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# Antimicrobial Activity and *In Vitro* Bioaccessibility of Antioxidant Compounds in Chestnut Honeys from Yalova (Türkiye) Region

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## Abstract

This study evaluated the antimicrobial, antioxidant, and total phenolic profiles of nine chestnut honey samples from Yalova, Türkiye, and the bioaccessibility of these compounds using a simulated *in vitro* gastrointestinal digestion model. Agar well diffusion and broth microdilution methods were used to evaluate antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella Enteritidis, Staphylococcus epidermidis*, *Listeria monocytogenes*, *Salmonella Typhi*, *Bacillus cereus*, *Enterococcus faecalis*, and *Candida albicans*. Honey samples (100% and 50% v/v) were effective against all microorganisms except *C. albicans*. The most sensitive strains were *S. aureus* and *S. epidermidis* while *E. coli* and *E. faecalis* were less susceptible, respectively. The total phenolic content of honey samples was between 59.9 and 77.5 mg GAE/100 g honey. The DPPH radical scavenging activity ranged from 8.46 to 13.27 µmol TE/g, while the ABTS radical scavenging activity was determined to be between 15.40 and 30.47 µmol TE/g. The CUPRAC reduced capacity ranged from 17.68 to 26.79 µmol TE/g. The antioxidant capacity of honey samples underwent significant fluctuations during *in vitro* gastrointestinal digestion ($p < 0.05$). While certain parameters declined during the gastric phase, samples H4 and H6 demonstrated remarkable resilience, achieving the highest post-digestive recovery with superior TPC stability and antioxidant retention.

**Keywords:** Chestnut honey, Antimicrobial, Antioxidant capacity, Phenols, Bioaccessibility

## INTRODUCTION

Chestnut honey is a monofloral honey produced from the nectar of the sweet chestnut tree (*Castanea sativa* L.) and cultivated as a dominant species around the Mediterranean and central region of Europe (Oddo et al. 2004). In addition to its nutritional value, chestnut honey has attracted attention for its potential bioactive properties. It contains a variety of enzymatic and non-enzymatic antioxidant compounds, such as glucose oxidase, catalase, ascorbic acid, carotenoid derivatives, organic acids, amino acids, and proteins (Andrade et al. 1997, Ferreira et al. 2009).

Chestnut honey has been reported to exhibit notable antioxidant and antimicrobial activities (Borum et al. 2026, Ishenbaeva et al. 2025). It has been associated with anticancer (Beretta et al. 2021), anti-inflammatory (Cianciosi et al. 2021), and wound-healing effects (Sevin and Yarsan 2022). The health-promoting properties are mainly attributed to its components, particularly phenolic compounds and flavonoids (Kolayli et al. 2016). The correlation between antioxidant activity and phenolic content has been reported by various studies (Al-Mamary et al. 2002, Alvarez-Suarez et al. 2010, Dimitrova et al. 2007, Liu et al. 2013). Honey exhibits broad-spectrum antimicrobial activity, which is attributed to phenolic acids as well as bioactive compounds derived from the hypopharyngeal secretions of bee (Çınar 2020, Escuredo et al. 2012, Estevinho et al. 2008). Compared with other types of honey, chestnut honey is distinguished by its high and diverse levels of phenolic and flavonoid content (Escuredo et al. 2013, Pichichero et al. 2009). It demonstrates higher antioxidant and antimicrobial activity than other monofloral honeys (Bertoncelj et al. 2007, Combarros-Fuertes et al. 2018, Küçük et al. 2007, Perna et al. 2013). Moreover, chestnut honey is among the few types of honey that can be meaningfully compared with Manuka honey, which is well known for its strong antimicrobial activity and therapeutic potential (Budak and Çakıroğlu 2022, Roberts et al. 2012).

Determining the bioaccessibility of phenolic compounds is important for understanding the potential bioactive effects of chestnut honey (Alvarez-Suarez et al. 2013). While extensive research exists on the bioaccessibility of phenolic compounds and antioxidant activity after digestion (Cianciosi et al. 2020, Magoshi et al. 2023, O'Sullivan et al. 2013, Seraglio et al. 2017), specific data on chestnut honey remains limited. Although a limited number of studies have investigated the

antioxidant activity and total phenolic content of chestnut honey after in vitro digestion (Mutlu et al. 2025, Simonetti et al. 2020), comprehensive research on samples from Türkiye remains scarce.

Türkiye holds a significant position in the global beekeeping industry, particularly in producing distinctive honeys, including chestnut honey. The Yalova region is considered a primary production area for this type of honey. Evaluating the bioaccessibility and bioactive properties of chestnut honey from this region may provide valuable insights into its potential functional and therapeutic value in comparison with internationally recognized bioactive honey types. Therefore, this study aimed to evaluate the antimicrobial and antioxidant profiles of Yalova chestnut honey as well as changes in its phenolic content and antioxidant capacity after *in vitro* digestion.

## MATERIAL AND METHODS

Chestnut honey samples (H1–H9) were collected from local beekeepers in different chestnut forest areas of Yalova, Türkiye. The samples were stored in glass containers at room temperature in the dark. The samples originated from local apiaries in the Yalova region, which are characterized by co-dominant chestnut and linden (*Tilia* spp.) vegetation. Although these samples are characterized as chestnut-dominated based on harvest period and organoleptic properties, they reflect the multi-floral nectar profile typical of the region's natural vegetation.

### Antimicrobial Activity

Antimicrobial activities of honey samples were assayed against *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 29212), *Salmonella Enteritidis* (ATCC 13076), *Staphylococcus epidermidis* (ATCC 12228), *Listeria monocytogenes* (ATCC 7644*), Salmonella Typhi* (ATCC 14028), *Bacillus cereus* (ATCC 4312), *Enterococcus faecalis* (NCTC 8213) and *Candida albicans (*A clinical isolate obtained from the Department of Microbiology, Bursa Uludağ University*).* Antimicrobial activity was initially screened using the well diffusion method (Perez et al. 1990). Bacterial strains were cultured in Mueller-Hinton Broth (MHB) at 35°C (18–24 h), while *C. albicans* was grown in Malt Extract Broth (MEB) at 25°C (24–48 h). A standardized inoculum equivalent to 0.5 McFarland was spread onto Mueller-Hinton agar (MHA). Undiluted and diluted chestnut honey samples (100%, 50%, and 25% v/v in 0.85% saline) were added (50 µL) into 5 mm diameter wells. Sterile 0.85% saline was used as the negative control. After incubation under aerobic conditions, inhibition zone diameters (mm) were measured. Because the high viscosity of honey may restrict diffusion through agar media and lead to underestimation of antimicrobial activity, MIC values were additionally determined using the broth microdilution method (Patton et al. 2006). Filtration through 0.20 µm membranes (Minisart, Sartorius) was selected as a cold sterilization method to preserve the heat-sensitive bioactive constituents of honey. Prior to filtration, the honey samples were diluted in sterile 0.85% saline (1.6%–50% v/v), thereby substantially reducing viscosity and ensuring efficient passage through the membrane without clogging. Following incubation with standardized inoculum at 35°C for 18–24 h, optical density (OD) was measured at 595 nm (Bio-Rad iMark). The MIC was defined as the lowest honey concentration producing ≥95% inhibition of $OD_{595}$ relative to the growth control (Antonio et al. 2010).

### Total Phenolic Content and Antioxidant Capacity

Phenolic compounds were extracted according to the method of Can et al. (2015) with minor modifications. Briefly, 5 g of honey was extracted with 25 mL of methanol/water (80:20, v/v) by continuous stirring for 24 h at room temperature in the dark, in accordance with the protocol of Can *et al.* (2015). After centrifugation at 1370 *g* for 10 min, the supernatant was filtered, and the final volume was adjusted to 25 mL with the same solvent system. Total Phenolic Content (TPC) was determined using the Folin–Ciocalteu method (Güneş et al. 2017), which reflects the sample's total reducing capacity rather than phenolics exclusively. Results were expressed as mg Gallic Acid Equivalents (GAE) per 100 g of sample. Antioxidant potential was evaluated via ABTS [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], DPPH (1,1-diphenyl-2-picrylhydrazyl), and CUPRAC (cupric reducing antioxidant capacity) assays. For the ABTS assay (Re *et al.* 1999), the radical cation was generated by reacting 7 mM ABTS with 2.45 mM potassium persulfate in the dark at room temperature for 12–16 h, and the resulting solution was diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm; 50 µL of extract was then mixed with 1.95 mL of the diluted $ABTS^{\cdot+}$ solution, and absorbance was recorded at 734 nm after 6 min. For the DPPH assay (Sánchez-Moreno et al. 1998), 100 µL of extract was mixed with 3.9 mL of methanolic DPPH solution ($6 \times 10^{-5}$ M), and absorbance was read at 517 nm after 30 min of incubation in the dark. For the CUPRAC assay (Apak *et al.* 2004), 0.1 mL of extract was added to a mixture of 1 mL Cu (II) (10 mM), 1 mL neocuproine (7.5 mM in ethanol), 1 mL ammonium acetate buffer (1 M, pH 7.0) and 1 mL distilled water, and absorbance was recorded at 450 nm after 30 min. For all three assays, a reagent blank (solvent in place of sample) and a Trolox calibration curve were run in parallel with every batch, and all measurements were performed in triplicate.

### Bioaccessibility of Phenolic Contents and Antioxidant Capacity After Digestion

Gastrointestinal digestion was simulated as described by Vitali et al. (2009). Briefly, 2 g of honey was diluted in 10 mL distilled water, acidified to pH 2.0, and incubated with 0.5 mL pepsin (20 g $L^{-1}$ in 0.1 M HCl; ≥250 U/mg solid, Sigma-Aldrich) at 37°C for 1 h. The gastric phase was terminated by

adjusting the pH to 7.0–7.5 with 1 M $NaHCO_3$. Subsequently, 2.5 mL of bile/pancreatin solution (12 g $L^{-1}$ bile salts and 2 g $L^{-1}$ pancreatin in 0.1 M $NaHCO_3$) and an electrolyte mixture (120 mM NaCl, 5 mM KCl) were added. After 2.5 h of intestinal incubation, the digesta was centrifuged at 3500 g for 10 min. The resulting bioaccessible fractions (supernatants) were analyzed for Total Phenolic Content (TPC) and antioxidant capacity to determine the bioaccessibility of phenolic compounds and their associated antioxidant capacity after simulated gastrointestinal digestion.

### Statistical analysis

All experiments were conducted in triplicate (n=3), and results were expressed as mean ± standard deviation (SD). The Shapiro-Wilk test (α=0.05) was employed to assess the normality of data distribution. Normally distributed data were analyzed using one-way ANOVA with Tukey's post hoc test ($p<0.05$) to determine significant differences between formulations. Statistical analyses were performed using IBM SPSS Statistics (IBM Corp., Armonk, NY, USA).

## RESULTS

### Antimicrobial Activity

The antimicrobial activity of chestnut honey samples at different concentrations (100%, 50%, and 25% v/v) is presented in Table 1.

**Table 1.** Inhibition zone diameters (mm) of chestnut honey samples against test microorganisms determined by agar well diffusion method.

| Test Microorg. | Conc. (%) | Honey Sample 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| *Sta. epi.* | 100 | 40.33±2.08$^{a,A}$ | 42.67±2.08$^{a,A}$ | 40.00±4.00$^{a,A}$ | 36.00±1.00$^{b,A}$ | 37.33±2.31$^{a,A}$ | 42.33±2.89$^{a,A}$ | 41.00±0.00$^{a,A}$ | 40.00±2.65$^{a,A}$ | 36.67±2.08$^{a,A}$ |
| | 50 | 38.67±0.58$^{a,B}$ | 30.33±1.53$^{b,B}$ | 31.00±1.00$^{b,B}$ | 34.67±1.53$^{b,B}$ | 31.67±1.53$^{b,B}$ | 33.33±1.15$^{b,B}$ | 39.67±0.58$^{a,B}$ | 32.00±2.65$^{b,B}$ | 32.00±2.65$^{b,B}$ |
| | 25 | 32.00±1.00$^{a,c}$ | 26.33±0.58$^{b,c}$ | 28.00±1.00$^{b,c}$ | 31.33±2.08$^{a,c}$ | 28.33±1.15$^{a,c}$ | 28.00±1.00$^{b,c}$ | 31.00±1.73$^{a,c}$ | 27.00±1.00$^{b,c}$ | 27.67±2.08$^{ab,c}$ |
| *E. coli* | 100 | 35.33±1.15$^{a,A}$ | 32.00±0.00$^{b,A}$ | 31.33±2.52$^{ab,A}$ | 37.00±2.00$^{a,A}$ | 32.67±0.58$^{b,A}$ | 30.33±0.58$^{c,A}$ | 32.67±1.15$^{b,A}$ | 29.33±0.58$^{c,A}$ | 36.00±0.00$^{a,A}$ |
| | 50 | 29.33±1.53$^{a,B}$ | 27.67±1.53$^{a,B}$ | 28.67±1.53$^{a,B}$ | 34.33±3.51$^{a,B}$ | 29.00±0.00$^{a,B}$ | 28.33±0.58$^{a,B}$ | 28.67±0.58$^{a,B}$ | 28.67±0.58$^{a,B}$ | 27.00±1.00$^{a,B}$ |
| | 25 | 28.67±2.08$^{a,c}$ | 23.33±1.53$^{b,c}$ | – | 28.67±1.15$^{a,c}$ | 23.67±0.58$^{b,c}$ | 25.67±1.15$^{a,c}$ | 25.33±1.15$^{a,c}$ | 26.67±0.58$^{a,c}$ | 23.00±1.00$^{b,c}$ |
| *E. fae.* | 100 | 28.00±0.00$^{d,A}$ | 32.67±0.58$^{b,A}$ | 33.67±2.89$^{a,A}$ | 36.00±0.00$^{a,A}$ | 29.67±0.58$^{c,A}$ | 31.33±0.58$^{b,A}$ | 31.67±0.58$^{b,A}$ | 21.33±0.58$^{e,A}$ | 26.67±0.58$^{d,A}$ |
| | 50 | 23.67±0.58$^{d,B}$ | 28.33±0.58$^{b,B}$ | 27.00±0.00$^{c,B}$ | 34.00±0.00$^{a,B}$ | 24.33±0.58$^{d,B}$ | 28.00±1.00$^{b,B}$ | 28.00±0.00$^{b,B}$ | 15.67±0.58$^{f,B}$ | 19.67±0.58$^{e,B}$ |
| | 25 | 21.67±2.08$^{bc,B}$ | 26.00±1.00$^{a,B}$ | 25.00±0.00$^{b,B}$ | 28.00±1.00$^{a,B}$ | 22.33±0.58$^{c,B}$ | 22.33±1.15$^{bc,B}$ | 25.33±0.58$^{a,B}$ | 14.33±0.58$^{e,B}$ | 17.33±0.58$^{d,B}$ |
| *B. cer.* | 100 | 34.00±0.00$^{b,A}$ | 35.33±1.15$^{a,A}$ | 23.00±1.73$^{d,A}$ | 35.67±1.15$^{a,A}$ | 33.00±2.00$^{ab,A}$ | 34.33±0.58$^{b,A}$ | 32.00±0.00$^{c,A}$ | 37.67±0.58$^{a,A}$ | 30.33±1.15$^{c,A}$ |
| | 50 | 30.33±0.58$^{a,B}$ | 27.00±1.73$^{ab,B}$ | 19.67±0.58$^{d,B}$ | 29.67±2.08$^{a,B}$ | 30.33±0.58$^{a,B}$ | 29.00±0.00$^{b,B}$ | 24.00±1.73$^{c,B}$ | 30.67±0.58$^{a,B}$ | 24.33±1.15$^{c,B}$ |
| | 25 | 21.33±2.08$^{ab,c}$ | 22.33±0.58$^{b,c}$ | 15.67±1.15$^{c,c}$ | 24.33±0.58$^{a,c}$ | 24.00±1.00$^{a,c}$ | 23.67±0.58$^{a,c}$ | 20.33±1.15$^{b,c}$ | 24.67±0.58$^{a,c}$ | 26.00±1.00$^{a,c}$ |
| *Sal. Typhi* | 100 | 34.00±0.00$^{b,A}$ | 35.00±1.00$^{b,A}$ | 36.00±1.00$^{b,A}$ | 29.67±2.08$^{c,A}$ | 39.00±0.00$^{a,A}$ | 38.00±1.00$^{a,A}$ | 28.33±1.15$^{c,A}$ | 31.00±1.00$^{c,A}$ | 30.00±0.00$^{c,A}$ |
| | 50 | 28.33±1.53$^{a,B}$ | 24.67±0.58$^{c,B}$ | 31.00±1.73$^{a,B}$ | 23.67±2.08$^{bc,B}$ | 30.67±1.15$^{a,B}$ | 25.00±1.00$^{b,B}$ | 25.67±1.53$^{b,B}$ | 27.00±0.00$^{b,B}$ | 26.33±0.58$^{b,B}$ |
| | 25 | 24.00±1.00$^{a,c}$ | 21.00±1.00$^{b,c}$ | 23.33±1.15$^{a,c}$ | 16.67±0.58$^{c,c}$ | 27.00±1.00$^{a,c}$ | 22.67±0.58$^{b,c}$ | 15.67±0.58$^{c,c}$ | 25.00±0.00$^{a,c}$ | 20.33±1.53$^{b,c}$ |
| *Sal. Ent.* | 100 | 33.00±0.00$^{b,A}$ | 30.00±1.00$^{c,A}$ | 35.67±0.58$^{a,A}$ | 31.67±0.58$^{b,A}$ | 32.00±2.65$^{ab,A}$ | 31.67±0.58$^{b,A}$ | 31.00±2.65$^{ab,A}$ | 35.33±0.58$^{a,A}$ | 32.33±1.53$^{ab,A}$ |
| | 50 | 25.33±1.53$^{a,B}$ | 25.00±1.00$^{a,B}$ | 24.67±1.53$^{a,B}$ | 26.67±1.15$^{a,B}$ | 25.67±0.58$^{a,B}$ | 25.33±0.58$^{a,B}$ | 25.67±1.15$^{a,B}$ | 24.33±1.15$^{a,B}$ | 23.67±0.58$^{a,B}$ |
| | 25 | – | – | 17.33±0.58$^{a,c}$ | 16.67±0.58$^{a,c}$ | 13.00±1.00$^{b,c}$ | 16.33±0.58$^{a,c}$ | – | 14.33±0.58$^{b,c}$ | 14.00±0.00$^{b,c}$ |
| *Sta. aur.* | 100 | 21.33±0.58$^{a,A}$ | 19.00±1.00$^{a,A}$ | 19.67±0.58$^{a,A}$ | 21.67±1.15$^{a,A}$ | 15.67±1.15$^{b,A}$ | 18.67±0.58$^{b,A}$ | 17.67±0.58$^{b,A}$ | 18.33±0.58$^{b,A}$ | 20.33±0.58$^{a,A}$ |
| | 50 | 18.33±0.58$^{a,B}$ | 15.33±1.15$^{ab,B}$ | 15.67±0.58$^{b,B}$ | 15.33±0.58$^{b,B}$ | 13.00±0.00$^{c,B}$ | 15.00±0.00$^{b,B}$ | 14.67±0.58$^{b,B}$ | 13.00±0.00$^{c,B}$ | 12.67±0.58$^{c,B}$ |
| | 25 | – | – | – | – | – | – | – | – | – |
| *L. mon.* | 100 | 31.00±0.00$^{b,A}$ | 23.67±1.53$^{c,A}$ | 26.67±1.15$^{c,A}$ | 25.33±0.58$^{c,A}$ | 24.67±0.58$^{c,A}$ | 35.33±1.53$^{a,A}$ | 31.67±0.58$^{a,A}$ | 31.33±0.58$^{a,A}$ | 28.00±2.00$^{b,A}$ |
| | 50 | 15.00±1.00$^{c,B}$ | 15.33±0.58$^{c,B}$ | 18.00±0.00$^{b,B}$ | 15.33±1.15$^{c,B}$ | – | 25.67±0.58$^{a,B}$ | 19.33±0.58$^{b,B}$ | 24.33±0.58$^{a,B}$ | 20.67±1.15$^{b,B}$ |
| | 25 | – | – | – | – | – | 18.00±0.00$^{a,B}$ | – | – | – |
| *C. alb.* | All | No inhibition zone observed at any tested concentration | | | | | | | | |

Abbreviations: *Sta. epi.* = *Staphylococcus epidermidis*; *E. coli* = *Escherichia coli*; *E. fae.* = *Enterococcus faecalis*; *B. cer.* = *Bacillus cereus*; *Sal.* Typhi = *Salmonella* Typhi; *Sal.* Ent. = *Salmonella* Enteritidis; *Sta. aur.* = *Staphylococcus aureus*; *L. mon.* = *Listeria monocytogenes*; *C. alb.* = Candida albicans. None of the honey samples exhibited inhibitory activity against *C. albicans* at any of the tested concentrations. Values are expressed as mean ± standard deviation (n=3). "–" indicates no detectable zone of inhibition. Different lowercase superscript letters (a–f) within the same row denote significant differences between honey samples at a specific concentration ($p < 0.05$). Different uppercase superscript letters (A–C) within the same column for each microorganism indicate significant differences between tested concentrations ($p < 0.05$).

Honey samples exhibited antibacterial activity against all tested bacterial strains, whereas no inhibition was observed against *Candida albicans* at any tested concentration. Inhibition zones ranged from 12.67 to 42.67 mm, with Sample H2 (100% v/v) producing the largest inhibition zone against *S. epidermidis*. Among the tested microorganisms, Gram-positive bacteria, particularly *S. epidermidis*, showed higher sensitivity than other strains at 100% and 50% concentrations. Although most honey samples maintained their antibacterial effects at the 25% (v/v) concentration, a complete loss of inhibitory activity was observed for samples H1, H2, H7, and H9 against *S. aureus* and *S. Enteritidis*. While H3 and H4 generally outperformed other samples across most strains, H8 and H6 produced exceptionally large inhibition zones against *B. cereus* and *L. monocytogenes*.

MIC values showed variability among samples across the tested pathogens (Table 2), ranging from 3.1% to 25% (v/v). Lower MIC values, particularly those observed for *Staphylococcus aureus* and *Staphylococcus epidermidis*, indicated stronger antimicrobial activity. Sample H3 showed the lowest MIC value (3.1% v/v) against *Staphylococcus aureus*.

**Table 2.** Minimum inhibitory concentrations of chestnut honey samples against test microorganisms

| Honey Sample | *E. coli* | *S. aureus* | *L. monocytogenes* | *S. Enteritidis* | *E. faecalis* | *S.* Typhi | *B. cereus* | *S. epidermidis* | *C. albicans* |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 25 | 6.3 | 12.5 | 12.5 | 25 | 12.5 | 25 | 6.3 | >50 |
| 2 | 25 | 12.5 | 12.5 | 12.5 | 25 | 12.5 | 25 | 6.3 | >50 |
| 3 | 12.5 | 3.1 | 12.5 | 12.5 | 12.5 | 25 | 12.5 | 6.3 | >50 |
| 4 | 12.5 | 6.3 | 12.5 | 12.5 | 12.5 | 25 | 12.5 | 6.3 | >50 |
| 5 | 25 | 12.5 | 25 | 25 | 25 | 25 | 25 | 12.5 | >50 |
| 6 | 25 | 6.3 | 12.5 | 12.5 | 25 | 12.5 | 25 | 6.3 | >50 |
| 7 | 25 | 6.3 | 12.5 | 12.5 | 25 | 12.5 | 12.5 | 6.3 | >50 |
| 8 | 25 | 6.3 | 12.5 | 12.5 | 25 | 12.5 | 25 | 6.3 | >50 |
| 9 | 25 | 6.3 | 12.5 | 12.5 | 25 | 12.5 | 12.5 | 6.3 | >50 |

MIC values are expressed as % (v/v) honey concentration. Samples were tested at serial two-fold dilutions: 50, 25, 12.5, 6.3, 3.1, and 1.5% (v/v). “>50” indicates no inhibitory activity observed at the highest tested concentration.

### Total Phenolic Content and Antioxidant Capacity

The chestnut honey samples showed variation in total phenolic content (TPC) and antioxidant capacity (Table 3). Among the nine samples, H6 and H4 exhibited the highest phenolic contents, reaching 77.52 and 76.48 mg GAE/100 g, respectively. These samples also showed higher CUPRAC values compared to the other samples. In contrast, H9 exhibited the lowest TPC (59.95 mg GAE/100 g) and lower antioxidant values compared to the other samples. While H 8 did not have the highest phenolic content, it exhibited the highest ABTS radical-scavenging activity (30.47 µmol TE/g).

**Table 3.** Changes in TPC and antioxidant capacity of chestnut honey samples before and after *in vitro* digestion.

| Sample | TPC (mg GAE/100g) | | DPPH (µmol TE/g) | | ABTS (µmol TE/g) | | CUPRAC (µmol TE/g) | |
|---|---|---|---|---|---|---|---|---|
| | BD | AD | BD | AD | BD | AD | BD | AD |
| H1 | 66.12 ± 1.40$^{cd}$ | 54.62 ± 1.19$^{d}$ | 13.23 ± 0.33$^{a}$ | 12.90 ± 0.08$^{e}$ | 19.92 ± 0.61$^{e}$ | 23.65 ± 0.40$^{c}$ | 20.74 ± 0.98$^{c}$ | 9.87 ± 0.12$^{cd}$ |
| H2 | 62.89 ± 0.78$^{de}$ | 59.66 ± 1.29$^{c}$ | 13.28 ± 0.41$^{a}$ | 13.34 ± 0.42$^{e}$ | 28.70 ± 0.69$^{b}$ | 21.25 ± 0.90$^{de}$ | 26.79 ± 0.77$^{a}$ | 10.17 ± 0.33$^{bc}$ |
| H3 | 73.34 ± 1.81$^{b}$ | 62.79 ± 0.33$^{bc}$ | 11.68 ± 0.33$^{b}$ | 16.39 ± 0.47$^{d}$ | 22.49 ± 0.53$^{d}$ | 28.26 ± 0.71$^{b}$ | 20.91 ± 0.88$^{c}$ | 10.39 ± 0.40$^{bc}$ |
| H4 | 76.48 ± 0.52$^{ab}$ | 67.64 ± 1.26$^{a}$ | 12.07 ± 0.32$^{b}$ | 21.79 ± 0.34$^{c}$ | 24.89 ± 0.44$^{c}$ | 29.14 ± 0.72$^{b}$ | 26.47 ± 1.06$^{a}$ | 12.41 ± 0.19$^{a}$ |
| H5 | 68.88 ± 1.85$^{c}$ | 67.64 ± 2.19$^{a}$ | 11.96 ± 0.30$^{b}$ | 23.17 ± 0.63$^{b}$ | 15.40 ± 0.30$^{f}$ | 15.40 ± 0.13$^{f}$ | 21.88 ± 0.61$^{bc}$ | 10.10 ± 0.30$^{bc}$ |
| H6 | 77.52 ± 0.83$^{a}$ | 64.70 ± 1.44$^{ab}$ | 13.46 ± 0.34$^{a}$ | 26.29 ± 0.92$^{a}$ | 27.28 ± 1.22$^{b}$ | 32.96 ± 0.68$^{a}$ | 26.72 ± 0.49$^{a}$ | 13.13 ± 0.47$^{a}$ |
| H7 | 62.51 ± 1.60$^{de}$ | 60.99 ± 2.20$^{bc}$ | 10.18 ± 0.30$^{cd}$ | 12.46 ± 0.18$^{e}$ | 24.27 ± 0.30$^{c}$ | 22.94 ± 0.58$^{cd}$ | 20.24 ± 0.61$^{c}$ | 10.73 ± 0.25$^{bc}$ |
| H8 | 61.75 ± 0.99$^{e}$ | 61.84 ± 2.10$^{bc}$ | 11.10 ± 0.14$^{bc}$ | 13.62 ± 0.29$^{e}$ | 30.47 ± 0.52$^{a}$ | 28.97 ± 0.51$^{b}$ | 24.00 ± 1.02$^{b}$ | 10.91 ± 0.35$^{b}$ |
| H9 | 59.95 ± 1.85$^{e}$ | 58.90 ± 1.66$^{cd}$ | 9.86 ± 0.49$^{d}$ | 13.73 ± 0.41$^{e}$ | 15.84 ± 0.36$^{f}$ | 20.01 ± 0.40$^{e}$ | 17.68 ± 0.05$^{d}$ | 9.04 ± 0.21$^{d}$ |

Values are expressed as mean ± standard deviation (n=3). Different superscript letters (a–f) within the same column indicate statistically significant differences ($p < 0.05$). BD: Before *in vitro* digestion; AD: After *in vitro* digestion; TPC: Total phenolic content; GAE: Gallic acid equivalent; TE: Trolox equivalent.

**Bioaccessibility of Phenolic Contents, and Antioxidant Capacity After Digestion**

*In vitro* gastrointestinal digestion induced changes in the bioactive profiles of the honey samples (Table 3). A general downward trend in TPC was observed, with mean values decreasing from 67.71 to 62.09 mg GAE/100 g after digestion. CUPRAC values decreased by approximately 53% after digestion. Samples H4 and H5 maintained relatively high phenolic contents after digestion (67.64 mg GAE/100 g). However, the highest stability was observed in H6. H6 exhibited a marked increase in DPPH radical-scavenging activity after digestion; however, this apparent increase may be attributable to matrix transformation during gastrointestinal simulation. On the other hand, H8 showed minimal changes in ABTS values after digestion.

## DISCUSSION

### Antimicrobial Activity

In the present study, honey samples showed measurable antimicrobial activity as reported in other studies (Ayvaz et al. 2018, Kolayli et al. 2016, Ronsisvalle et al. 2017). *S. epidermidis* and *S. aureus* had MIC values closest to the results reported by Koloh et al. (2024), who found MIC values of 4% for Methicillin-Resistant *S. aureus* (MRSA) and *S. epidermidis* in Hungarian samples. Also, the larger inhibition zones observed in *S. epidermidis* (up to 42.67 mm) compared to those reported by Kolayli et al. 2016 (15 mm) and Cviljević et al. 2020 (21 mm) are directly attributed to our use of undiluted (100%) honey, whereas their maximum tested concentrations were 50% and 75%, respectively.

Among the tested Gram-negative pathogens, *E. coli* and *S. Enteritidis* exhibited moderate sensitivity, with MIC values predominantly in the 12.5%-25% (v/v) range. These findings are closely consistent with the reports of Ronsisvalle et al. (2017), who demonstrated that Sicilian chestnut honey is as effective as Manuka honey against ATCC strains of *E. coli* and *E. faecalis*. However, a pronounced concentration-dependent decline in antibacterial activity was evident at the 25% (v/v) dilution. This pronounced loss of efficacy points to a critical threshold effect within the honey matrix. Upon significant dilution, the synergistic action of hydrogen peroxide and complex non-peroxide components is reported to fall below the absolute minimum concentration required to sustain microbial inhibition (Brudzynski et al. 2011, Kwakman et al. 2010).

However, the ability of certain samples to preserve this synergistic action even at higher dilutions underscores the critical role of their inherent phytochemical strength. The potent activity of our samples, particularly Honey 3 (lowest MIC of 3.1% against *S. aureus*), is consistent with previous reports showing a strong correlation between phenolic content and antioxidant activity in chestnut honey (Çol Ayvaz et al. 2018), which, in turn, may contribute to antimicrobial effects. However, in the absence of compound-level identification (e.g., LC-MS) and direct correlation analysis in our samples, the precise contribution of individual phenolic compounds to the observed antimicrobial activity remains to be elucidated. As indicated by Ronsisvalle et al. (2017), advanced characterization often reveals that specific flavonoids are responsible for these effects. Such targeted bioactive profiles are also essential when addressing opportunistic bacteria that evade standard treatments. The fact that our chestnut honey maintained significant activity against *E. faecalis* (MIC 12.5–25%) is particularly noteworthy, as this strain is intrinsically resistant to many conventional antibiotics, a point also emphasized by Čengić *et al.* (2025) regarding Bosnian samples.

While the chestnut honey samples demonstrated remarkable efficacy against difficult-to-treat bacterial isolates, no inhibitory effect was detected against *C. albicans*. Although some reports in the literature suggest that chestnut honey possesses anti-Candida properties (Boutrou et al. 2024, Fernandes et al. 2021), this lack of antifungal efficacy strongly aligns with the consistent findings of Čengić et al. (2025), Kolayli et al. (2016), and Sagdic et al. (2013).

**Phenolic Composition, Antioxidant Capacity, and Bioaccessibility Following *In Vitro* Digestion**

These substantial values fit perfectly within the broad spectrum (19.05–108.21 mg GAE/100 g) documented by Sagdic et al. (2013) and closely mirror the 46.3–101.3 mg GAE/100 g range reported by Ayvaz et al. (2018) for Turkish chestnut honeys. This consistency is further supported by recent findings from the Artvin region (52.19–92.42 mg GAE/100 g) (Saral 2023), collectively confirming the robust and high-phenolic character of Anatolian samples. While our findings demonstrate a notably higher phenolic density compared to Bosnian samples (26.49–40.60 mg GAE/100 g) (Oraščanin et al. 2023), they remain more moderate in comparison to the exceptionally high concentrations reported for Greek chestnut honeys (149.9 mg GAE/100 g) (Tananaki et al. 2024).

Variations in antioxidant activity may depend more on the specific phenolic profile of each honey than on the total phenolic content alone. Previous studies indicate that the bioactivity of Turkish samples is largely linked to gallic acid, rutin, and caffeic acid phenethyl ester (Saral 2023, Taş-Küçükaydın et al. 2023). In contrast, European samples are characterized by major constituents such as pinobanksin and chrysin (Vujčić Bok et al. 2024), while South Korean variants are distinguished by p-coumaric and ferulic acids (Kim et al. 2023). Such compositional disparities likely explain the fluctuations in biological activity observed among honeys with similar total phenolic levels. However, since compound-level identification (e.g., LC-MS) was not conducted in this study, the specific

contribution of individual phenolic constituents to the bioactivity of the current samples remains to be determined.

The distinct phenolic matrices of honey samples lead to varying antioxidant efficiencies across different assays. Although direct numerical comparisons are often hindered by inconsistent reporting units and extraction protocols, our average DPPH and ABTS values align with the high inhibition levels reported by Perna et al. (2013) and the IC50 benchmarks established by Bertoncelj et al. (2007) for regional chestnut honeys. This convergence among samples from Yalova, southern Italy, and Slovenia suggests that the radical-scavenging capacity of monofloral chestnut honey is primarily governed by the conserved phenolic backbone of *Castanea sativa* nectar, which comprises gallic acid, kaempferol, and quercetin derivatives, rather than regional environmental variability.

The strong correlation between TPC and CUPRAC reducing power is consistent with the findings of Sarikaya et al. (2009). This alignment is mechanistically plausible, as the CUPRAC assay measures the electron-donating capacity of phenolic hydroxyl groups, which directly scales with the abundance of single-electron-transfer-active species typical of chestnut nectar. Accordingly, the narrow CUPRAC range observed in this study suggests that unblended chestnut honey is a compositionally homogeneous matrix regarding these active phenolics. In contrast, the substantially higher CUPRAC values (264–378 µmol/g) reported by Ulusoy et al. (2010) for multifloral honeys likely reflect the contribution of additional electron-donating constituents from *Rhododendron* and *Tilia* spp., which are absent in monofloral chestnut honeys. This divergence highlights the assay's sensitivity to floral origin and its utility in discriminating between monofloral and polyfloral honeys.

The bioaccessibility of honey phytochemicals is a critical parameter for evaluating the quantity and quality of antioxidants in the human body, as well as for determining the antioxidant potential of food materials. *In vitro* digestion models, although unable to replicate the full complexity of *in vivo* metabolism, remain the most widely accepted screening approach for estimating bioaccessibility and permit direct comparison across sample types under standardized conditions (Minekus et al. 2014). Therefore, evaluating changes in antioxidant activity measured by different methods following digestion is expected to provide a clearer understanding of these processes. As illustrated in Figure 1, the response to simulated digestion varied depending on both the sample type and the analytical method employed. This suggests that the stability and transformation of antioxidant constituents during gastrointestinal transit are governed by a complex set of mechanisms.

TPC followed a broadly similar downward trajectory for most samples (fold change 0.7–0.9). However, sample H4 deviated significantly, showing a marked increase in post-digestion. This outlier response likely stems from the enzymatic liberation of phenolics initially bound to the honey's proteinaceous or carbohydrate matrix. Such a mechanism was previously proposed by Mutlu et al. (2025), who observed a 2.5-fold increase in TPC during the intestinal phase in Turkish honeys, which they attributed to the disruption of phenol–macromolecule complexes.

The most consistent observation was a substantial reduction in CUPRAC values, with all samples exhibiting fold changes below unity and an average decline of approximately 50%. Since the CUPRAC assay is highly sensitive to the intact hydroxyl architecture of flavonoids and hydroxycinnamic acids (Apak et al. 2004), this pronounced loss suggests structural modifications or degradation of electron-donor moieties under the near-neutral to mildly alkaline conditions of the intestinal phase. This trend aligns with the findings of Seraglio et al. (2017), who attributed similar declines in honeydew honey to alkaline-induced ionization and oxidative polymerization of catechol- and galloyl-type systems. Furthermore, the 21–28% decrease in ABTS and FRAP values reported for Italian chestnut honeys by Simonetti et al. (2020) supports the view that the electron-transfer capacity of honey polyphenols is inherently vulnerable to intestinal pH.

In contrast to the CUPRAC results, radical-scavenging assays (DPPH and ABTS) demonstrated a divergent pattern. DPPH values exceeded unity in samples H3 through H6, with the most significant enhancements observed in H4 and H5. Meanwhile, ABTS values remained largely stable or showed slight increases across the same samples. This heterogeneity contrasts with the uniform declines reported by O'Sullivan et al. (2013) for Irish floral honeys, a discrepancy that may be explained by the unique chemotype of chestnut honey. Characterized by high levels of gallic, caffeic, and p-coumaric acids (Perna et al. 2013), the enzymatic cleavage of glycosylated or esterified precursors in chestnut honey may generate free aglycones with superior hydrogen-atom-donating capacity. This could enhance DPPH and ABTS responses without a corresponding increase in the electron-transfer capacity measured by CUPRAC.

Furthermore, the sample-to-sample variability observed underscores the matrix heterogeneity of chestnut honey. As noted by Matkovits et al. (2023), factors such as altitude, micro-climate, and botanical purity prevent the reduction of these honeys to a single standardized fingerprint. Finally, it is critical to emphasize that a decrease *in in vitro antioxidant capacity does not necessarily indicate* a loss of biological function. Digested polyphenols often undergo biotransformation into low-molecular-weight catabolites with potent anti-inflammatory or

cytotoxic effects (Cianciosi et al. 2020, Mutlu et al. 2025).

## Conclusion

This study evaluates the functional properties and gastrointestinal stability of chestnut honeys from the Yalova region of Türkiye. The results indicate that these honeys exhibit antimicrobial activity, particularly against Gram-positive bacteria, and contain measurable levels of phenolic compounds with associated antioxidant capacity. Although several studies have investigated chestnut honey, limited data are available on the changes in phenolic content following digestion. The present study provides such data for chestnut honeys from the Yalova region. By examining how digestion affects its bioactivity, these findings provide a preliminary characterization of the bioactive potential of Anatolian chestnut honeys. Our results further suggest a potential trend: the in vitro functional behavior of honey may be influenced more by the stability of its phenolic compounds during digestion than by its initial raw composition. However, this observation should be interpreted with caution, as more comprehensive analyses are required to clearly establish this relationship. Future studies that identify specific metabolites generated during digestion may help clarify the biological relevance of these observations.

The in vitro digestion model used in this study specifically evaluates the bioaccessibility of phenolic compounds, representing their release from the food matrix for potential absorption, rather than their full bioavailability. Therefore, these findings provide preliminary insights into the gastrointestinal stability and antioxidant retention of chestnut honey phenolics, rather than direct evidence of in vivo physiological effects. Further research involving cell-based models and in vivo studies is necessary to translate these observations into definitive health-related conclusions.

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