier of microorganisms. Of course, the effects of propolis even at a concentration of 0.1% can be seen after 72 hours suggesting that propolis needs time to be effective (Fig. 3).

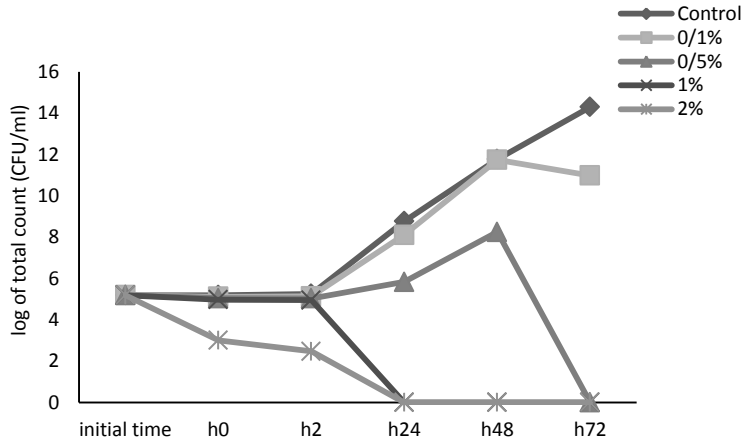

Fig. 3- The effect of different concentrations of ethanolic extract of propolis on the logarithm of microbial total count (log CFU/ml) in raw milk during storage at 25 °C

As can be seen in Fig. 4, concentrations of 3% and 5% of the aqueous extract can have significant effects on the microbial load of raw milk at 25 °C. Of course, after 48 hours, the effects on reducing microbial load are also observed at lower concentrations. It can be concluded that the antimicrobial activity of the propolis extracts is more observed after 24, 48, and 72 hours, and no changes are observed at the initial times (Fig. 4).

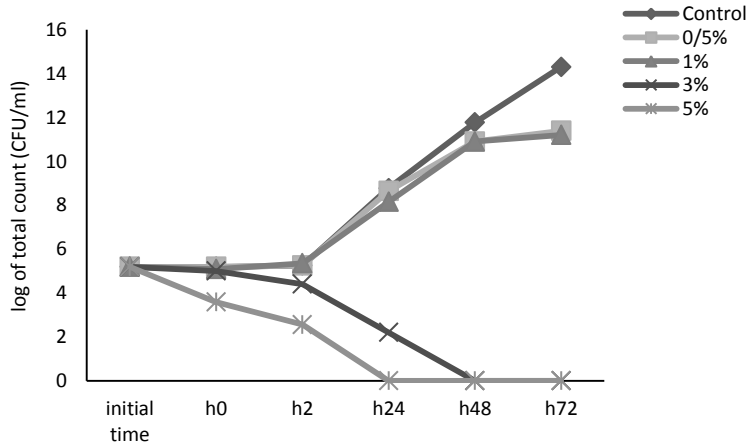

Fig. 4- The effect of different concentrations of aqueous extract of propolis on the logarithm of microbial total count (log CFU/ml) in raw milk during storage at 25 °C

There is a significant difference between the concentration of 2% ethanolic extract with other concentrations, and also between 3% and 5% aqueous extracts with other concentrations. Thus, a significant antimicrobial activity could be observed at these concentrations.

The role of time is quite evident regarding the effect of aqueous and ethanolic extracts of propolis at 4 °C and 25 °C. The results showed that at both temperatures, the maximum reduction in the microbial load of raw milk was observed at 72 hours and no significant antimicrobial activity was observed at 0 and 2 hours. As the storage time of raw milk containing propolis extracts increases, the microbial load decreases further, and this suggests that propolis needs time to exhibit a high antimicrobial activity.

Identification of Chemical Compounds: Considering the antimicrobial effects of propolis extract, the analysis and identification of the active compounds of this substance is an indispensable necessity. The identification of mixtures of natural products by chromatography is rather difficult due to the number of isomers and minor differences in them. Hence, the peak numbers in Table 1 are presented only according to the retention time of the major peaks. The main types of compounds were identified as listed in Table 1. Forty-one compounds with the dominance of phenolic and flavonoid compounds were identified.

Table 1- Identified Compounds of Propolis

Peak number	RT (minutes)	Substances
1	3.630	2(3H)-Furanone, 5-butyldihydro
2	4.446	3,8-Dioxatricyclo[5.1.0.0(2,4)]
3	9.614	1 2-Bromopropionyl bromide
4	10.099	1 Benzene, 1,5-difluoro-2,4-dinitro
5	10.313	1,2-Cyclobutanedicarboxylic acid
6	16.503	1 Tridecanoic acid
7	19.498	Pyrazine, ethoxy
8	20.119	Heneicosane
9	20.300	2-Nonadecanone
10	21.497	Osthole
11	21.723	OleicAcid
12	21.833	(E)-1-Indan-1-ylethanone oxime
13	23.709	2,2-Dimethyl-N'-[2-(trifluoromet
14	24.757	2,5-Dimethoxyterephthalic acid
15	26.627	Heptadecane
16	29.693	2-Propen-1-one, 1-(2,6-dihydroxy
17	32.215	Cyclopentanemethanamine, 2-amino
18	33.244	Octadecane
19	34.033	Phenol, 4,4'-(1-methylethylidene
20	35.301	4H-1-Benzopyran-4-one, 5-hydroxy
21	36.103	Hexacosane
22	36.394	Propenone, 1-(benzo[b]-1,4-dioxa
23	36.840	2H,8H-Benzo[1,2-b:3,4-b']dipyran
24	37.080	Phenazopyridine P358
25	37.436	2-Trimethylsilyl-3-trimethylsily
26	38.251	(S)-8,8-Dimethyl-2-oxo-7,8-dihyd
27	38.923	Tetracosane
28	39.053	But-2-enoic acid, 2-methyl-,
29	39.279	1-Docosene
30	39.447	Triacontyl trifluoroacetate
31	39.823	(S)-8,8-Dimethyl-2-oxo-7,8-dihyd
32	40.456	Carbamic acid, (cyanoacetyl)-, e...

33	40.638	15,19-Dimethylpentatriacontane
34	40.903	Tetracosane
35	42.436	Carbamic acid, (cyanoacetyl)-, e...
36	42.520	1-Nonadecene
37	43.031	Nonacosane
38	43.361	Cyanic acid, ethyl ester
39	44.150	Triacontane
40	44.668	Difluoromalonic acid,diethyl ester
41	44.842	9-Tricosene, (Z)-

Discussion and Conclusion

Many studies have been done to find natural ingredients to add to foods. In a general view, the results of this study showed that propolis, due to its high antimicrobial effects, has the potential to be added to food to reduce the microbial load. Generally, the effect of different concentrations of propolis extracts on the microbial load of raw milk was clear in this study, and by increasing the concentration of extracts, a significant decrease was observed in the number of microorganisms in raw milk (P=0.05). In a similar vein, Fu Lian et al. (2009) reported the effect of propolis extract with the concentration of 0.2% on increasing the shelf life of pasteurized milk (11).

There is a significant difference between the concentration of 2% ethanolic extract with other concentrations, and also between 3% and 5% aqueous extracts with other concentrations. So, a significant antimicrobial activity has been observed at these concentrations.

The role of time is quite evident in the effect of aqueous and ethanolic extracts of propolis at 4 °C and 25 °C. The results showed that at both temperatures, the maximum reduction in the microbial load of raw milk was observed at 72 hours and no significant antimicrobial activity was observed at 0 and 2 hours. As the storage time of raw milk containing propolis extracts increased, the microbial load decreased further, and this suggests that propolis needs time to exhibit a high antimicrobial activity.

El-Bassiony et al. (2012) investigated the effect of time on the antimicrobial activity of propolis. They investigated the antimicrobial activity of ethanolic extracts of propolis against *S. aureus* inoculated in ice cream prepared in the laboratory. The results of their research showed that the antibacterial effects of propolis extract increased over time. This is consistent with the present research results (9).

The antimicrobial activity of aqueous and ethanolic extracts of propolis was higher at 25 ° C. The aqueous and ethanolic extracts of propolis at 25 °C compared to 4 °C showed the antimicrobial activity at lower concentrations. At 4 °C, the effects of aqueous and ethanolic extracts of propolis on the microbial load of raw milk were limited to 2% ethanolic extract and 3% and 5% aqueous extract.

The effect of propolis at 25 °C has also been reported in other studies. Bahtiti (2013) investigated the protective effect of Jordanian propolis on the quality of potato mashed. He reported that when the microorganisms were treated with propolis, the population of food spoilage microorganisms decreased by 2.6-3.7 log cycles. The inhibitory effect increased in more acidic conditions (12).

Payandan et al. (2017), in a study on the efficacy of different extracts of Iranian propolis on the microbiological parameters of minced Cyprinus carpio meat at 4 °C storage reported that the antimicrobial activity of the ethanolic extract in concentrations of 3%, 5%, and 7% was higher than the aqueous extract. This is in line with the results of this study (13). Considering the antimicrobial effects of

propolis extract, the analysis and identification of the active compounds of this substance is a necessity. The chemical compounds of propolis depend on the vegetation of the region where the propolis is collected (6).

The compounds responsible for the biological activity of propolis are different and variable in samples of different origins. Analyzing the extract of propolis samples by gas chromatography could help know its composition and promote biological properties. Of course, due to a large number of compounds and isomers and their variety in natural products, it is very difficult to identify all the compounds by chromatography (14). The identified compounds were similar to previous compounds found in other studies; however, the variability and differences of constituents of propolis in different studies showed that they were collected by the honeybee from different plants depending on the geographic location.

The antibacterial activity of propolis extracts is attributed to the presence of high levels of flavonoids and caffeate esters compounds in aqueous and ethanolic extracts of propolis (15, 16). Yaghoubi et al. (2007) determined the chemical composition of ethanol extract of Iranian propolis. They found the presence of pinocembrine, caffeic acid, kaempferol, phenethyl caffeate, chrysin, and galangin in Iranian propolis (17). The results of a higher antibacterial activity of propolis ethanolic extract are consistent with other findings that attributed the antimicrobial activity of propolis ethanolic extract to its components such as pinostrobin, naringenin, chrysin, dihydrochrysin, and caffeic acid esters (16, 18, 19, 20, 21, 22).

Generally, the comparison of the microbial load in all treatments indicated the effect of the tested propolis on the reduction of microbial load. Among the treatments, the treatment containing 2% ethanolic extract and 3% and 5% aqueous extract at the temperature of 4 °C showed a significant influence on the inactivation of the growth of the microbial load. At 25 °C, all concentrations of the aqueous and ethanolic extracts affected the microbial load of the raw milk, but at low concentrations, these effects were observed after 72 hours. Aqueous and ethanolic extracts of propolis at 25 °C compared with 4 °C in fewer concentrations showed a greater antimicrobial activity and propolis needed time to exhibit a high antimicrobial activity.

Conflicts of Interest

There was no conflict of interest in this study.

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