



Antimicrobial and Antioxidant Activities of Commercialized Turkish Propolis Extract, and Application to Beef Meatballs

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ABSTRACT

Propolis is a resinous substance produced by bees that is rich with phenolic and flavonoid compounds. Extract of propolis has a high antioxidant and antimicrobial properties due to the presence of these compounds. Therefore, the main objectives of this study were to examine the antibacterial and the antioxidant activities of standardized, commercial water extract of propolis in vivo assays, and to assess its impact on preservation of raw beef meatballs aerobically packaged and stored at 4°C for 7 days. The results showed that the propolis extract demonstrated the highest antibacterial activity against *Staphylococcus epidermidis* in vivo test. Furthermore, meatballs prepared with the propolis extract had an extended shelf life about a week in comparison to the regular meatballs. Besides, the propolis extract was a very effective natural antioxidant agent for controlling the oxidative changes in meatballs. The propolis treatment provided 64.6% reduction in the malondialdehyde formation at the final day of storage. Color lightness and yellowness values of meatballs were not affected by the propolis treatment, only difference was observed in redness values. The pH of the meatballs prepared with the propolis extract almost stayed constant during storage, while the pH of the control meatball samples increased. In summary, propolis extract exhibited a strong antimicrobial and antioxidant activity in vivo assays and in a meat product. Accordingly, it should be used in meat product formulations to enhance preservation of meat products.

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Introduction

Bees collect resinous and balsamic substances from various parts of plants and trees including flower buds, leaves and barks. Then, propolis is produced through bee salivary secretions and enzymatic changes (Lotti et al., 2010). Bees use propolis to support structural integrity of the hive, and to protect against insects and microorganisms. Meanwhile, people have been using propolis as antiseptic, antioxidant and anti-inflammatory since the ancient times (Toreti et al., 2013).

Various bioactive chemicals have been identified in propolis such as flavonoids (pinostrobin), flavonols (galangin), flavones (chrysin), flavanone (pinocembrin), and phenolic acids (caffeic acid) (Schnitzler et al., 2010; Rivero-Cruz et al., 2020). In numerous studies, antioxidant (Rivero-Cruz et al., 2020), antiviral (Liao et al., 2021), antifungal (Marwa Ezz El-Din Ibrahim and Randah Miqbil Alqurashi, 2022), and antibacterial activity (Przybyłek and Karpinski, 2019) of different propolis extracts have been demonstrated.

Bioactivity of propolis depends on many factors such as geography, season, plant resource, composition and concentration of phenolic compounds, and extraction methodology (Toreti et al., 2013; Rivero-Cruz et al., 2020; Bakkaloglu et al., 2021). It has been noted that Turkish propolis in particular with the presence of wide range of flavonoids (Coskun et al., 2018) and phenolics (Bakkaloglu et al., 2021) has an elevated antioxidant and antimicrobial activity (Ristivojevic et al., 2018).

In the food industry, there is a major interest to find natural ingredients that are non-toxic to humans, effective against pathogenic and/or spoilage organisms, and delay the physicochemical changes occurs in food products. Especially in meat industry, there isn't a single ingredient used in meat formulations to provide both antimicrobial and antioxidant properties. On the other hand, propolis extract with its high antioxidant and antimicrobial activity could be a great functional ingredient in meat products. Despite of its high bioactivity there are only a few studies

investigated the antioxidant and antimicrobial potential of propolis extract in meat products (Soare's dos Reis et al., 2017; Mahdavi-Roshan et al., 2022). Therefore, the aim of this work was, first to measure the antibacterial and antioxidant activities of Turkish propolis extract, and second to determine its effectiveness for preserving raw ground beef meatballs.

Materials and Methods

Extract Preparation

Standardized water extract of propolis was obtained from a retail (148 mg/mL pure propolis).

Antibacterial Testing

A disc diffusion method was used to detect the antibacterial properties, which was described in a previous study by Gedikoğlu et al. (2019). Propolis extract was very turbid, therefore, the extract was diluted with distilled water (1:1, v/v) twice, providing pure propolis concentration of 0.74 mg/20 µL, before its use in the assay. The extract was tested against 9 bacteria. The test was performed in duplicate.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

The 96-well multiplate method of Prieto (2012) was employed with modifications. First, a 100 µL of methanol was placed in to the each well. Then, a 100 µL of propolis extract (2 mg/mL) was placed into the first well in the 1st column of the plate. After proper mixing of extract by hold and release multiple times, A 100 µL of the methanolic propolis extract was transferred to the next well, providing 50% dilution of the extract concentration for the next well. The 12th column was left for the blank (Methanol-DPPH solution). Later, a 100 µL of a 0.2 mM DPPH methanolic solution was placed into the each well allowing incubation at room temperature for 30 min in the dark. The absorbance values of extract was obtained at 517 nm. Using the following formula, inhibition (%) of the DPPH radical was calculated.

$$I (\%) = \frac{(A_{\text{blank}} - A_{\text{sample}})}{A_{\text{blank}}} \times 100$$

A_{blank} : Absorbance of methanol and DPPH radical

A_{sample} : Absorbance of propolis extract with the DPPH radical

The amount of propolis extract (µg/mL) producing 50% inhibition (IC_{50}) was determined by plotting extract concentrations against inhibitions (%). The study was carried out in three independent replications.

The Ferric Reducing Antioxidant (FRAP) Assay

The method of Riahi et al. (2013) was used with modifications. In order to prepare the FRAP reagent, a 300 mM acetate buffer (pH 3.6), a 10 mM 2,4,6-Tris(2-pyridyls-triazine) (TPTZ) solution in 40 mM HCl, and a 20 mM $FeCl_3 \cdot 6H_2O$ solution were mixed together in order of 10:1:1 (v:v:v). After that, the propolis extract was mixed

with the FRAP reagent in the ratio of 1:90 (v:v), incubated at 37°C for 15 min, then, the absorbance was measured at 595 nm. The FRAP antioxidant capacity was determined by using the $FeSO_4 \cdot 7H_2O$ standard curve. The results were stated as µM of Fe^{+2} /g of extract. The tests were carried in three replications.

Meat Sample Preparation

Fresh ground beef was obtained at the day of the analysis from three local stores (to provide three independent replications) in Konya, Türkiye. Two treatment groups were prepared, which were the control treatment without the extract and the propolis treatment with the extract. For all three replications, ground beef was randomly separated into a half. First half of the ground beef, which was the control treatment, was shaped, placed on the Styrofoam tray, filmed, and labeled for the treatment and the day of storage. While, the other half was mixed with Turkish (Anatolian) water extract of propolis at the concentration of 13.15 mg pure propolis/g of meat sample to provide the propolis treatment. Then, packages were prepared following the same procedure as the control treatment. The meat samples tested for total mesophilic count (TMC), Enterobacteriaceae count, lipid oxidation, heme iron, pH, and color L^* , a^* , b^* values during refrigerated storage at day 1, 3, 5 and 7.

Microbiological Assessment

The microbial changes of aerobically packaged raw ground beef meatball treatments prepared with and without propolis extract were evaluated at day 1, 3, 5, and 7. Total mesophilic count of meat samples was determined according to the pour plate method. (ISO, 2003). The enumeration of Enterobacteriaceae was carried out according to ISO (2004). Logarithms of colony forming units per gram of meat (\log_{10} CFU/g) was used to calculate the number of microorganisms.

Thiobarbituric Acid Reactive Substances (TBARS) Assay

The testing for lipid oxidation was adapted from Witte et al. (1970). Briefly, a 10 g of raw ground beef sample was blended with 20 mL of 10% trichloroacetic acid. The slurry was centrifuged at 3000 rpm for half an hour at 4°C, then it was filtered to extract lipid fractions. After that, a 2 mL of the filtrate and the same amount of 20 mM thiobarbituric acid went through a reaction at 97°C for 20 min. The absorbance of the reactant was measured at 532 nm using a spectrometer at room temperature. The standard curve of the 1,1,3,3, tetra methoxypropane (precursor of malondialdehyde) was used to calculate the TBARS value, and the results were expressed in mg MDA/g sample.

Heme Iron Content

The method of Clark et al. (1997) was used to assess the total heme iron content of samples. A 2 g of ground beef sample was mixed with 9 mL of acidified acetone (90% acetone, 8% deionized water, 2% HCl). The mixture was incubated for an hour at ambient temperature in the dark. Then, the absorbance of the filtered solution was measured at 640 nm. The following formulation was used to calculate the heme iron content.

Heme iron ($\mu\text{g/g}$ of meat) = $A_{640} \times 680 \times 0.0882$

pH and Fat Content

Ground beef samples were mixed with distilled water (1:10, w/v), and the pH of the mix was tested using a digital pH meter at room temperature at day 1, 3, 5, and 7. The fat content of ground beef samples was determined at day 1 according to TSE (2003) standards using a semi-automated soxhlet extraction system (Velp Scientifica SER 148, Italy).

Color Measurements

A ser-lab SL400 colormeter was used to determine Lightness (L^*), redness (a^*), and yellowness (b^*) values of ground beef meatball samples. White and black standard plates were used to calibrate the instrument. Then, the color measurements were taken at randomly chosen locations of the flattened surface of meat samples.

Statistical Analysis

The data was expressed as the mean \pm standard deviation. There were three independent experimental replications. A two-way ANOVA (Stata IC 14, Stata Corp., USA) was used to test the effect of treatment and storage on TMC, Enterobacteriaceae count, TBARS value, heme iron content, pH, and color values. The Tukey test ($P < 0.05$) was used to test the difference in mean values between the treatments.

Results and Discussion

Antibacterial Effect of Propolis Extract

The results of the disk diffusion assay are shown in Table 1. Turkish propolis extract with 37 mg/mL (740 $\mu\text{g/disk}$) pure propolis concentration showed antimicrobial properties against all the tested bacteria. For the Gr+ bacteria, propolis was the most effective against *Staphylococcus epidermidis* and followed by *Enterococcus faecalis* and *Listeria monocytogenes*. Also, propolis extract showed stronger antibacterial activity against the Gr+ bacteria (10 – 26.5 mm) than the Gr- bacteria (6 – 13.5 mm). Comparable results were found by Bakkaloglu et al. (2021). They also reported that ethanol as an extraction solvent provided higher antimicrobial activity for the Turkish propolis extracts in comparison to propylene glycol, dimethyl sulfoxide, and distilled water. Uzel et al. (2005) stated that Anatolian propolis extract demonstrated a high antibacterial activity against *Streptococcus sobrinus* and *E. faecalis*. Furthermore, Ristivojevic et. al. (2018) noted that Turkish propolis extract showed a strong antimicrobial activity against oral infection causing bacteria *S. pyogenes*. Regarding to the results of Gr- bacteria in this study, it was found that propolis extract displayed a good antimicrobial effect against one of the major foodborne pathogens *Salmonella typhimurium* with 13 mm inhibition zone and followed by *Escherichia coli* and *S. enteritidis* with 9 and 6 mm, respectively. The results were in an agreement with earlier studies (Przybylek and Karpinski, 2019; Letullier et al., 2020) that Gr- bacteria was more resistant to antibacterial activity of the propolis extract due to the difference in their membrane structure.

Antioxidant Activity of Propolis Extract

It has been stated that antioxidant activity of propolis extract depends on essentially on the phenolic and flavonoid content. These compounds provide antioxidant activity through interrupting free radical chain reactions by donating protons to radicals (Irigoitia et al., 2021). The result of the antioxidant assay proved that Turkish propolis water extract had a high antioxidant activity. The IC_{50} value of propolis extract was $38.025 \pm 0.135 \mu\text{g/mL}$. Guzelmeric et al. (2018) suggested that radical scavenging activity of Turkish propolis extracts is due to the presence of o-dihydroxy phenyl structure included phenolics such as kaempferol, quercetin, and caffeic acids. The results of the DPPH assay were within the range with the earlier studies conducted with the Turkish propolis extracts (Ozdal et al., 2019; Bakkaloglu et al., 2021). In addition, propolis extract used in this study had a much higher antioxidant activity than the Mexican propolis extract (Vargas-Sanchez et al., 2019). Furthermore, When the results of the FRAP assay was used for comparison of the antioxidant activity, the Turkish propolis extract demonstrated much higher antioxidant activity ($23.27 \pm 0.26 \mu\text{M}$ of Fe^{+2}/g) than the encapsulated Brazilian propolis extract ($3.39 \pm 0.01 \mu\text{M}$ of Fe^{+2}/g) (Reis et al., 2017).

Changes in Microbial Quality

The effect of propolis extract and storage on the total mesophilic count of raw beef meatballs is presented in Figure 1. The results of the statistical analysis showed that the TMC was significantly ($P < 0.05$) influenced by the main effects (treatment \times storage). The TMC increased during storage particularly for the control treatments. During day 1 and 3, there was no significant difference ($P > 0.05$) between the treatments. On the contrary, at day 5 and 7, antimicrobial properties of propolis extract were observed against the mesophilic bacteria. Propolis extract diminish the TMC with the increase in its effectiveness during the storage ($P < 0.05$). The TMC count of the propolis treatment was reduced by 15.4% (1.39 log CFU/g) at day 5, and 24.9% (2.42 log CFU/g) at day 7 in comparison to the control treatment. It has been suggested that antimicrobial action mechanism of propolis is due to inhibition of cell division and causing bacteriolysis (Takaisi-Kikuni and Schilcher, 1994). Vargas-Sanchez et al. (2014) stated that propolis treatment provided 1 log CFU/g reduction in the TMC count for beef patties after 8 days. Despite the fact that the initial microbial quality of the product was low and above the hygiene standards (6 log CFU/g) (ICMSF). Nevertheless, the TMC of the propolis treatment remained same at the final day of storage as the initial day of storage, and delayed the increase in the TMC about a week, showing a strong antibacterial action.

The results of the Enterobacteriaceae count are displayed in Figure 2. Both the treatment and the storage had a significant ($P < 0.05$) effect on the Enterobacteriaceae count. Coliforms, fecal coliforms, *Escherichia coli* and *Salmonella* spp. are part of the Enterobacteriaceae family. The presence of Enterobacteriaceae in high numbers could be used as a criterion for fecal contamination and to evaluate the hygiene standards (Halkman and Halkman, 2014). The antimicrobial effect of propolis extract against Enterobacteriaceae count increased over time.

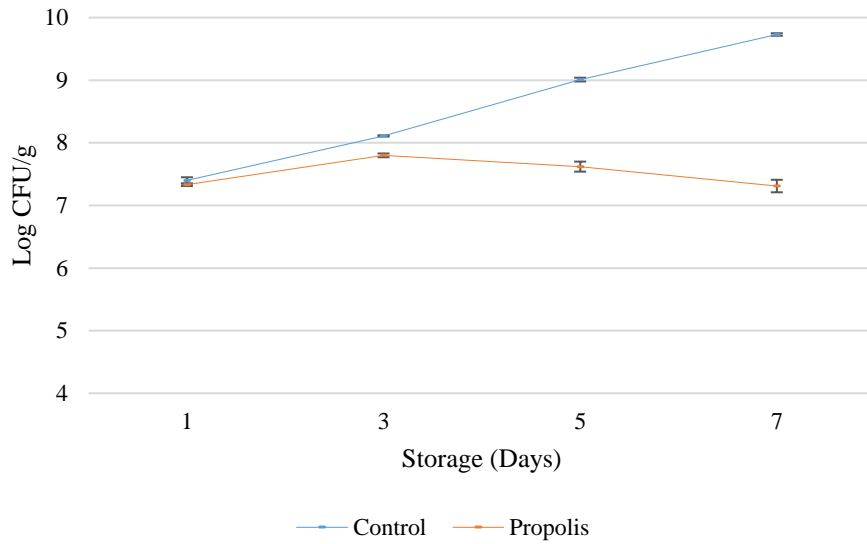


Figure 1. The changes in the total mesophilic count of ground beef treatments stored aerobically at 4°C.

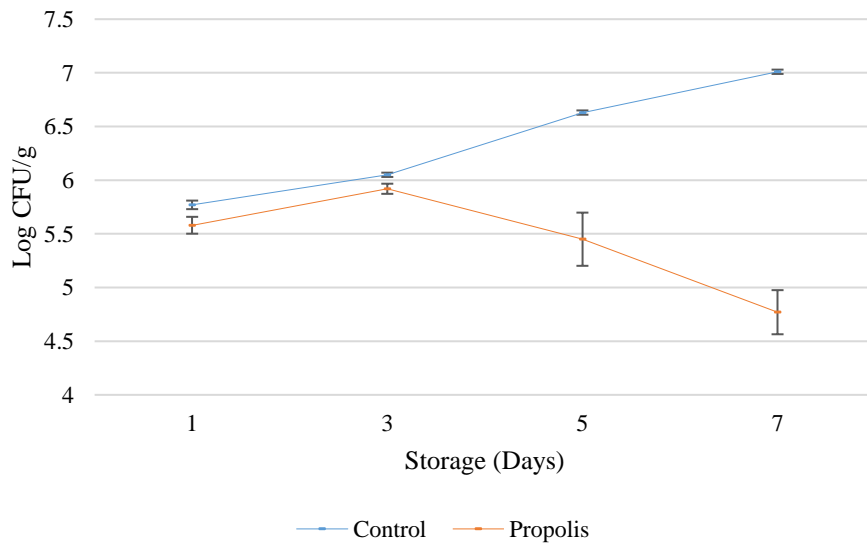


Figure 2. The changes in the Enterobacteriaceae count of ground beef treatments stored aerobically at 4°C.

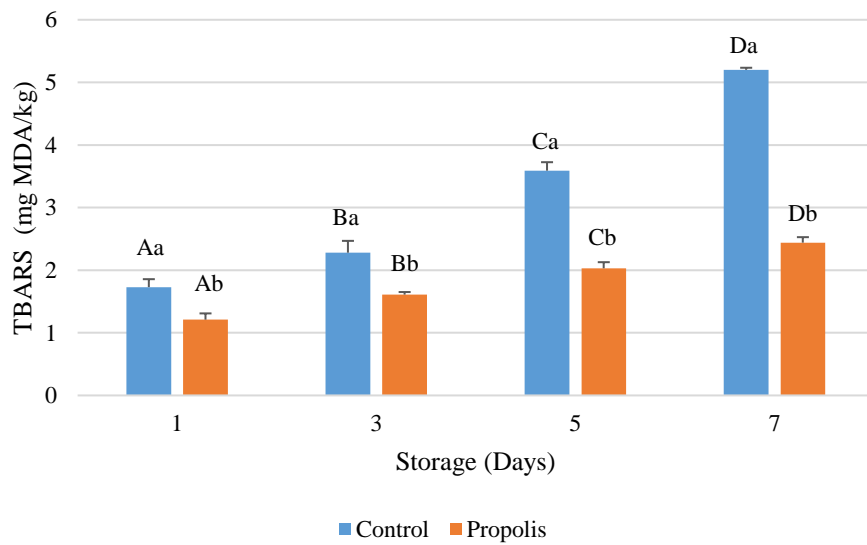


Figure 3. The changes in the lipid oxidation of ground beef treatments stored aerobically at 4°C.

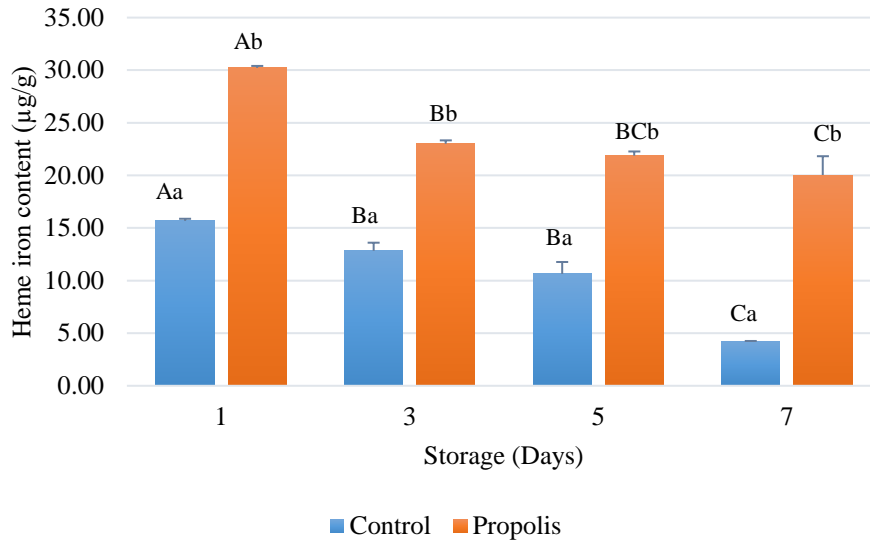


Figure 4. The changes in the heme-iron content of ground beef treatments stored aerobically at 4°C.

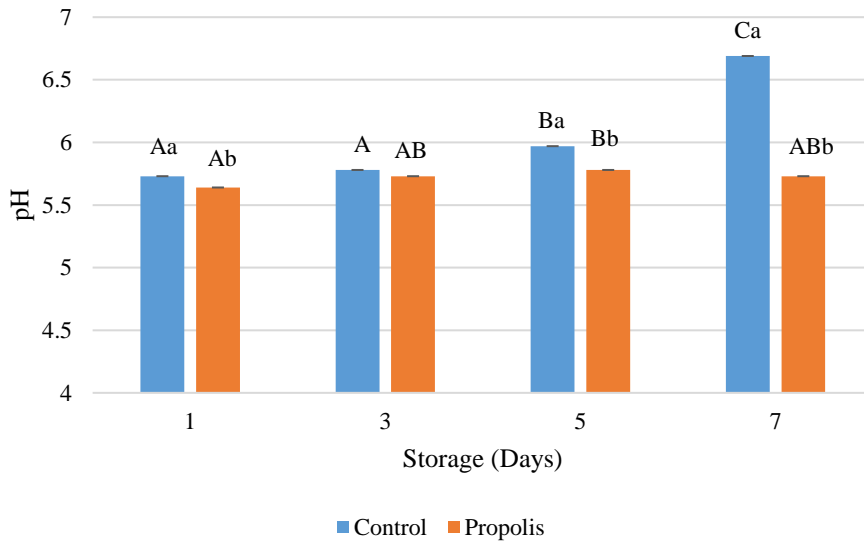


Figure 5. The changes in the pH of ground beef treatments stored aerobically at 4°C.

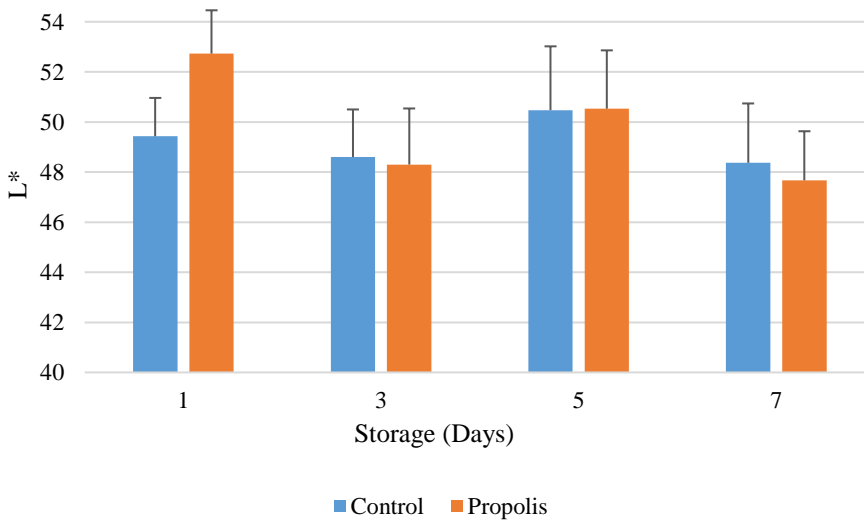


Figure 6. The changes in the lightness values of ground beef treatments stored aerobically at 4°C.

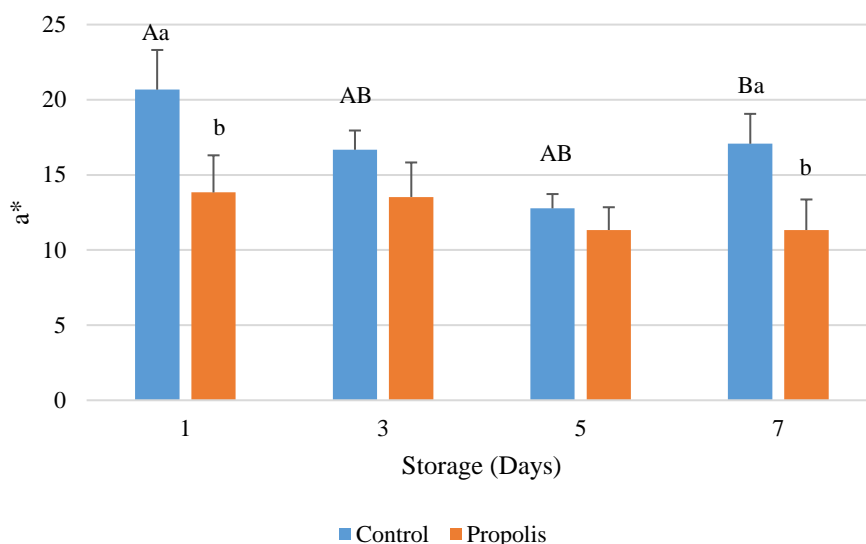


Figure 7. The changes in the redness values of ground beef treatments stored aerobically at 4°C.

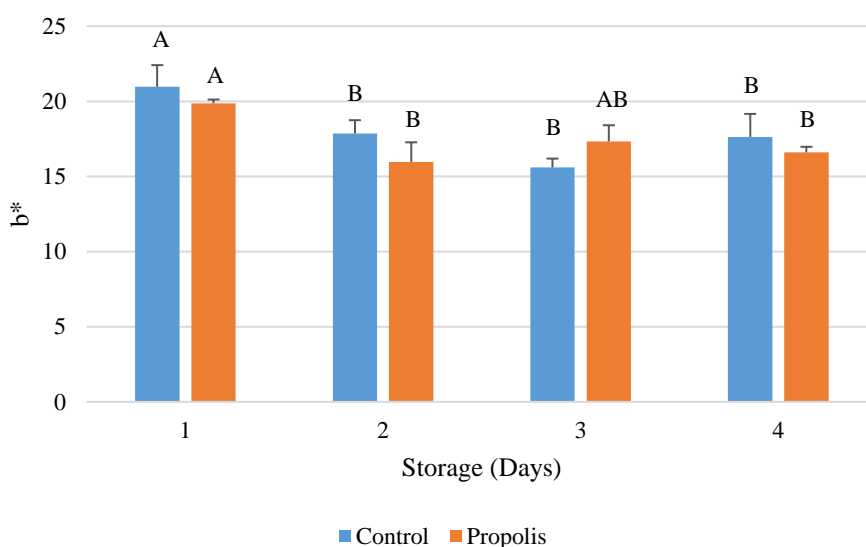


Figure 8. The changes in the yellowness values of ground beef treatments stored aerobically at 4°C.

Table 1. Effects of Turkish propolis water extract tested against bacteria using a disk diffusion method (mm).

Bacterial species	Inhibition Zone (mm)*
Bacillus cereus NRRL B3711	14.50 ± 0.71
B. subtilis PY79	10.00
Enterococcus faecalis ATCC 29212	16.00 ± 1.41
Listeria monocytogenes ATCC 19115	15.00 ± 1.41
Staphylococcus aureus ATCC 9144	14.00
S. epidermidis ATCC 12228	26.50 ± 3.54
Escherichia coli ATCC 25922	9.00 ± 1.41
Salmonella enteritidis ATCC 13076	6.00 ± 1.41
S. typhimurium ATCC 14028	13.50 ± 0.71

*Values are expressed as mean ± standard deviation

At first, initial Enterobacteriaceae count was high for both the control (5.77 log CFU/g) and the propolis treatments (5.58 log CFU/g), and it was not significantly ($P>0.05$) different between the treatments. But later on, the propolis treatment exhibited significant ($P<0.05$) reduction in the Enterobacteriaceae count with 1.18 log CFU/g (17.8%) for day 5 and 2.24 log CFU/g (31.9%) for day 7. Besides the major findings of this study, antimicrobial

properties of propolis extracts in meat applications have been demonstrated in different studies. Jonaidi Jafari et al. (2018) reported that beef patties coated with chitosan with 2% propolis extract exhibited antimicrobial activity against the coliform bacteria. In another study, chicken kebab marinated with water propolis extract slow down the growth of *Escherichia coli* (Mahdavi-Roshan et al., 2022).

Changes in Physicochemical Properties

TBARS value is commonly used as an index to assess lipid oxidation in meat products. The secondary oxidation product – a malondialdehyde (MDA) reacts with thiobarbituric acid (TBA) producing color compound that can be measured at a certain wavelength (Abeyrathne et al., 2021). The results of the ANOVA analysis showed that the use of propolis treatment and the day of storage significantly ($P < 0.05$) influenced the oxidative changes in meat samples. The amount of MDA presences in the ground beef treatments was significantly ($P < 0.05$) reduced with the addition of propolis extract (Figure 3). While, the TBARS values of the control treatment showed 3.47 mg MDA/kg increase from the initial day of storage to the final day of storage, the propolis treatment increased only by 1.23 mg MDA/kg. In addition, the propolis treatment provided 64.6% reduction in the TBARS values in comparison to the control treatment at day 7. This reveals that phenolic compounds in the propolis extract had a strong anti-oxidative effect in a meat matrix. Similarly, successful use of propolis extracts to prevent oxidative changes in meat products have been reported in the literature. For instance, propolis extract in beef and pork patties (Vargas-Sanchez et al., 2014; Vargas-Sanchez et al., 2019), propolis marinate in chicken kebab (Mahdavi-Roshan et al., 2022), microencapsulated propolis extract in burger meat, (Soare's dos Reis et al., 2017), and propolis coated throat fillets (Ucak et al., 2020).

Iron is found predominantly as heme iron in animal products. During processing or storage, heme iron can be released from the porphyrin ring structure, then, free ferrous iron can induce the oxidation process (Macho-Gonzalez et al., 2020). The changes in heme iron content of ground beef treatments are displayed in Figure 4. Both the treatment and the storage had a significant ($P < 0.05$) effect on the heme iron content. The heme iron content decreased over time. The propolis treatment had a significantly ($P < 0.05$) higher heme iron content than the control treatment throughout the storage period. This indicates that possibly the phenolic compounds in the propolis extract blocks the release of iron from porphyrin ring by binding to oxygen-myoglobin binding site. In one study, Zahid et al. (2020) compared the effect of clove extract, butylated hydroxytoluene (BHT) and ascorbic acid on the heme iron content of fresh beef patties. They found that patties prepared with the clove extract had a higher heme iron content than the patties prepared with BHT or had no antioxidant.

The effect of propolis extract and the storage on the pH values of the ground beef meatball treatments is displayed in Figure 5. The pH results were significantly ($P < 0.05$) affected by both the treatment and the storage. The pH of the control treatment increased from 5.73 at initial day of storage to 6.69 at final day of storage and it was significantly ($P < 0.05$) affected by the storage after the 3rd day. Meanwhile, the propolis treatment displayed a slight increase in the pH value from 5.64 at day 1 to 5.73 at day 7. This shows that the propolis extract keep the pH of the product almost constant. When the treatments compared within the storage period, there was a significant ($P < 0.05$) difference between treatments at day 1, 5, and 7. These findings coincide with Vargas-Sanchez et al. (2014) and Vargas-Sanchez et al. (2019), who reported minimal

changes in pH of beef patties treated with propolis extract. The fat content of the control and the propolis treatment were 16.59% and 14.72%, respectively, which is relatively low-fat content for meatballs.

The results of the color L^* , a^* , and b^* values are illustrated in Figure 6, 7 and 8, respectively. Color is a great indicator for the freshness of meat product and it is an important parameter for consumer preferences. Microbial and oxidative changes impact the color of meat causing discoloration via metmyoglobin formation (Vargas-Sanchez et al., 2014). In this study, the all the color values decreased at the final testing period to metmyoglobin formation. Lightness values of ground beef meatball samples was not significantly ($P > 0.05$) affected by the treatment and the storage. In addition, the redness value was significantly ($P < 0.05$) affected by the treatment and decreased with the addition of propolis extract. Furthermore, despite the significant ($P < 0.05$) difference was noted between the treatments at initial and final day of storage, the redness value of propolis treatment did not significantly ($P > 0.05$) change during the storage. The results were in agreement with the previous studies (Vargas-Sanchez et al., 2014; Vargas-Sanchez et al., 2019). The yellowness values were not affected ($P > 0.05$) by the treatment. However, the storage had a significant ($P < 0.05$) effect on the b^* values. The addition of propolis extract decrease the yellowness values of the ground beef meatballs. There were also reports of increase in the yellowness values in beef patties with the addition of propolis extract (Vargas-Sanchez et al., 2014; Vargas-Sanchez et al., 2019).

Conclusion

This work demonstrated that the Turkish propolis extract exhibited a strong antimicrobial and antioxidant properties both in vitro and in situ assays. The addition of propolis extract to ground beef meatballs significantly ($P < 0.05$) reduced the growth of total mesophilic and Enterobacteriaceae counts, and enhanced the shelf life for a week in comparison to the regular meatballs. Similarly, the propolis treatment prevented the oxidative deterioration in meatballs and yielded 64.6% reduction in the TBARS value in comparison to the control treatment at the final day of storage. Only, the redness value decreased with the addition of propolis extract. The pH of the propolis treatment remained almost constant throughout the storage. All these results reveal that the propolis extract is a powerful natural ingredient that offers improvement in shelf life and oxidative changes in meat products.

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