IN VITRO ANTIMICROBIAL AND ANTIOXIDANT POTENCY OF ETHANOLIC PROPOLIS EXTRACT ACROSS VARIABLE CONCENTRATIONS AND STORAGE INTERVALS

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ABSTRACT

Propolis, the resinous material collected by honeybees, is high in phenolic compounds and flavonoids and is well documented for its antimicrobial and antioxidant activity. Nearly all of this evidence, however, comes from extracts tested soon after preparation, so it is not clear whether propolis keeps its activity once the extract has been stored, which is how it would be used in practice. The present study therefore examined how concentration and storage time affect the in vitro antimicrobial and antioxidant activity of ethanolic propolis extract (EEP). Working solutions of 1%, 2% and 3% (v/v), together with synthetic controls (benzoic acid and BHT), were held at 4 °C and tested at four points over 21 days in a completely randomized design with three technical replicates from a single extract batch. Antimicrobial activity was measured by agar well diffusion against four dairy-associated bacteria and five fungal species, and antioxidant activity by TAC, TFC, DPPH, ABTS and lipid peroxidation inhibition assays. Baseline screening of crude ethanolic propolis extract yielded total phenolic and flavonoid contents of 182.42 ± 3.15 mg GAE/g and 68.30 ± 1.84 mg QE/g, respectively. RP-HPLC-DAD profiling detected chrysin (~30%) and pinocembrin (~26%) as major constituents, with quantifiable phenolic levels increasing over 21 days of refrigerated storage. Concentration and storage time interacted significantly ($P < 0.01$). Rather than weakening with time, as would normally be expected, the antimicrobial and antioxidant activity of EEP increased during storage, consistent with the gradual release of phenolic constituents from the resin into the ethanol over 21 days, while the antioxidant capacity of the control declined with storage. The 3% extract on day 21 was the most active overall, producing inhibition zones of 18.7-21.8 mm against the bacteria and 18.1-21.2 mm against the fungi, a flavonoid content of 0.97 mg QE/g, and 66-79% inhibition in the radical-scavenging assays. These results suggest that ethanolic propolis extract need not be used immediately after preparation, and that a 3% extract is worth evaluating further as a natural preservative, once tested in milk itself.

Key words: Propolis; Natural antioxidant; Antimicrobial agent

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INTRODUCTION

Propolis is a natural adhesive material that honey bees collect from buds, bark, and other plant materials (Güneş *et al*., 2024) and then modify by adding wax, enzymes, and secretions from their salivary glands (Liberio *et al*., 2011). Compositionally, it generally contains around 50% plant resins and balsams, 30% waxes, 10% essential oils, 5% pollen, and 5% other compounds (Anjum *et al*., 2019). More than 500 chemical components have been discovered in propolis, among which are aromatic acids, amino acids, coumarins, steroids, terpenes, polyphenols, phenolic acids, and flavonoids (Fokt *et al*., 2010; Toreti *et al*., 2013). Propolis is also a major source of minerals, including sodium, potassium, calcium, copper, magnesium, zinc, and iron (Abdullah *et al*., 2019, 2020).

Milk and milk products provide an ideal nutrient-rich environment that supports the rapid growth of spoilage and pathogenic microorganisms. Common bacterial genera associated with dairy contamination include *Escherichia*, *Staphylococcus*, *Salmonella*, *Streptococcus*, and *Enterococcus*, while fungal species such as *Candida* and *Aspergillus* are also frequently isolated (Dash *et al*., 2022). Several studies have found that the unpasteurized cow and buffalo milk sold in the Punjab region of Pakistan often fails to meet the acceptable microbiological safety standards, owing to high levels of *Escherichia coli*, *Staphylococcus aureus* and other pathogens. This clearly indicates the importance of developing efficient natural antimicrobials (Riaz *et al*., 2026). In mozzarella cheese, discoloration defects have been associated with bacteria of the genus *Pseudomonas*, which yield blue pigmentation and cause spoilage during storage (Carrascosa *et al*.,

2021). In addition, *Listeria monocytogenes* is known as a main contaminant in milk, cheese, and ice cream. Large-scale contamination of ice cream production lines in the United States with *Listeria monocytogenes* has also been reported, highlighting its persistence in dairy processing environments (Chowdhury and Anand, 2023).

Cretenet *et al.* (2011) described the occurrence of *Staphylococcus aureus* and its enterotoxins formed during the production and ripening of different cheeses, highlighting the need for strict sanitation in dairy processing areas. Carminati *et al.* (2024) reported that the late blowing defect in Grana Padano cheese, which is usually associated with the development of cavities, fissures, and cracks, results from the action of *Clostridium* species. These bacteria yield butyric acid, acetate, hydrogen, and CO2 through lactate metabolism (McSweeney and Fox, 2004). *Salmonella*, *Listeria monocytogenes*, and *Escherichia coli O157:H7* have been identified in samples of unprocessed cow milk in Ethiopia at the median prevalence levels of 6%, 9% and 10%, respectively, highlighting an important presence of bacterial pathogens in raw milk (Keba *et al.*, 2020). Milk and milk products are also influenced by fungi, which not only deteriorate the organoleptic properties but also produce mycotoxins, mainly spoilage molds such as *Penicillium* and *Aspergillus* spp. (Hymery *et al.*, 2014).

Several studies have reported that propolis shows a wide range of biological effects, including antiviral, antibacterial, anticancer, antifungal, antioxidant, and antimutagenic activities (Kujumgiev *et al*., 1999; Elnakady *et al*., 2017; Ezzat *et al*., 2019; Silva *et al*., 2019). In Brazilian propolis, antifungal activity is mainly due to the disturbance of fungal cell wall and membrane organization, causing swelling and subsequent cell rupture (Mello *et al*., 2006). Moreover, the effect of propolis on *Candida albicans* growth inhibition is attributed to cell wall and membrane damage, as well as cell death. This is the result of synergistic effects of flavonoids and phenolics in propolis (Corrêa *et al.*, 2020).

Although propolis is generally known for its potent bioactivity, a critical gap remains in understanding how its therapeutic properties perform over time. Because extraction conditions govern both the recovery and the stability of phenolic and flavonoid constituents (Ahamed *et al*., 2025), the composition of an ethanolic extract does not necessarily remain constant once prepared, and its activity may therefore change upon standing. Any preservative intended for practical use must keep its efficacy throughout storage, yet the stability of ethanolic propolis extract during refrigerated storage has seldom been examined, and antimicrobial activity and antioxidant markers do not appear to have been tracked simultaneously in the same stored extract. Consequently, it remains unknown whether the activity of propolis extract is lost, retained, or altered during storage, and whether this behavior differs between extract concentrations.

The dairy industry relies extensively on synthetic additives to improve product properties and extend storage stability, including preservatives, antibacterials, antioxidants, nutritional improvers, coloring, flavors, and texture modifiers (Chandan, 2011; Caleja *et al*., 2016). However, excessive intake of synthetic food additives has been associated with negative effects on the digestive, respiratory, skin, and nervous systems (Csáki, 2011).

Food quality loss during storage is primarily driven by microbial activity, oxidation of lipids, and enzyme-induced spoilage (Oulahal and Degraeve, 2022). Compounds combining antimicrobial and antioxidant activity are especially attractive, since they address microbial spoilage and oxidative deterioration simultaneously (Teshome *et al*., 2022).

Driven by the increasing demand for natural preservatives in the dairy sector, the current research evaluated the in vitro bioactivity and storage stability of ethanolic extract of propolis. Specifically, our objectives were to: determine the antibacterial and antifungal efficacy across variable concentrations (1%, 2%, and 3% v/v) against key dairy spoilage and pathogenic microorganisms including Gram-negative (*Escherichia coli, Pseudomonas fluorescens*) and Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) bacteria, as well as fungal strains (*Candida albicans, Candida glabrata, Aspergillus flavus, Aspergillus fumigatus, and Aspergillus terreus*); to assess the antioxidant performance by measuring the total antioxidant capacity (TAC), total flavonoid content (TFC), DPPH and ABTS radical-scavenging capacities, and lipid peroxidation inhibition; and to monitor the activity changes during 21 days of refrigerated storage to determine time- and concentration-dependent stability. It was hypothesized that antimicrobial and antioxidant activity would increase with extract concentration, and that both would decline with storage duration owing to degradation of phenolic constituents.

MATERIALS AND METHODS

Materials: Raw propolis was sourced from beehives at the University of Veterinary and Animal Sciences, Pattoki, Pakistan. Chemical reagents, including benzoic acid and butylated hydroxytoluene (BHT), along with culture media Eosin Methylene Blue agar, Mueller-Hinton agar, Mannitol Salt agar, PALCAM agar, Cetrimide agar, Sabouraud Dextrose agar and Nutrient agar were purchased from Sigma Scientific Store, Lahore. Test microorganisms were obtained from the departmental culture collection of the Department of Microbiology, UVAS, Lahore. Bacterial panels comprised Gram-negative (*Escherichia coli* and *Pseudomonas fluorescens*) and Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) species. Fungal strains included *Candida albicans, Candida glabrata, Aspergillus flavus, Aspergillus terreus* and *Aspergillus fumigatus.* All isolates were laboratory-maintained strains previously identified using standard microbiological methods; official accession numbers were unavailable as they were not procured from an international

culture repository. The research was carried out over a 13-month period from June 2024 to July 2025 in the Food Chemistry and Bioimaging Laboratories at the Central Laboratory Complex, UVAS, Ravi Campus, Pattoki.

Extraction and preparation of ethanolic propolis extract: The propolis sample was stored under hygienic conditions and crushed into a fine powder using a laboratory grinder, following the method of Haghdoost *et al.* (2016). To prepare the ethanolic extract, 10 grams of powdered propolis were mixed with 100 mL of 70% ethanol and stirred continuously for 24 hours at room temperature using a magnetic stirrer. The mixture was incubated at 50 °C in a water bath for 2 hours to increase the extraction of bioactive compounds. After the extraction process, the mixture was filtered through Whatman No. 1 filter paper, and the filtrate was collected and stored in amber-colored airtight bottles at 4 °C for subsequent analyses (Wieczorek *et al.*, 2022). The single batch of crude ethanolic extract of propolis (EEP) was considered the stock solution.

Experimental design and sampling: The experiment used a Completely Randomized Design (CRD) with repeated measures, consisting of four treatment groups: CON (synthetic control), T1 (1% EEP), T2 (2% EEP), and T3 (3% EEP). Working solutions of 1%, 2%, and 3% (v/v) EEP were prepared by diluting the stock extract with sterile distilled water prior to antimicrobial and antioxidant assays. These contained about 0.7%, 1.4% and 2.1% (v/v) ethanol, respectively. Benzoic acid and butylated hydroxytoluene (BHT) served as the synthetic controls (CON) for the antimicrobial and antioxidant assays, respectively, to validate the comparative efficacy of the propolis extract against standard synthetic preservatives. Each treatment was prepared in three different amber vials (replicates) stored at 4 °C. The samples from each vial were taken aseptically on days 1, 7, 14, and 21; the vial was so the experimental unit. These were technical replicates resulting from the same stock solution, not independently prepared biological replicates. All treatments and replicates were analyzed at four storage intervals, yielding 48 observations per tested parameter (4 treatments × 3 replicates × 4 storage days = 48). Statistical procedures are described under Statistical Analysis.

Total phenolic and flavonoid content: The total phenolic content was determined following the method described by Miłek *et al*. (2024). Before the biological assays, the total phenolic content of the stock solution of the ethanolic propolis extract was measured using the Folin-Ciocalteu method, with gallic acid as the calibration standard; TPC was expressed as mg gallic acid equivalents (GAE)/g extract. The total flavonoid content (TFC) of the stock solution was measured by the aluminum chloride colorimetric method described below under "Determination of Total Flavonoid Content (TFC)".

HPLC-DAD analysis: For the determination of compounds from ethanolic propolis extract, the extract was filtered through a membrane filter and analyzed using RP-HPLC-DAD on a C18 column. Acidified water and acetonitrile were used for the separation under gradient elution at a flow rate of 1.0 mL/min. Phenolic compounds were monitored at 280-370 nm and identified by comparing retention times and UV spectra with standards. Quantification was done using external calibration curves for individual standards. Caffeic acid, *p*-coumaric acid, ferulic acid, pinocembrin, chrysin, galangin and caffeic acid phenethyl ester (CAPE) were the target compounds (Miłek *et al*., 2024). Each sample was analyzed in triplicate (n = 3).

Evaluation of antibacterial activity: The antibacterial activity of the ethanolic propolis extract was measured against *Escherichia coli*, *Pseudomonas fluorescens*, *Staphylococcus aureus* and *Listeria monocytogenes*, using the agar well diffusion method described by Balouiri *et al.* (2016), with slight modifications. Suitable selective media were used for each strain, to confirm strain identity during the repeated assays: EMB agar for *E. coli*, Cetrimide agar for *P. fluorescens*, Mannitol Salt Agar for *S. aureus*, and PALCAM agar for *L. monocytogenes*. Media were autoclaved at 121 °C for 15-20 min to ensure sterility. After cooling to ~45-50 °C, the pH of the media was adjusted to 4.7 using sterile 1 N HCl to simulate acidic environment for (fermented) dairy products and because benzoic acid, the reference preservative, is mostly active below its pKa (≈4.2). Untreated growth-control plates presented merging growth of all strains at this pH. The media were then poured aseptically into sterile Petri plates. After solidification, the plates were spread with bacterial suspensions adjusted to a 0.5 McFarland standard using sterile swabs. Wells of 7 mm diameter were made and sealed with a thin layer of molten agar to promote even diffusion. Each well received 100 µL of ethanolic propolis extract at 1%, 2%, and 3% concentrations. Benzoic acid (200 µg/mL) served as the synthetic control. The inoculated plates were incubated at 36 ± 1 °C for 24 h. Analyses were run on days 1, 7, 14 and 21 to determine the stability and persistence of the antibacterial activity during storage, and the inhibition zone diameter was documented in millimeters (mm).

Determination of Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentration (MIC) of the ethanolic extract of propolis (EEP) against the tested bacterial strains was determined using the broth microdilution technique in sterile 96-well microtiter plates, as described by Balouiri *et al*. (2016). Serial two-fold dilutions of EEP were prepared in Mueller–Hinton broth to obtain a range of extract concentrations. For each well, 100 µL of the diluted extract was mixed with 100 µL of bacterial inoculum prepared from a 0.5 McFarland suspension and diluted to give a final density of approximately 5×10^5 CFU/mL in each well. Control wells containing Mueller–Hinton broth with bacterial inoculum (growth control) and broth without inoculum (sterility control). were also included to confirm bacterial growth and ensure

medium sterility. Solvent-control wells (ethanol without extract) were also involved and did not inhibit growth. The MIC was determined on day 21 of storage. The microplates were then incubated at 36 ± 1 °C for 24 hours. After incubation, bacterial growth was assessed visually and by turbidity. The MIC was considered the lowest concentration of EEP that completely inhibited visible bacterial growth.

Conversion of MIC Values from % (v/v) to mg/mL: The MIC values, stated as % (v/v), were converted to mg/mL on the basis of the amount of crude propolis used to make the stock solution. During extraction, 10 g of powdered crude propolis was macerated in 100 mL of 70% ethanol, giving a stock solution equivalent to 10% (w/v), i.e. 100 mg/mL of crude propolis. Because a 1% (v/v) dilution contains 1/100 of the stock, 1% (v/v) corresponds to 1 mg/mL of crude propolis equivalent. The converted values therefore indicate crude propolis equivalents instead of the dry-weight concentration of the extract.

Conversion Formula

MIC (mg/mL) = MIC (% v/v) × (stock concentration in mg/mL) / 100
= MIC (% v/v) × 100 mg/mL / 100
= MIC (% v/v) × 1 mg/mL per %

Evaluation of Antifungal Activity by Agar Well Diffusion Assay: The antifungal effect of EEP at various concentrations (1%, 2%, and 3%) was analyzed against five types of fungi: *Candida albicans, Candida glabrata, Aspergillus flavus, Aspergillus terreus, and Aspergillus fumigatus*. The study used the agar well diffusion technique, modified from that of Balouiri *et al*. (2016). Sabouraud Dextrose Agar (SDA) was used for the growth of all fungi, and its pH was adjusted to 4.7 as for the bacterial media. The medium was prepared according to the manufacturer's guidelines, autoclaved at 121 °C for 30 min and poured aseptically into Petri dishes. After the media had hardened, each Petri dish was inoculated with a freshly prepared fungal culture using a sterilized cotton swab. Aseptically, seven-millimeter holes were punched in the agar medium and sealed with agar plugs. Each hole was filled with 100 µL of EEP (concentrations: 1%, 2%, and 3%).

The synthetic antifungal control used was benzoic acid (200 µg/mL). The plates were incubated at 28 ± 1 °C for 48 hours, after which the inhibition zone diameters (mm) were measured. The assay was repeated on days 1, 7, 14, and 21 to measure the persistence of the antifungal activity of the propolis extracts and the synthetic control during storage. The zone of inhibition (ZOI) was recorded in millimeters using digital vernier calipers.

Estimation of Total Antioxidant Capacity (TAC): The total antioxidant capacity (TAC) of ethanolic propolis extract (EEP) was determined using the phosphomolybdenum assay, following the modified method described by Prieto *et al*. (1999). The assay is based on the reduction of molybdenum (VI) to molybdenum (V) by antioxidant compounds, leading to the formation of a green phosphate–molybdenum (V) complex under acidic conditions. Briefly, 0.3 mL of propolis extract (1%, 2%, and 3%) or BHT (synthetic antioxidant control) was mixed with 3 mL of phosphomolybdenum reagent containing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The mixture was incubated at 95 °C in a water bath for 90 min and then cooled to room temperature. Absorbance was subsequently recorded at 695 nm using a UV-Vis spectrophotometer against a reagent blank. Measurements were taken on days 1, 7, 14, and 21 to assess changes in antioxidant capacity during storage.

Determination of Total Flavonoid Content (TFC): The TFC of the stock extract and of the EEP working solutions was measured by the aluminum chloride colorimetric method, with quercetin as the calibration standard, and results were expressed as mg quercetin equivalents (QE)/g; the method is based on the formation of a colored complex between aluminum and flavonoids. Briefly, 0.1 mL of extract (stock, or 1%, 2% or 3% EEP) was mixed with 0.2 mL of 5% sodium nitrite solution for 5 min.

Following this, 0.2 mL of 10% aluminum chloride in methanol and 1.0 mL of 1 M sodium hydroxide were added. The resulting mixture was allowed to stand at room temperature for 15 min, and absorbance was subsequently recorded at 510 nm against a reagent blank. Measurements were taken on days 1, 7, 14, and 21 to monitor variations in flavonoid content during storage (Shraim *et al.*, 2021).

DPPH Radical Scavenging Activity: The free radical scavenging activity of EEP was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, following a modified protocol (Ozgen *et al.*, 2006). In this method, 1 mL of propolis extract (1%, 2% or 3%) or 1 mL of BHT (synthetic antioxidant control) was added to 2.5 mL of freshly prepared 0.1 mM DPPH solution in ethanol. The reaction mixture was vortexed briefly and incubated in the dark at room temperature for 30 minutes. The reduction in absorbance was measured at 517 nm using a UV–Vis spectrophotometer. The percentage of DPPH inhibition was calculated by comparing the absorbance of the sample with that of the blank (DPPH solution + ethanol). The assay was repeated on days 1, 7, 14, and 21 to assess the time-dependent antioxidant stability of the extracts (Jimenez-Pulido *et al.*, 2022).

ABTS Radical Cation Decolorization Assay: The ABTS radical scavenging capacity of EEP was assessed using a modified method described by Elfalleh *et al.* (2009). The $ABTS^{+}$ radical was produced by combining a 7 mM ABTS solution with 2.45 mM potassium persulfate and incubating the mixture in the dark at room temperature for 12–16 hours. The resulting solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. For the assay, 1 mL of EEP (1%, 2% or 3%) or 1 mL of BHT (synthetic antioxidant control) was added to 2 mL of the diluted $ABTS^{+}$ solution, and the mixture was allowed to incubate at room temperature for ten minutes. Absorbance was recorded at 734 nm, and the percentage inhibition was calculated relative to the blank ($ABTS^{+}$ solution without sample). This analysis was performed on days 1, 7, 14, and 21.

Lipid Peroxidation Inhibition Assay (TBARS Method): Lipid peroxidation inhibition activity of EEP was determined using the Thiobarbituric Acid Reactive Substances (TBARS) assay, with slight modifications of the method of Vijayavel *et al*. (2004). In brief, 1 mL of 10% (v/v) egg yolk homogenate in PBS was mixed with 1 mL of EEP (1%, 2% or 3%) or 1 mL of BHT (synthetic antioxidant control) in a test tube. To this, 1.5 mL of 20% trichloroacetic acid (TCA) and 1.5 mL of 0.67% thiobarbituric acid (TBA) were added. The mixture was vortexed and incubated at 95°C for 30 minutes in a water bath. Following cooling, the samples were centrifuged at 3000 rpm for 10 min, after which the absorbance of the supernatant was recorded at 532 nm. The extent of lipid peroxidation inhibition was calculated in comparison with a blank containing no antioxidant. Measurements were taken on days 1, 7, 14, and 21 to evaluate antioxidant activity over time.

Statistical analysis: The normality of the data was tested using the Shapiro-Wilk test. Data were analyzed using the Completely Randomized Design (CRD) in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The model included fixed effects for treatments, days, and their interaction. Storage day was included in the REPEATED statement, with the replicate vial within treatment as the subject. When significant effects were detected, treatment means were compared using Tukey's adjustment; different superscript letters in Tables 3-5 indicate significant differences among means. In all the mixed-effects models, the denominator degrees of freedom for the F tests were estimated using the Kenward-Roger method. Statistical significance was set at $P < 0.05$. Because all replicates came from one extract batch, the results reflect variability within this extract only. The following statistical model was used:

$Yijk=\mu+Ti+Sj+(TS)ij+eijk$, where:

Yijk = observed value of the dependent variable

μ = overall mean

Ti = effect of the treatment (i = CON, T1, T2 and T3)

Sj = repeated effect of the storage time (j = 1, 7, 14 and 21)

(TS)ij = interaction effect between treatment and storage time (i × j = combined effect of ith treatment at the jth storage time)

eijk = random error associated with each observation, assumed to be independently and normally distributed with mean zero and constant variance σ^2 (i = effect of treatment, j = effect of storage time and k = replication)

RESULTS

Bioactive content in the ethanolic extract of propolis at baseline: Before the biological assays, the total phenolic content (TPC) and total flavonoid content (TFC) of the crude ethanolic extract of propolis were measured to establish its baseline bioactive content. The crude ethanolic extract of propolis possessed a baseline TPC of 182.42 ± 3.15 mg GAE/g and a TFC of 68.30 ± 1.84 mg QE/g.

HPLC profiling of ethanolic extract of propolis during refrigerated storage: HPLC analysis of ethanolic extract of propolis was carried out and major compounds (caffeic acid, p-coumaric acid, ferulic acid, pinocembrin, chrysin, and galangin) were monitored at 280-370 nm and identified by comparing retention times and UV spectra with standards (Table 1). Caffeic acid, p-coumaric acid, ferulic acid, pinocembrin, chrysin, and galangin were identified, while CAPE was not detected. Chrysin and pinocembrin were the major measured compounds throughout storage, constituting about 30% and 26% of the total identified phenolic markers, respectively. The concentrations of identified compounds increased progressively from Day 1 to Day 21, indicating changes in the quantifiable phenolic compounds of the extract during refrigerated storage. The relative abundance of most compounds remained comparatively stable despite this increase, indicating that storage increased the measurable levels of the major constituents without changing the phenolic profile. The increase in caffeic acid was comparatively higher than that of other compounds, whereas the relative contributions of chrysin, pinocembrin, p-coumaric acid, ferulic acid, and galangin remained relatively constant. CAPE was not detected under the chromatographic conditions used, which may reflect its absence from the propolis sample or a concentration below the detection limit.

Antibacterial activity of propolis against bacteria: The antimicrobial potential of propolis was assessed by measuring the zone of inhibition at different propolis concentrations and storage days. Statistical analysis indicated that propolis concentration and storage days had a significant effect ($P < 0.01$) on antimicrobial activity. A significant interaction ($P < 0.01$) was also noted between propolis concentration and storage days for the antibacterial activity of all bacterial strains. On day 1, inhibition zones were uniform and only slightly larger than the 7 mm well diameter (7.30-7.45 mm) against both Gram-negative (*E. coli* and *Pseudomonas fluorescens*) and Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) bacterial strains for all concentrations of propolis, indicating no immediate concentration-dependent effect. However, with extended storage, the inhibition zones increased with both propolis concentration and storage time.

The mean values for antimicrobial activity against Gram-negative *E. coli* indicated that the inhibition zone increased with both propolis concentration and storage duration. On day 7, the EEP with 3% propolis concentration produced the highest inhibition ($P < 0.01$; 14.55 vs 11.54 vs 10.52 mm) among the treatments. The synthetic control, benzoic acid, showed a larger zone of inhibition (19.36 mm) than the propolis-containing treatments. Similarly, on day 21, EEP with 3% propolis showed the greatest inhibition ($P < 0.01$; 19.54 vs 17.08 vs 16.07 mm) compared with the other treatments, whereas the inhibition zone of benzoic acid increased slightly but non-significantly during storage, reaching 19.52 mm on day 21, which did not differ significantly from the 3% EEP (Table 3).

For *Pseudomonas fluorescens*, the inhibition zone of the synthetic preservative changed only slightly during storage (19.18-19.25 mm), and these small differences were statistically non-significant. On day 7, EEP with 3% propolis produced an inhibition zone of 12.59 mm, whereas benzoic acid exhibited a zone of 19.18 mm. By day 21, EEP with 3% propolis demonstrated the strongest inhibition among the natural treatments ($P < 0.01$; 18.71 vs. 17.16 vs. 16.15 mm), while the synthetic control maintained a slightly larger inhibition zone of 19.25 mm on day 21 (Table 3).

The mean values for the antimicrobial activity against the Gram-positive bacterium *Staphylococcus aureus* revealed a consistent increase in inhibition zones with higher propolis concentrations and longer storage durations. On day 7, EEP containing 3% propolis exhibited the strongest inhibitory effect among the natural treatments ($P < 0.01$; 14.56 vs. 11.32 vs. 10.55 mm), whereas the synthetic control, benzoic acid, produced a larger inhibition zone (22.57 mm). A similar trend was observed on day 21, where EEP with 3% propolis produced the largest inhibition zone among the natural treatments ($P < 0.01$; 21.81 vs. 17.31 vs. 16.20 mm). The inhibition zone of the synthetic preservative increased slightly but not significantly during storage, reaching 22.64 mm on day 21 (Table 3). The mean values for the antimicrobial activity of propolis against the Gram-positive bacterium *Listeria monocytogenes* showed a steady increase in inhibition zones with increasing propolis concentration and storage duration. On day 7, EEP containing 3% propolis exhibited the highest inhibition among the natural treatments (13.28 mm), whereas the synthetic control, benzoic acid, produced a larger inhibition zone (21.20 mm). By day 21, inhibition zones increased significantly across all propolis concentrations ($P < 0.01$; 20.21 vs. 18.15 vs. 17.14 mm). The inhibition zone of the synthetic preservative increased slightly but not significantly during storage, reaching 21.27 mm on day 21 (Table 3).

Minimum inhibitory concentration (MIC) of ethanolic extract of propolis: The MICs of ethanolic propolis extract (EEP) against the Gram-positive bacteria *Staphylococcus aureus* and *Listeria monocytogenes*, as well as the Gram-negative bacteria *Escherichia coli* and *Pseudomonas fluorescens*, were determined by broth microdilution on day 21 of storage. Gram-positive bacteria were more sensitive to EEP, exhibiting MIC values of 0.25% (v/v), equivalent to 0.25 mg/mL, while Gram-negative bacteria showed MIC values of 0.5% (v/v), equivalent to 0.5 mg/mL (Table 2). These results indicate that EEP exhibits strong antimicrobial activity even at low concentrations, with greater inhibitory activity against Gram-positive strains than against Gram-negative strains. The MIC values are consistent with the agar well diffusion data, in which inhibition zones increased with increasing EEP concentration, and support the antibacterial potential of the extract against both Gram-positive and Gram-negative bacteria.

Antifungal activity of propolis against fungi: Mean inhibition zone diameters recorded on day 1 for *Candida albicans, Candida glabrata, Aspergillus flavus, Aspergillus fumigatus* and *Aspergillus terreus* did not differ significantly ($P > 0.05$) among the various propolis concentrations, indicating no immediate concentration-dependent antifungal effect at the initial observation. However, as the concentration and storage time increased, the zone of inhibition increased significantly ($P < 0.01$) for all fungal species. Antifungal activity of ethanolic extract of propolis (EEP) against *Candida albicans* and *Candida glabrata* increased progressively with higher propolis concentrations and longer storage durations. On day 7, EEP containing 3% propolis produced the largest inhibition zones among the natural treatments, measuring 15.88 mm for *C. albicans* and 15.78 mm for *C. glabrata*, whereas the synthetic control, benzoic acid, exhibited larger zones of 21.37 mm and 19.42 mm, respectively. By day 21, the antifungal activity of EEP increased significantly across all concentrations ($P < 0.01$; 21.21 vs. 19.09 vs. 18.22 mm for *C. albicans* and 19.18 vs. 18.09 vs. 17.22 mm for *C. glabrata*), with the 3% treatment showing the strongest inhibitory effect. The inhibition zones of the synthetic preservative increased slightly but not significantly during storage, reaching 21.46 mm and 19.51 mm on day 21 for *C. albicans* and *C. glabrata*, respectively; on day 21, the 3% EEP did not differ significantly from benzoic acid against *C. albicans* (Table 4).

The antifungal activity of the synthetic preservative, benzoic acid, changed little during storage, with mean inhibition zones of 18.38, 18.25, and 18.36 mm for *A. flavus*, *A. fumigatus*, and *A. terreus*, respectively, showing no significant variation over time (Table 4). EEP also exhibited notable antifungal activity against all three species, which increased with higher propolis concentrations and longer storage durations. On day 7, the 3% EEP treatments produced inhibition zones of 13.50 mm (*A. flavus*), 13.48 mm (*A. fumigatus*), and 12.47 mm (*A. terreus*). On day 21, the highest concentration of propolis showed the most potent inhibition, showing highly significant differences ($P < 0.01$) from the 1% treatment for all species and from the 2% treatment for *A. flavus* and *A. terreus*, as follows: *A. flavus* (18.31 mm), 17.92 mm, 16.87 mm; *A. fumigatus* (18.20 mm), 17.88 mm, 16.86 mm; *A. terreus* (18.13 mm), 16.88 mm, 15.85 mm. On day 21, the 3% propolis did not differ significantly from benzoic acid against *A. flavus* and *A. fumigatus* (Table 4).

Antioxidant capacity of propolis: Antioxidant performance was examined using TAC (absorbance at 695 nm), TFC (mg quercetin equivalent/g), DPPH radical scavenging activity (% inhibition), ABTS radical inhibition (% inhibition), and lipid peroxidation inhibition (% inhibition).

Total Antioxidant Capacity (TAC): Statistically, the influence of propolis concentrations and storage times on the total antioxidant capacity (TAC) was significant ($P < 0.01$). The day 1 results indicated that the synthetic antioxidant (BHT) had the highest TAC level among all the examined samples (0.96 AU). In propolis samples, the TAC levels increased with the increasing concentration of the propolis, with TAC levels of 0.52 AU at 1% concentration and 0.60 AU at 3% concentration. The storage time had distinct impacts on TAC levels for all examined samples, where TAC constantly increased in propolis samples and TAC constantly decreased in the synthetic antioxidant sample. After 21 days of storage, the TAC level of 3% propolis was 0.87 AU (Table 5).

Total flavonoid content (TFC): The total flavonoid content (TFC) of the propolis treatments was significantly influenced by propolis concentration and storage duration ($P < 0.01$). TFC was not measured for the synthetic control, because BHT is not a flavonoid. On day 1, TFC was 0.62 mg QE/g for 1% propolis and 0.71 mg QE/g for 3% propolis. Over the storage period, all propolis treatments showed a significant increase, and by day 21 the 1% and 3% treatments reached 0.79 mg QE/g and 0.97 mg QE/g, respectively, indicating a clear concentration- and time-dependent enhancement of flavonoid content (Table 5).

ABTS Radical Scavenging Activity: The ABTS radical scavenging activity was significantly ($P < 0.01$) affected by both propolis concentration and storage duration. On day 1, the control sample (containing the synthetic antioxidant) exhibited the highest activity (77.13%), whereas the propolis-treated samples showed lower initial values: 22.06% for 1% propolis and 34.17% for 3% propolis. However, there was a significant reduction in the ABTS activity of the control over time, with a value of 22.12% on day 21. On the other hand, there was an increase in the activity of both propolis samples over time, with values of 41.53% for 1% propolis and 66.08% for 3% propolis (Table 5).

DPPH Radical Scavenging Activity: The DPPH radical scavenging activity was significantly influenced by propolis concentration and storage duration ($P < 0.01$). On day 1, the synthetic antioxidant control (CON) exhibited the highest activity (88.87%), which was markedly higher than the propolis-containing treatments. Among the propolis treatments, activity increased with concentration, measuring 25.05% for 1% propolis and 33.87% for 3% propolis. Regarding storage, the DPPH activity in the control sample decreased steadily to 24.94% on day 21, whereas samples treated with propolis showed a dose-dependent increase. After storage, the activities for 1% and 3% propolis were recorded at 52.93% and 78.89%, respectively (Table 5).

Lipid Peroxidation Inhibition Assay (TBARS Method): The LPI activity of propolis was significantly influenced by both the concentration of propolis and duration of storage ($P < 0.01$). On day 1 of storage, the control sample had a noticeably higher LPI activity (84.56%) than all propolis-containing samples, whose initial LPI activity was significantly lower (18.87% at 1% and 32.93% at 3%). For propolis samples, the treatment that had 3% propolis showed a higher inhibitory effect on LPI than the treatment that had 1%. With the progression of storage, the control treatment continued to show a consistent decrease in LPI activity down to 28.76%. In contrast, both propolis samples increased gradually with storage, resulting in LPI activity of 44.32% for 1% propolis and 74.48% for 3% propolis (Table 5).

Table 1. HPLC profile of major phenolic compounds in ethanolic extract of propolis

Compounds	Retention time (min)	Day 1 (mg/g)	Relative abundance (%)	Day 7 (mg/g)	Relative abundance (%)	Day 14 (mg/g)	Relative abundance (%)	Day 21 (mg/g)	Relative abundance (%)
Caffeic acid	7.16	3.40 ± 0.18	7.33	3.56 ± 0.19	7.36	3.95±0.22	7.73	4.36 ± 0.22	8.09
p-Coumaric acid	5.19	8.00 ± 0.35	17.24	8.28 ± 0.37	17.12	8.71±0.41	17.05	9.12 ± 0.42	16.91
Ferulic acid	6.93	3.50 ± 0.22	7.54	3.64 ± 0.22	7.53	3.82±0.23	7.48	4.04 ± 0.24	7.49
Pinocembrin	10.12	12.00 ± 0.48	25.86	12.52 ± 0.61	25.89	13.16±0.63	25.76	13.82 ± 0.67	25.63
Chrysin	13.03	14.00 ± 0.60	30.17	14.60 ± 0.72	30.19	15.34±0.73	30.03	16.10 ± 0.76	29.86
Galangin	14.15	5.50 ± 0.30	11.85	5.76 ± 0.32	11.91	6.11±0.32	11.96	6.48 ± 0.33	12.02
CAPE	-	ND	ND	ND	ND	ND	ND	ND	ND
Total quantified markers	**—**	**46.40**	**100.00**	**48.36**	**100.00**	**51.09**	**100.00**	**53.92**	**100.00**

Values are expressed as mean ± standard deviation (n = 3). Compounds were identified by comparing retention times with authentic standards.

Table 2. Minimum Inhibitory Concentration (MIC) of Ethanolic Propolis Extract Against Tested Microorganisms

Microorganism	MIC (% v/v of EEP)	MIC (mg/mL equivalent*)
Escherichia coli	0.5	**0.5**
Pseudomonas fluorescens	0.5	**0.5**
Staphylococcus aureus	0.25	**0.25**
Listeria monocytogenes	0.25	**0.25**

MIC: Minimum inhibitory concentration; % (v/v) values indicate the volume/volume concentration of EEP in the broth; mg/mL equivalent is based on the crude propolis content in the extract, calculated from the stock solution concentration of 100 mg/mL (10 g crude propolis extracted in 100 mL of 70% ethanol), such that 1% v/v ≈ 1 mg/mL, 0.5% v/v ≈ 0.5 mg/mL, and 0.25% v/v ≈ 0.25 mg/mL.

Table 3: Effect of propolis concentration and storage days on the bacterial growth.

TRT	Day	*E. coli* (mm)	*Listeria* (mm)	*Pseudomonas* (mm)	*Staphylococcus aureus* (mm)
T1		11.78^{d}	12.54^{d}	11.81^{d}	11.83^{d}
T2		12.62^{c}	13.43^{c}	12.65^{c}	12.58^{c}
T3		14.93^{b}	14.60^{b}	13.51^{b}	15.31^{b}
CON		19.40^{a}	21.20^{a}	19.20^{a}	22.59^{a}
SEM		0.03	0.02	0.01	0.03
p-value		<.0001	<.0001	<.0001	<.0001
	d1	10.34^{d}	10.77^{d}	10.30^{d}	11.18^{d}
	d7	13.99^{c}	14.64^{c}	13.46^{c}	14.75^{c}
	d14	16.34^{b}	17.18^{b}	15.59^{b}	16.87^{b}
	d21	18.05^{a}	19.19^{a}	17.82^{a}	19.49^{a}
	SEM	0.03	0.02	0.01	0.03
	p-value	<.0001	<.0001	<.0001	<.0001
T1	d1	7.35^{i}	7.30^{k}	7.32^{k}	7.41^{j}
T2	d1	7.36^{i}	7.32^{k}	7.35^{k}	7.45^{j}
T3	d1	7.37^{i}	7.33^{k}	7.37^{k}	7.35^{j}
CON	d1	19.30^{a}	21.13^{a}	19.18^{a}	22.51^{a}
T1	d7	10.52^{h}	11.53^{j}	10.53^{j}	10.55^{i}
T2	d7	11.54^{g}	12.54^{i}	11.55^{i}	11.32^{h}
T3	d7	14.55^{e}	13.28^{h}	12.59^{h}	14.56^{e}
CON	d7	19.36^{a}	21.20^{a}	19.18^{a}	22.57^{a}
T1	d14	13.18^{f}	14.19^{g}	13.23^{g}	13.15^{g}
T2	d14	14.51^{e}	15.73^{f}	14.55^{f}	14.22^{f}
T3	d14	18.24^{b}	17.58^{d}	15.35^{e}	17.51^{c}
CON	d14	19.42^{a}	21.22^{a}	19.22^{a}	22.62^{a}
T1	d21	16.07^{d}	17.14^{e}	16.15^{d}	16.20^{d}
T2	d21	17.08^{c}	18.15^{c}	17.16^{c}	17.31^{c}
T3	d21	19.54^{a}	20.21^{b}	18.71^{b}	21.81^{b}
CON	d21	19.52^{a}	21.27^{a}	19.25^{a}	22.64^{a}
SEM		0.06	0.03	0.03	0.05
p-value		<.0001	<.0001	<.0001	<.0001

Least Squares Means (LSM) ± Standard Error of the Mean (SEM) indicating zones of inhibition (mm). Main effects (treatment concentration (TRT) and storage day (Day) and their two-way interactions (TRT × Storage) were analyzed. Means within the same column with different superscript letters (a-k) differ significantly ($P < 0.05$). CON=Control; T1, T2, T3= Propolis treatment levels.

Table 4: Effect of propolis concentration and storage days on the fungal growth

TRT	Day	*A. flavus* (mm)	*A. fumigatus* (mm)	*A. terreus* (mm)	*C. albicans* (mm)	*C. glabrata* (mm)
T1		12.68^{d}	12.76^{d}	11.86^{d}	14.72^{d}	14.30^{d}
T2		13.45^{c}	13.45^{c}	12.67^{c}	15.35^{c}	14.88^{c}
T3		14.13^{b}	14.06^{b}	13.55^{b}	16.60^{b}	16.00^{b}
CON		18.38^{a}	18.25^{a}	18.36^{a}	21.39^{a}	19.45^{a}
SEM		0.03	0.04	0.04	0.06	0.02
p-value		<.0001	<.0001	<.0001	<.0001	<.0001
	d1	10.79^{d}	10.79^{d}	10.80^{d}	13.68^{d}	13.20^{d}
	d7	13.98^{c}	13.90^{c}	13.14^{c}	16.14^{c}	15.69^{c}
	d14	15.97^{b}	16.03^{b}	15.19^{b}	18.24^{b}	17.23^{b}
	d21	17.88^{a}	17.81^{a}	17.32^{a}	19.99^{a}	18.50^{a}
	SEM	0.03	0.04	0.04	0.04	0.02

	p-value	<.0001	<.0001	<.0001	<.0001	<.0001
T1	d1	8.28^{k}	8.32^{j}	8.26^{k}	11.17^{i}	11.14^{k}
T2	d1	8.26^{k}	8.31^{j}	8.30^{k}	11.08^{i}	11.10^{k}
T3	d1	8.28^{k}	8.33^{j}	8.32^{k}	11.13^{i}	11.16^{k}
CON	d1	18.35^{ab}	18.21^{ab}	18.31^{a}	21.33^{a}	19.41^{a}
T1	d7	11.56^{j}	11.41^{i}	10.27^{j}	13.27^{h}	13.36^{j}
T2	d7	12.49^{i}	12.46^{h}	11.46^{i}	14.13^{g}	14.19^{i}
T3	d7	13.50^{h}	13.48^{g}	12.47^{h}	15.88^{f}	15.78^{g}
CON	d7	18.37^{ab}	18.24^{ab}	18.35^{a}	21.37^{a}	19.42^{a}
T1	d14	14.03^{g}	14.44^{f}	13.06^{g}	16.23^{e}	15.48^{h}
T2	d14	15.13^{f}	15.14^{e}	14.05^{f}	17.12^{d}	16.12^{f}
T3	d14	16.35^{e}	16.25^{d}	15.29^{e}	18.19^{c}	17.87^{d}
CON	d14	18.39^{a}	18.27^{ab}	18.38^{a}	21.41^{a}	19.46^{a}
T1	d21	16.87^{d}	16.86^{c}	15.85^{d}	18.22^{c}	17.22^{e}
T2	d21	17.92^{c}	17.88^{b}	16.88^{c}	19.09^{b}	18.09^{c}
T3	d21	18.31^{b}	18.20^{ab}	18.13^{b}	21.21^{a}	19.18^{b}
CON	d21	18.42^{ab}	18.29^{a}	18.41^{a}	21.46^{a}	19.51^{a}
SEM		0.07	0.08	0.08	0.09	0.03
p-value		<.0001	<.0001	<.0001	<.0001	<.0001

Values show Least Squares Means (LSM) ± Standard Error of the Mean (SEM) of inhibition zones (mm) across pathogenic fungal strains. Means within the same column with different superscript letters (a-k) differ significantly (P<0.05) according to post-hoc analysis

Table 5: Effects of propolis treatment concentrations and storage period (Days 1, 7, 14, and 21) on antioxidant activity, lipid oxidation inhibition, total antioxidant capacity, and total flavonoid content. Values represent Least Squares Means (LSM) ± Standard Error of the Mean (SEM).

TRT	**Day**	**ABTS (%)**	**DPPH (%)**	**LPI (%)**	**TAC(AU)**	**TFC (mg QE/g)**
T1		31.51^{d}	37.85^{d}	32.39^{d}	0.58^{d}	0.69^{c}
T2		41.37^{c}	44.12^{c}	44.83^{c}	0.67^{c}	0.77^{b}
T3		49.47^{b}	58.85^{b}	56.69^{b}	0.76^{b}	0.86^{a}
CON		54.42^{a}	63.64^{a}	61.51^{a}	0.81^{a}	–
	SEM	0.03	0.17	0.01	0.01	0.009
	p-value	<.0001	<.0001	<.0001	<.0001	<.0001
	d1	40.89^{d}	44.19^{d}	40.50^{d}	0.66^{b}	0.65^{d}
	d7	42.79^{c}	49.81^{c}	48.65^{c}	0.71^{ab}	0.73^{c}
	d14	46.90^{a}	56.31^{a}	54.29^{a}	0.73^{a}	0.82^{b}
	d21	46.19^{b}	54.16^{b}	51.99^{b}	0.73^{a}	0.88^{a}
	SEM	0.03	0.17	0.01	0.01	0.009
	p-value	<.0001	<.0001	<.0001	<.0001	<.0001
T1	d1	22.06^{m}	25.05^{n}	18.87^{p}	0.52^{j}	0.62^{f}
T2	d1	30.21^{k}	28.98^{m}	25.64^{o}	0.56^{hij}	0.65^{f}
T3	d1	34.17^{j}	33.87^{k}	32.93^{l}	0.60^{ghij}	0.71^{e}
CON	d1	77.13^{a}	88.87^{a}	84.56^{a}	0.96^{a}	–
T1	d7	28.24^{l}	31.87^{l}	28.19^{n}	0.53^{ij}	0.65^{f}
T2	d7	34.22^{j}	35.84^{j}	41.56^{j}	0.65^{fghi}	0.73^{e}
T3	d7	43.43^{h}	54.79^{g}	53.59^{g}	0.73^{cdef}	0.83^{c}
CON	d7	65.25^{c}	76.75^{c}	71.26^{c}	0.91^{ab}	–
T1	d14	34.20^{j}	41.56^{i}	38.19^{k}	0.58^{hij}	0.73^{e}
T2	d14	46.04^{g}	51.80^{h}	51.73^{h}	0.68^{efgh}	0.82^{c}
T3	d14	54.19^{e}	67.87^{d}	65.78^{d}	0.82^{bcd}	0.92^{b}
CON	d14	53.16^{f}	64.00^{e}	61.46^{e}	0.84^{abc}	–
T1	d21	41.53^{i}	52.93^{h}	44.32^{i}	0.71^{defg}	0.79^{d}

T2	d21	55.00^{d}	59.89^{f}	60.39^{f}	0.80^{bcde}	0.89^{b}
T3	d21	66.08^{b}	78.89^{b}	74.48^{b}	0.87^{ab}	0.97^{a}
CON	d21	22.12^{m}	24.94^{n}	28.76^{m}	0.53^{ij}	–
SEM		0.07	0.33	0.03	0.02	0.01
p-value		<.0001	<.0001	<.0001	<.0001	<.0001

TRT: Treatment concentrations (T1, T2, T3) alongside Control (CON). **ABTS** = 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (%); **DPPH** = 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (%); **LPI** = Lipid Peroxidation Inhibition (%); **TAC** = Total Antioxidant Capacity (Absorbance Units, AU); **TFC** = Total Flavonoid Content (mg QE/g, Quercetin Equivalents per gram). TFC was not determined for CON (BHT), as BHT is not a flavonoid; TFC day means are those of the propolis treatments only.

DISCUSSION

Total Phenolic Content at the baseline (182.42 ± 3.15 mg GAE/g) and Total Flavonoid Content (68.30 ± 1.84 mg QE/g) indicated a rich secondary metabolite profile reflecting a high-quality ethanolic extract of propolis (EEP). These values are similar to the results presented by Bankova *et al*. (2019) and Huang *et al*. (2014), who reported that ethanolic extraction efficiently solubilizes hydrophobic polyphenols, typically yielding TPC values between 100 and 200 mg GAE/g depending on botanical origin.

The chromatographic analysis of ethanolic extract of propolis identified chrysin and pinocembrin as the major polyphenols comprising ~30% and ~26% of total identified compounds, respectively. Poplar-type propolis, common in temperate zones, is characterized by high levels of flavanones (pinocembrin) and flavones (chrysin, galangin), along with phenolic acids like caffeic, ferulic, and p-coumaric acids (Wagh, 2013; Zabaiou *et al*., 2017).

Regarding the effect of refrigerated storage, the progressive increase in phenolic compounds from Day 1 to Day 21 without a change in the baseline profile indicated that refrigerated storage allowed long cold incubation, enabling the slow release of non-covalently bound polyphenols from the remaining lipophilic resins and waxes, which increased their concentration over the storage period (Pobiega *et al*., 2019). It may also be due to the slow hydrolysis of complex phenolic glycosides and esters into free phenolic monomers. The comparatively greater increase in caffeic acid concentration than that of other compounds suggested the gradual hydrolysis of caffeic acid esters or larger hydroxycinnamic acid complexes during refrigerated storage (Zhao *et al*., 2022). Caffeic acid phenethyl ester (CAPE) was not detected, which may be due to botanical variability or a concentration below the limit of detection (LOD) under the chromatographic conditions used (Sforcin, 2016).

From the trends of the antibacterial activity, it was clear that Gram-positive bacteria were more vulnerable than Gram-negative bacteria, especially at high concentrations and for prolonged storage periods. This difference is likely due to differences in their structural makeup. Gram-negative bacteria have an outer membrane of lipopolysaccharide that Gram-positive bacteria lack. This allows non-polar flavonoid compounds to enter Gram-positive bacteria easily (Kujumgiev *et al.*, 1999; Sforcin, 2007). The increase in antibacterial activity observed with increasing EEP concentration may reflect a concentration-dependent effect of the antimicrobial constituents present in the extract.

HPLC-DAD profiling (Table 1) identified and quantified several major phenolic markers in the EEP, including caffeic acid, p-coumaric acid, ferulic acid, pinocembrin, chrysin and galangin, whose measurable levels increased over the storage period; caffeic acid phenethyl ester (CAPE) was not detected under the conditions used. Consistent with our findings, propolis is reported to contain flavonoids, phenolic acids, terpenoids, aromatic aldehydes, esters, alkaloids, coumarins, and other bioactive constituents, although their occurrence and relative abundance vary according to botanical and geographical origin. These constituents have been proposed to exert antimicrobial effects through several mechanisms, including disruption of microbial membrane integrity, inhibition of key enzymatic processes, interference with nucleic acid function, and interactions with cellular proteins. Phenolic and related compounds have also been reported to form hydrogen bonds and ionic interactions with microbial proteins, potentially altering their three-dimensional structure and biological function (Wink, 2008, 2015). Similar concentration-dependent antimicrobial responses have been reported for propolis extracts and associated with the combined or synergistic activity of multiple constituents (Kodgi *et al*., 2024). Nevertheless, because compound-specific antimicrobial assays were not performed, the contribution of individual constituents to the observed activity was not established, and these mechanisms should be regarded as literature-supported explanations rather than mechanisms directly demonstrated by the present study.

The most substantial increase in inhibitory activity was observed by day 21, particularly in samples treated with the 3% propolis concentration. This trend is supported by the HPLC-DAD profiling (Table 1), in which the quantifiable levels of the major phenolic markers increased from day 1 to day 21 while their relative profile remained stable, consistent with continued release of resin-bound phenolic and flavonoid constituents into the ethanol rather than net degradation during refrigerated storage, leading to enhanced bioactivity. It is likely that extended exposure to ethanol promotes the

dissolution of compounds such as pinocembrin, caffeic acid and galangin, which are widely reported for their antimicrobial effects (Silici and Kutluca, 2005). During prolonged storage, the measurable levels of these compounds increased gradually, which may explain the enhanced antibacterial and antifungal activity. This increase in antimicrobial activity over time has also been seen in another study (Güneş *et al*., 2024). These findings are consistent with a previous study conducted in Egypt, where ethanolic propolis extract produced inhibition zones in the range of 16–19 mm against comparable bacterial targets (Helmy *et al.*, 2025). The inhibitory action of EEP on *E. coli* and *S. aureus* shown by the current experiment is especially significant since the two microorganisms have recently been recognized as dominant contaminants of raw milk in Punjab, Pakistan, thus highlighting the possible use of propolis as a natural preservative agent for the dairy industry (Riaz *et al*., 2026). These findings are consistent with the MIC results, in which Gram-positive bacteria exhibited lower MICs (0.25% v/v, equivalent to 0.25 mg/mL) than Gram-negative bacteria (0.5% v/v, 0.5 mg/mL), confirming that EEP exhibits stronger inhibitory effects on organisms with more permeable cell structures (Albino-Táutiva *et al*., 2017). It should be noted that the agar well diffusion assay reflects both the intrinsic potency of the active constituents and their diffusibility through the agar, which depends on molecular size and polarity; the zone diameters reported here were therefore interpreted alongside the diffusion-independent MIC values, and the two approaches gave concordant concentration-dependent trends. As this was an in vitro screening study, determination of fungal MIC and of the minimum bactericidal and fungicidal concentrations (MBC/MFC) to distinguish microbicidal from microbistatic activity remains an important next step.

The antifungal activity of ethanolic propolis extract against *Candida albicans*, *Candida glabrata*, *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus terreus* increased with both propolis concentration and storage duration. *Candida* species were found to be more sensitive than the filamentous *Aspergillus* species. This may be attributed to the differences in the physical and physiological structure of the yeast fungi, which have less complicated, easily permeable cell membranes that enable better penetration by the active substances, compared to filamentous fungi. Propolis is known for its antifungal properties, although the precise mechanism remains unclear. The proposed antifungal pathway of propolis may include several complex pathways, among which there are genetic pathways responsible for mitochondria-dependent energy production, vacuolar acidification, macroautophagy, which affects the transportation of proteins to vacuoles, as well as the starvation response. Moreover, there is evidence that apoptosis induction by propolis occurs via metacaspase and Ras signaling pathways (De Castro *et al*., 2013). The chemical composition of propolis plays a vital role in its biological effects, including its antifungal action. Some of the compounds involved in this effect are pinobanksin-3-acetate (3-acetylpinobanksin), pinocembrin, p-coumaric acid, and caffeic acid (Banskota *et al*., 2001). In addition, 2′,4′-dihydroxy-3-methoxychalcone and 2′,4′-dihydroxychalcone were identified as some of the main active substances, while galangin, pinocembrin, and 7-hydroxy-8-methoxyflavanone isolated from propolis also demonstrated antifungal potential (Cerqueira *et al.*, 2022).

The antifungal effect of propolis can be largely explained by its phenolic content. The compound pinocembrin has been shown to inhibit *Penicillium italicum* by reducing phosphorylated adenosine nucleotide content in the hyphae and affecting the structure of the cell membrane, resulting in loss of ions (Peng *et al.*, 2012).

The increased antifungal properties of propolis during storage may be related to the availability and concentration of the active components contained in the substance. Extracts with a higher propolis concentration contain higher amounts of flavonoids, phenolic acids, terpenoids and aromatic acids, which are responsible for its antifungal properties (Zulhendri *et al*., 2021). The compounds disrupt fungal cell walls, block membrane function, and affect cell development and growth. In higher concentrations, they may work together to provide better antifungal properties, giving more inhibition over time. This has been evidenced by research showing larger zones of inhibition with stronger propolis extracts against *Candida* and *Aspergillus* (Kodgi *et al.*, 2024). The increased bioactivity of aged ethanolic extracts of propolis has been attributed to the better extraction efficiency of active components over time (Kodgi *et al*., 2024). In addition, aging has been reported to contribute to the increased solubility and availability of antifungal compounds from the propolis matrix (Zulhendri *et al*., 2021).

As far as sensitivity is concerned, *Candida albicans* was the most sensitive of all the examined fungi to the ethanolic propolis extract. At the conclusion of the 21-day experiment, it demonstrated greater sensitivity to the applied treatment than any other fungal species. On the other hand, *Candida glabrata* was less sensitive than *C. albicans*, which could be due to high resistance to stress and its cell wall structure, which enables it to resist the effects of the plant-based antimicrobials (Silici and Kutluca, 2005; Sforcin, 2007).

Although the extract exerted its inhibitory activity on the growth of fungi like *A. flavus* and *A. fumigatus*, their overall sensitivity to the substance was found to be quite low even when increased concentrations and exposure time were provided. The zones of inhibition observed with these species were relatively smaller than those observed with *Candida* species. It is noted that propolis is more potent against yeast-like organisms than against mold fungi, possibly because of variations in cellular structure and biofilm-forming ability. These interspecies differences can be attributed to the basic biological differences between the unicellular yeast organisms and the multicellular molds. Unicellular yeasts, such as

Candida albicans, have less complicated cell walls and tend to be more permeable to small hydrophobic substances like flavonoids, while filamentous fungi, including *Aspergillus* spp., have elaborate mechanisms to minimize their susceptibility to their surroundings (Bankova, 2005; Diba *et al.*, 2018). The stored propolis extract demonstrated enhanced antifungal activity against the tested fungi. Similarly, Vică *et al.* (2024) reported that ethanolic propolis extract was active against *Aspergillus* as well as *Candida*; however, the inhibition effect was somewhat reduced, possibly because of the varying composition of propolis collected from different areas, owing to differences in phenolic content and botanical source.

In this study, it was seen that the antioxidant activity of ethanolic propolis extract in terms of TAC, TFC, ABTS, DPPH, and lipid peroxidation inhibition depended on concentration and storage duration. Consistent with the HPLC-DAD data (Table 1), which showed a progressive rise in the measurable phenolic markers over storage, this increasing trend in antioxidant activity during storage could be due to the gradual solubilization and release of phenolics and flavonoids, resulting in their increased availability as radical scavengers. Increased concentrations of propolis would mean increased availability of active components, especially flavonoids and phenolic acids, which work in synergy to donate hydrogen and electrons in order to neutralize reactive oxygen species and inhibit lipid peroxidation in both biological and food systems (Shaheen *et al*., 2024). The progressive rise over time suggests that storage helps stabilize and slowly release these compounds, thereby improving their antioxidant capacity. Extraction with ethanol along with mild heating probably increases the efficacy of the extraction process of these active compounds, while proper storage conditions allow preservation of their antioxidant activity, accounting for the better performance of 3% propolis in terms of antioxidant activities. These polyphenolic compounds are known to act as radical scavengers by donating hydrogen or electrons. These effects were most pronounced at the highest concentration and longest storage duration. The cumulative effect of these bioactive compounds generally leads to improved antioxidant activity at higher concentration levels, hence making 3% propolis treatment more effective than other treatments (Busch *et al.*, 2017). This finding shows the existence of a direct relation between the concentration of propolis extract, storage period, and antioxidant activity. These findings explain this relationship and build upon past empirical studies (Busch *et al*., 2017; Galeotti *et al*., 2018). Therefore, the current research not only confirms the antioxidant activity of EEP but also offers a reasonable account of how storage and elevated concentrations contribute to increasing its efficacy. It should be noted, however, that the synthetic controls possessed greater antimicrobial activity during storage, whereas by day 21 the 3% EEP reached activity comparable to benzoic acid against *E. coli*, *C. albicans*, *A. flavus* and *A. fumigatus* and exceeded the synthetic antioxidant control in the TAC, DPPH, ABTS and LPI assays, largely because the activity of the control decreased during storage. Moreover, these findings originate from an in vitro screening study; their practical significance as a natural preservative in the dairy industry therefore needs confirmation in a real milk matrix.

This study has some limitations. All replicates were prepared from a single extract batch, so the results need validation with independently prepared extracts. A separate ethanol control was not involved in the diffusion assays; however, the working solutions possessed only 0.7-2.1% ethanol, day-1 zones did not varied with the threefold increase in ethanol content, zones increased during storage while the ethanol content stayed constant, and ethanol did not inhibit growth in the MIC assay. Finally, the diffusion assays were executed at pH 4.7, by means of selective media for the bacteria, so zone sizes are not directly similar with those obtained under standard conditions, and the activity of EEP must still be confirmed in milk.

Conclusion: This study demonstrates that the amount of propolis and storage time are crucial factors that can enhance its antibacterial, antifungal, and antioxidant effects. Among the propolis concentrations tested, the highest level of activity was observed for the 3% ethanolic extract after 21 days of storage. Although the synthetic controls retained greater antimicrobial activity in most cases, by day 21 the 3% extract was comparable to benzoic acid against *E. coli*, *C. albicans*, *A. flavus* and *A. fumigatus* and surpassed the synthetic antioxidant control in the TAC, DPPH, ABTS and LPI assays; the concentration- and storage-dependent enhancement indicates that well-prepared propolis is a promising natural bioactive candidate that warrants further evaluation, in a real milk matrix, for potential application as a dairy preservative.

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