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Chemical Characterization and Biological Activities of Ankara Propolis: Antioxidant, Antimicrobial and Cytotoxic Effects on Colorectal Cancer Cell Lines

Ümmügülsüm Yurteri

gulsumyurteri@gmail.com

Middle East Technical University

Mustafa Çiçek

Karamanoğlu Mehmetbey University

Şükran Yılmaz

Foot-and-Mouth Disease Institute

Faruk Bozoğlu

Middle East Technical University

Gülçin Celep

Gazi University

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Abstract

Propolis, a resinous product produced by bees, is recognized for its antioxidant, antimicrobial, and antiproliferative properties; however, its composition and bioactivity are influenced by geographic origin and processing methods. This study systematically characterized ethanolic extracts of Ankara propolis (Türkiye) before and after thermal treatment, employing chemical, antioxidant, antimicrobial, and cytotoxic analyses. Extraction with 70% ethanol resulted in a yield of 20.30 ± 0.01% (w/w) of the raw material. The antioxidant capacity, determined by the DPPH assay, produced an IC50 of 4.101 ± 0.014 mg/mL and a maximum radical-scavenging activity of 96.0 ± 0.1%. The Trolox-equivalent antioxidant capacity was measured at 2.970 mmol TE/g extract. Total phenolic and flavonoid contents were quantified as 10.712 ± 0.007 mg gallic acid equivalents/g and 0.742 ± 0.014 mg quercetin equivalents/g extract, respectively. The extract demonstrated inhibitory effects against Staphylococcus aureus and Candida albicans, with the most pronounced activity observed against C. albicans (minimum inhibitory concentration 4.0 mg/mL); no activity was observed against Escherichia coli. Mass spectrometry analysis identified p-coumaric acid (341.06 ± 1.43 ppm) as the predominant phenolic acid, followed by caffeic acid (223.50 ± 0.44 ppm) and vanillic acid (0.4875 ± 0.0014 ppm). Thermal treatment at 60°C and 120°C significantly reduced antioxidant, phenolic, flavonoid, and antimicrobial activities, indicating the thermolability of the bioactive constituents. The extract also exhibited dose- and time-dependent cytotoxicity against Caco-2 and HT-29 colorectal cancer cells, with Caco-2 cells displaying greater sensitivity. These results suggest that Ankara propolis is a rich source of phenolic acids with notable antioxidant, antimicrobial, and antiproliferative properties. Still, its bioactivity is highly sensitive to thermal processing, a critical factor in product standardization.

1. Introduction

Propolis is a resinous material produced by honeybees (*Apis mellifera*) from plant-derived exudates and beeswax. Bees utilize propolis to seal hive openings and cracks, thereby maintaining an environment that restricts microbial contamination (Anjum et al., 2019; Kasote et al., 2022). Propolis has been employed in traditional medicine since antiquity; however, its chemical composition and biological activity exhibit considerable variability among samples (Ghisalberti, 1979; Crane, 1999).

This variability is primarily influenced by botanical and geographical origin, seasonal factors, and the plant sources accessible to bees (Anjum et al., 2019; Kasote et al., 2022). Propolis comprises a complex mixture of resins and balsams, waxes, essential oils, pollen, and a diverse array of phenolic and other organic compounds. Over 800 compounds have been identified in various types of propolis, although the composition of individual samples is strongly dependent on their origin (Kasote et al., 2022). Phenolic acids and flavonoids represent major bioactive constituents, including compounds such as caffeic acid, *p*-coumaric acid, pinocembrin, chrysin, and galangin (Irigoiti et al., 2021; Kurek-Górecka et al., 2013).

The chemical differences among propolis samples are reflected in their biological activities. Propolis has been studied for its antimicrobial, antioxidant, anti-inflammatory, immunomodulatory, and

antiproliferative effects (Kurek-Górecka et al., 2013; Kasote et al., 2022). Its antimicrobial properties have been demonstrated against various bacteria and fungi, including oral pathogens. For instance, propolis extracts can inhibit biofilm formation by *Streptococcus mutans* and exhibit bactericidal activity against *Porphyromonas gingivalis* (Veloz et al., 2019; Yoshimasu et al., 2018). The antioxidant activity of propolis is primarily attributed to its phenolic constituents and has been confirmed through multiple radical-scavenging and oxidative stress assays (Kurek-Górecka et al., 2013; Nagai et al., 2003; Sobočanec et al., 2006). Additionally, propolis has been reported to modulate inflammatory responses, such as cytokine production by macrophages (Machado et al., 2012).

The chemical profile of propolis varies significantly across geographical regions. For example, Brazilian green propolis is derived from *Baccharis dracunculifolia* and is characterized by prenylated phenolic compounds, including artepillin C. In contrast, Cuban propolis is associated with *Clusia* species and contains polyisoprenylated benzophenones such as nemorosone (Anjum et al., 2019; Cuesta-Rubio et al., 2002). Extraction methods introduce additional variability, as ethanol and other solvents extract different proportions of phenolic and other bioactive constituents. Consequently, the composition of a propolis extract may not accurately represent that of the raw material (Wagh, 2013; Irigoiti et al., 2021).

This chemical variability constitutes a major obstacle to the standardization of propolis-based products. Variations in botanical origin, geographical location, and processing methods hinder the establishment of universal chemical markers and consistent quality-control criteria (Kasote et al., 2022). Processing conditions further influence the recovery and stability of phenolic compounds in propolis extracts, depending on the specific extraction procedures employed (Malenica et al., 2024). Nevertheless, the extent to which thermal processing alters the chemical composition and biological activity of defined propolis preparations remains insufficiently characterized.

This study investigated Ankara propolis from Türkiye before and after thermal processing. Changes in chemical composition were analyzed using mass spectrometry, with a focus on selected polyphenolic compounds. Antioxidant, antimicrobial, and antiproliferative activities were evaluated concurrently to determine whether alterations in chemical composition corresponded to changes in biological activity. By examining these parameters collectively, the study seeks to provide a clearer foundation for assessing the stability of Ankara propolis during thermal processing and its implications for product standardization.

2. Materials and Methods

2.1. Sample Collection

Propolis samples were collected from a local beekeeper in Kıbrıs village, situated in the Mamak district of Ankara, Türkiye. This village, located at an altitude of 1124 meters, was designated a first-degree archaeological site by the Ministry of Culture in 1995. The area is characterized by distinctive vegetation, comprising nearly 1000 plant species, including several endemic taxa (Yurteri, 2015).

2.2. Extraction

A total of 64 g of raw propolis was fragmented into small pieces and stored at − 20°C. The frozen material was ground and extracted with 70% ethanol at a 1:20 (w/v) ratio. The mixture was kept under agitation in the dark for 10 days and then filtered. The residue was re-extracted with 70% ethanol for an additional 2 days, then filtered. To facilitate wax removal, the combined filtrates were stored overnight at 4°C and subsequently filtered through Whatman No. 1 paper. The solvent was evaporated, and the extract was lyophilized for 48 h. The dried extract was stored in Eppendorf tubes at − 20°C until further use (Trusheva et al., 2007). The extraction yield was calculated using the following equation:

$$\%\ Extraction\ Yield = \frac{Mass\ of\ raw\ propolis\ in\ solution}{Mass\ of\ total\ propolis} \times 100$$

2.3. DPPH Radical Scavenging Activity

The free radical scavenging activity of the extract was assessed using the DPPH• (1,1-diphenyl-2-picrylhydrazyl) assay. DPPH• is a stable molecule with an unpaired electron, known as a free radical, that gives it a deep purple color. It turns yellow when it gains an electron and becomes diphenylpicryl hydrazine, which occurs when antioxidants in the test compound react with it (Blois, 1958). A decrease in absorbance at 517 nm was measured as an indicator of antioxidant activity.

For the assay, 1.4 mL of a 0.05 mg/mL ethanolic DPPH solution was mixed with 0.1 mL of propolis extract at different concentrations (0.5–10 mg/mL) and vortexed. The mixture was incubated in the dark at room temperature for 20 min, and absorbance was measured at 517 nm. Ethanol was used as the control. All measurements were performed in triplicate with duplicate readings. Radical scavenging activity (RSA) was calculated as follows:

$$\%\ RSA = \frac{A_0 - A_1}{A_0} \times 100$$

where A0 is the absorbance of the control (DPPH in ethanol) and A1 is the absorbance with extract. RSA values were plotted against extract concentration (mg/mL), and IC50 was determined.

2.4. Trolox Equivalent Antioxidant Capacity (TEAC)

The antioxidant capacity was assed using the ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) assay, following the methods of Arnao et al. (2001) and Re et al. (1999), with minor modifications. A 7 mM ABTS solution was prepared and oxidized with 2.5 mM potassium persulfate. The mixture was kept in the dark for 16 h before use.

The ABTS•+ solution was diluted in ethanol until an absorbance of 1.1 ± 0.02 at 734 nm was reached. Different concentrations of extract (2.5–10 µg/mL) were mixed with 1 mL of ABTS•+ and incubated in the dark for 6 min. Absorbance was measured at 734 nm. Trolox was used as a reference standard.

Inhibition percentage was calculated as:

$$\%\ Inhibiton = \frac{A_0 - A_1}{A_0} \times 100$$

where A0 is the absorbance of ABTS•+ and A1 that of ABTS•+ with extract. TEAC values were determined by comparing the slopes of extract inhibition curves with those of Trolox, adjusted by the dilution factor (60).

2.5. Total Phenolic Content

The total phenolic content was quantified using the Folin–Ciocalteu method (Singleton & Rossi, 1965). Gallic acid (0.05–0.3 mg/mL) served as the calibration standard. In brief, 0.1 mL of extract or standard was mixed with 2 mL of 2% Na_2CO_3. After 3 min, 0.1 mL of 50% Folin–Ciocalteu reagent was added. The mixture was incubated in the dark for 30 min. Absorbance was read at 750 nm.

Results were expressed as gallic acid equivalents (mg GAE/g extract):

$$mg\ GAE/g\ extract = \frac{(Abs.\ of\ Sample - Abs.\ of\ blank)}{Slope} \times Dilution\ Factor$$

2.6. Total Flavonoid Content

Total flavonoids were quantified by the aluminum chloride colorimetric method (Marinova et al., 2005). Briefly, 1 mL of extract was mixed with 4 mL of water and 0.3 mL of 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. After 1 min, 2 mL of 1 M NaOH was added. The volume was adjusted to 10 mL with water. Absorbance was read at 510 nm. Quercetin was used as the standard.

Flavonoid content was expressed as quercetin equivalents (mg QE/g dry extract):

$$mg\ QE/g\ dry\ extract\ mass = \frac{(Abs.\ of\ Sample - Abs.\ of\ blank)}{Slope} \times Dilution\ Factor$$

2.7. Antimicrobial Activity Assays

The antimicrobial activity was evaluated by the Kirby–Bauer disk diffusion method (Bauer et al., 1966). Initially, *E. coli* and *S. aureus* cultures were inoculated in Mueller–Hinton broth and incubated at 37°C for 18 h. Following incubation, suspensions were diluted 1:100, and 100 µL (~ 10^7–10^8 cells/mL) was spread on Mueller–Hinton agar. Subsequently, sterile 6-mm disks impregnated with 20 µL of propolis extract (10 mg/mL) were placed on the plates, which were then incubated at 37°C for 18 h.

Tetracycline (10 µg) and amikacin (30 µg) served as positive controls; ethanol and distilled water were used as solvents and negative controls, respectively. Inhibition zones were measured and interpreted using NCCLS (now CLSI) guidelines (NCCLS, 2002a).

2.8. Antifungal Acitivity Assay

Candida albicans (~ 0.5×10^6 cells/mL) was first cultured on Malt Extract Agar. Next, sterile 6 mm discs containing 20 µL of extract (20 mg/mL) or controls were placed on the prepared plates, which were then incubated at 25°C for 36–48 h. Amikacin (0.30 mg/mL) and ethanol served as positive controls, while distilled water acted as the negative control. Results were interpreted according to CLSI standards (NCCLS, 2003).

2.9. Determination of MIC and MBC

MIC (Minimum Inhibitory Concentration) values were determined using the disk diffusion method with serial dilutions of extract (64–1 mg/mL) applied to sterile discs. Plates were incubated at 37°C for 24 h, and MIC was defined as the lowest concentration at which no visible growth occurred (NCCLS, 2000, 2002b). After determining MIC values, the analysis proceeded to characterize MBC (Minimum Bactericidal Concentration).

For MBC determination, overnight cultures of test microorganisms were adjusted to 10^5–10^6 CFU/mL in Mueller–Hinton broth. The test tubes were serially diluted with propolis extract (64–1 mg/mL). Each tube was inoculated with equal volumes of the microbial suspension. Positive controls (tetracycline, amikacin, chloramphenicol, ethanol) and a negative control (distilled water) were included. MBC is defined as the lowest concentration of an antimicrobial that kills ≥ 99.9% of the initial bacterial population.

2.10. Heat Stability Assay

To assess thermal stability, a 40 mg/mL extract in 70% ethanol was divided into two tubes. One was incubated at 60°C, and the other was autoclaved at 120°C. Antioxidant and antimicrobial assays (DPPH, ABTS, phenolics, flavonoids, and disk diffusion) were then repeated.

2.11. Cell Culture and Viability Assay

HT-29 and Caco-2 colorectal adenocarcinoma cells (from Foot-and-Mouth Disease Institute, Ankara, Türkiye) were cultured at 37°C in a humidified incubator with 5% CO_2. Cell suspensions (2×10^4 – 5×10^4 cells/mL) were seeded into 96-well plates (100 µL/well) and incubated for 24 h for attachment.

After the initial 24 h incubation for cell attachment, the medium was replaced with 100 µL of fresh medium containing propolis (0.01–100 µg/mL) in the relevant wells. Control wells received only fresh medium. Cells were then incubated for either 24, 48, or 72 h, depending on group assignment.

After each incubation period, the medium was removed, and 100 µL of fresh medium and 130 µL of MTT solution (0.5 mg/mL) were added to each well, followed by incubation for 4 h. Formazan crystals were dissolved with 100 µL isopropyl alcohol, and absorbance was measured at 570 nm using a microplate reader. Wells containing medium and MTT solution without cells served as blanks. All experiments were performed in triplicate as described by Holst-Hansen et al. (1996).

2.12. Mass Spectrometry

Phenolic compounds were analyzed at the METU Central Laboratory, Molecular Biology-Biotechnology Research and Development Center in Ankara, Türkiye, in the Mass Spectroscopy Laboratory using an AGILENT 6460 Triple Quadrupole System (ESI+ Agilent Jet Stream) with an AGILENT 1200 Series HPLC.

2.13. Statistical Analysis

Statistical analysis for the cell viability assay was conducted. Data are shown as mean values. Error bars represent standard deviation (SD, n = 3). GraphPad Prism (GraphPad Prism Software Inc., USA) was used. A two-way ANOVA was applied.

3. Results and Discussion

3.1. Yield of Extraction

The efficiency of propolis extraction is influenced by solvent type, extraction conditions, and extraction technique. In the present study, maceration with 70% ethanol yielded an extract recovery of 20.30 ± 0.01% (w/w).

Maceration usually yields lower yields than more intensive methods such as Soxhlet or supercritical fluid extraction, but it remains popular because it is simple and works well for heat-sensitive compounds (Bitwell et al., 2023). Similar yields for hydroethanolic extracts, such as 18.1% and 18.63% reported by Khacha-ananda et al. (2013) and Mahani et al. (2013), also show that ethanol-based extraction is effective.

Amin et al. (2023) reported a similar yield of 19.21 ± 0.75% with conventional ethanol extraction, whereas water extraction yielded 14.63 ± 0.56%. On the other hand, supercritical CO2 extraction yielded 24.26%, demonstrating the promise of advanced extraction methods.

Moreno et al. (2020) reported much higher yields (51%) with 80% ethanol and a longer extraction time, underscoring the importance of solvent concentration and processing conditions. Mokhtar et al. (2019) also showed that yield depends on solvent polarity and the sample-to-solvent ratio, with the best results using ethanol.

Overall, the yield found in this study matches previous reports using standard ethanol-based methods. Differences between studies are probably due to variations in propolis source, extraction settings, and post-processing. These results show that 70% ethanol maceration is a reliable and effective method for extracting propolis.

3.2. Antioxidant Activity of Propolis

The antioxidant activity of ethanolic propolis extract was measured using the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. For comparison, α-tocopherol was used as the reference

antioxidant (Table 1). The propolis extract showed an IC_{50} of 4.101 ± 0.014 mg/mL and a maximum RSA of 96.0 ± 0.1%. In contrast, α-tocopherol had a lower IC_{50} (0.306 ± 0.086 mg/mL) but a similar maximum RSA (97.0 ± 0.1%). Thus, α-tocopherol is about 13 times more potent than Ankara propolis extract for radical scavenging. However, both achieve nearly complete radical inhibition at higher concentrations, so their maximal antioxidant capacities are similar despite potency differences.

To further clarify, the IC_{50} value indicates the antioxidant's efficiency at low concentrations. Maximum RSA reflects the radical scavenging capacity at higher concentrations. The similar maximum RSA values for both samples indicate that propolis can achieve the same level of radical neutralization as α-tocopherol, albeit at much higher concentrations.

Table 1
Antioxidant activity of propolis and α-tocopherol determined by DPPH assay.

Sample	IC_{50} (mg/mL ± SD)	Maximum RSA % (± SD)
Propolis Extract	4.101 ± 0.014	96.0 ± 0.1
α-Tocopherol	0.306 ± 0.086	97.0 ± 0.1

Moreover, the propolis extract showed antioxidant activity of 1.26 ± 0.01 mg α-tocopherol equivalents per gram, indicating moderate antioxidant capacity. However, this classification requires caution. Comparisons with literature under similar assay conditions are necessary, as reported propolis antioxidant capacity varies widely with botanical origin, extraction procedure, and analytical method.

Similar trends have been documented in previous research. For example, Karagecili et al. (2023) reported strong DPPH radical scavenging activity for Turkish propolis. They found an IC50 of 20.55 µg/mL, comparable to that of butylated hydroxytoluene (BHT). Propolis samples from 39 regions across Türkiye demonstrated antioxidant capacities ranging from 55.98% to 86.17% (Böke Sarıkahya et al., 2021), showing significant intra-country variability. Al-Ani et al. (2018) also reported higher antioxidant potential in Turkish propolis compared to several European samples.

In contrast, Chinese propolis samples exhibited considerably weaker and more variable antioxidant activity. Their IC50 values ranged from 71.19 to 432.08 µg/mL (Jiang et al., 2020), showing the strong influence of regional and botanical factors. Earlier studies have also reported that Ankara propolis exhibits stronger radical scavenging activity than some Asian samples (Kunimasa et al., 2011), which agrees with these observations.

The moderate antioxidant activity of Ankara propolis is likely due to its phenolic and flavonoid content. These compounds are effective hydrogen donors and radical stabilizers (Kujumgiev et al., 1999). Differences in antioxidant performance among propolis samples can be attributed to variations in origin, climate, and metabolites. Overall, Ankara propolis exhibits substantial radical-scavenging capacity. This supports its use as a natural antioxidant, though it is less potent than α-tocopherol.

3.3. Trolox Equivalent Antioxidant Capacity (TEAC)

The antioxidant potential was further evaluated using the ABTS assay, expressed as Trolox Equivalent Antioxidant Capacity (TEAC). Results are shown in Table 2.

Table 2
Trolox equivalent antioxidant capacity (TEAC) of Ankara propolis

Concentration (µg / mL)	TEAC value (mmol TE / g extract ± SD)
10	2.970 ± 0.086
20	0.148 ± 0.043
30	0.099 ± 0.002
40	0.074 ± 0.021
50	0.060 ± 0.001

The TEAC value of the extract was as 2.970 ± 0.072 mmol TE/g. In comparison, the ethanol extract of Brazilian propolis was reported to yield 1.22 ± 0.14 mmol TE/g extract (Skaba et al., 2013). Such differences may be attributed to factors including the surrounding plant flora and the season during which the propolis was collected.

3.4. Total Phenolic Content

Total phenolic content (TPC) was measured by the Folin–Ciocalteu assay, using gallic acid for calibration ($y = 81.36x - 0.013$, $R^2 = 0.998$). The extract contained 10.712 ± 0.007 mg GAE/g extract (Table 3).

Table 3
Total phenolic content of propolis in gallic acid equivalents.

Concentration (mg/mL)	Total Phenolic Content (mg GAE/g extract ± SD)
0.25	4.218 ± 0.004
0.5	7.878 ± 0.007
1	13.333 ± 0.013
1.5	17.511 ± 0.005

The total phenolic content reported in this study is consistent with the findings of Khacha-Ananda et al. (2013), who observed values ranging from 17.17 ± 2.19 to 18.27 ± 3.30 mg GAE/g extract in propolis from Phayao province, Thailand. In contrast, propolis from other regions demonstrates substantially higher phenolic content. For instance, Kumazawa et al. (2004) reported 298.0 ± 8.7 mg GAE/g in Chinese propolis from Hubei province, while Moreira et al. (2008) documented values ranging from 151.00 to 329.00 mg GAE/g in Portuguese propolis.

In Türkiye, Karagecili et al. (2023) reported a total phenolic content of 53.0 mg GAE/g for propolis from the Berdav region of Ağrı province, which is moderate relative to both international and local samples. Despite similarities in geography and climate between Thailand and China, their respective propolis samples exhibit markedly different phenolic profiles. These findings underscore the significant influence of local plant species and floral diversity.

Overall, these results indicate that the chemical composition and biological activity of propolis are influenced by factors such as geographic origin, local flora diversity, climate, and harvest period. Each of these variables affects the accumulation of phenolic compounds in propolis extracts.

3.5. Total Flavonoid Content of Propolis

Flavonoid levels, measured by the aluminum chloride colorimetric assay with quercetin calibration, were calculated as 0.742 ± 0.014 mg QE/g extract (Table 4).

Table 4
Total flavonoid content of propolis extract in quercetin equivalents.

Concentration (mg/mL)	Total Flavonoid Content (mg QE/g extract ± SD)
0.25	0.223 ± 0.005
0.5	0.437 ± 0.001
1	1.127 ± 0.045
1.5	1.187 ± 0.003

This value indicates a moderate flavonoid content, which is considerably lower than levels reported in several previous studies. For example, Velázquez et al. (2007) found a total flavonoid content of 57.8 ± 3.6 mg QE/g extract in Sonoran propolis from Northwest Mexico. Cottica et al. (2011) documented a wide range of 2.5–176 mg QE/g extract among Brazilian propolis samples. Similarly, Karagecili et al. (2023) reported a total flavonoid content of 170.2 mg QE/g extract in propolis from the Berdav region of Ağrı province, Türkiye. These findings highlight substantial regional variability, even within a single country.

3.6. Antimicrobial Activity of Propolis

The antioxidant components of propolis, particularly phenolic compounds, contribute to its antimicrobial properties. In this study, we assessed the antimicrobial activity of Ankara propolis against *Staphylococcus aureus*, *Escherichia coli*, and the fungal strain *Candida albicans*, organisms selected for their clinical significance in human infections as representatives of Gram-positive bacteria, Gram-negative bacteria, and opportunistic fungal pathogens.

In evaluating the extract's effects, the ethanolic extract of propolis exhibited antibacterial activity exclusively against *S. aureus*, while no inhibitory effect was detected against *E. coli*. Notably, the greatest

inhibition was observed against *C. albicans*, where the antifungal effect exceeded that of the reference antibiotic amikacin (Table 5)

Table 5
Antifungal and antibacterial activity of Ankara propolis (n = 3)

Microorganism	Zone of Inhibition (mm)				
	Propolis Extract	**Antibiotic Control**	**Distilled Water**	**Ethanol**	**Reference Antibiotic Disc***
E. coli	NA	25.0	NA	NA	TE 30
S. aureus	13.3	23.0	NA	NA	AMC 30
C. albicans	55.0	52.0	NA	12.3	AMC 30

*NA: no activity; TE: tetracycline; AMC: amikacin.

Comparable findings have been documented in studies from other regions of Türkiye. For example, Aksoy and Dığrak (2006) found that ethanol extracts of propolis (EEP) from Bingöl province showed no inhibitory effect on *E. coli.* In contrast, chloroform extracts produced a 33.0 mm zone of inhibition. In the same study, EEP created inhibition zones of 23.0 mm and 16.0 mm against *S. aureus* and *C. albicans*, respectively. Similarly, Alan et al. (2014) reported that EEP from Muş province produced inhibition zones of 18.0 mm against *E. coli* and 20.0 mm against *S. aureus* but showed no activity against *C. albicans*. Building upon these regional results, recent findings further elucidate antimicrobial effects.

Consistent with these regional and current findings, Rivera-Yáñez et al. (2022) reported pronounced antifungal activity of propolis extracts against *Candida albicans*, attributing this effect to high concentrations of flavonoids and phenolic acids. The authors demonstrated that propolis compounds disrupt fungal cell membranes and inhibit both biofilm and hyphal formation, thereby supporting the strong antifungal effect observed in this study.

The findings demonstrate that Ankara propolis exhibits selective antimicrobial activity, showing efficacy against Gram-positive bacteria, particularly *Candida albicans*, but no activity against Gram-negative *Escherichia coli*. This pattern suggests that the antimicrobial efficacy of propolis is influenced by microbial cell structure, with phenolic constituents contributing more substantially to antifungal and Gram-positive antibacterial activity.

To further investigate and expand on these observations, the influence of growth medium composition on antimicrobial activity was assessed using various media. The resulting inhibition zones are presented in Table 6.

Table 6

Antimicrobial activity of propolis on different culture media*.

Microorganism / Antibiotic	Baird Parker	Mueller Hinton	Nutrient	MacConkey
E. coli	–	–	–	–
S. aureus	+	++	++	–
C. albicans	+	++	++	+
Amikacin	++	++	++	++
Tetracycline	++	++	++	++

*Symbols: "–" no inhibition; "+" moderate inhibition; "++" strong inhibition.

3.6.1. Determination of MIC Value of Propolis

The minimum inhibitory concentrations (MICs) of the propolis extract were determined using the Kirby–Bauer disc diffusion method, and the results are summarized in Table 7.

Table 7
MIC Values of Propolis Against Tested Microorganisms

Microorganism	MIC Value (mg/ml)
E. coli	16.0
S. aureus	8.0
C. albicans	4.0

The minimum inhibitory concentration (MIC) values of the extract were 16.0 mg/mL for *E. coli*, 8.0 mg/mL for *S. aureus*, and 4.0 mg/mL for *C. albicans*. These data demonstrate that *C. albicans* was the most susceptible microorganism to the extract, while *E. coli* exhibited the highest level of resistance.

Similar results have been documented in the literature. For example, a study on Brazilian propolis reported MIC values of 3.1 mg/mL for both *S. aureus* and *C. albicans*, with no measurable MIC for *E. coli* (Campos et al., 2014). Conversely, ethanol extracts of Turkish propolis from the Kayseri region exhibited substantially higher MIC values: 1024.0 mg/mL for *E. coli*, 64.0 mg/mL for *S. aureus*, and 128.0 mg/mL for *C. albicans* (Gunduz-Kaya et al., 2012).

Comparable observations have been documented in previous studies. For example, an investigation of Brazilian propolis reported MIC values of 3.1 mg/mL for both *S. aureus* and *C. albicans*, while no measurable MIC was obtained for *E. coli* (Campos et al., 2014). In contrast, ethanol extracts of Turkish propolis from the Kayseri region showed markedly higher MIC values: 1024.0 mg/mL for *E. coli*, 64.0 mg/mL for *S. aureus*, and 128.0 mg/mL for *C. albicans* (Gunduz-Kaya et al., 2012).

Sa-eed et al. (2023) also reported that propolis extracts from various regions of Ghana exhibited greater antimicrobial activity against *Staphylococcus aureus* than against *Escherichia coli*, with significantly higher MIC and minimum bactericidal concentration (MBC) values for Gram-negative bacteria. In their study, *E. coli* isolates showed limited susceptibility, whereas *S. aureus* isolates were more sensitive to the active fractions. These findings correspond with the present results and further emphasize the influence of geographical origin and extraction method on propolis's antimicrobial efficacy.

These differences across studies show the essential roles of geographic origin, botanical sources, and chemical composition in determining propolis's antimicrobial efficacy.

3.6.2. Determination of Minimum Bactericidal Concentration (MBC) of Propolis

The bactericidal and fungicidal properties of the extract were evaluated by determining the minimum bactericidal concentration (MBC). Microbial cultures were incubated in media containing varying concentrations of extract. After incubation, samples were subcultured on Mueller–Hinton broth (MHB) or malt extract agar, depending on the organism. The MBC was defined as the lowest concentration (mg/mL) at which no visible microbial growth occurred. This confirmed bactericidal or fungicidal activity relative to previously established minimum inhibitory concentration (MIC) values.

Based on these methods, the analysis indicated that the MBC values of propolis were 64.0 mg/mL for *E. coli*, 32.0 mg/mL for *S. aureus*, and 16.0 mg/mL for *C. albicans* (Table 8).

Table 8
MBC Values of Propolis Against Tested Microorganisms

Microorganism	MBC Value (mg/ml)
E. coli	64.0
S. aureus	32.0
C. albicans	16.0

Consistent with the minimum inhibitory concentration (MIC) results, *C. albicans* was identified as the most susceptible organism, whereas *E. coli* demonstrated the greatest resistance to the propolis extract. This trend is further supported by previous studies. For instance, Suleman et al. (2015) reported that the ethanol extract of South African propolis exhibited minimum bactericidal concentrations (MBCs) of 1.563 mg/mL for *S. aureus*, 1.563 mg/mL for *E. coli*, and 0.781 mg/mL for *C. albicans*. Together, these findings underscore the significant influence of geographical origin and botanical sources on propolis's antimicrobial efficacy.

3.7. Heat Stability of Propolis

The effects of heat treatment on antioxidant activity, total phenolic content, flavonoid content, and antimicrobial properties of propolis were evaluated. A 40 mg/mL extract in 70% ethanol was prepared and split into two tubes. One was heated to 60°C; the other to 120°C (Table 9).

Table 9
Effect of heat treatment on the antioxidant properties, total phenolic and flavonoid content of propolis extract (n = 3).

Propolis Sample	Antioxidant Activity (IC_{50} ± SD, mg/mL)	Maximum RSA (%) ± SD	TEAC (mmol TE/g extract ± SD)	Total Phenolic Content (mg GAE/mg extract ± SD)	Total Flavonoid Content (mg QE/mg extract ± SD)
Non-treated	4.101 ± 0.014	96.0 ± 1.090	0.290 ± 0.072	10.712 ± 0.007	0.742 ± 0.014
60°C	8.31 ± 0.01	43.0 ± 1.08	0.521 ± 0.063	4.199 ± 0.003	0.224 ± 0.019
120°C	10.06 ± 0.09	30.0 ± 0.183	2.003 ± 0.140	2.573 ± 0.006	0.097 ± 0.047

Bioactivity declined as temperature increased. The sample treated at 120°C demonstrated significantly lower antioxidant activity and reduced total phenolic and flavonoid contents compared to the extract treated at 60°C. This decrease is attributed to the thermal degradation of phenolic compounds and the reduced stability of other bioactive constituents in propolis under high-temperature conditions. The biological activity of propolis is primarily due to its high polyphenolic content, but these compounds are thermolabile. Exposure to temperatures above 50–60°C can cause oxidative degradation, structural changes, and loss of functional hydroxyl groups, which leads to decreased total phenolic content and diminished bioactivities, including antioxidant and antimicrobial effects (ElGamal et al., 2023). Thus, thermal processing conditions are critical for preserving the bioactivity of propolis.

Heat treatment significantly reduced propolis's antimicrobial efficacy. The 60°C and 120°C extracts showed no notable inhibitory effects against the tested microorganisms. An exception was a minimal inhibition zone of 8.00 ± 0.08 mm against *S. aureus* from the 60°C sample. No measurable minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) values were obtained from the heat-treated extracts. These findings confirm the loss of antimicrobial potential. They also emphasize the detrimental effects of thermal exposure on propolis's functional properties.

3.8. Effect of Ankara Propolis on Cell Viability

Previous studies have demonstrated that propolis exhibits cytotoxic activity against various cancer cell lines, primarily attributed to its rich phenolic composition and antioxidant properties (Vukovic et al., 2018). In this study, the cytotoxic potential of Ankara propolis extract was assessed against two colorectal cancer cell lines, Caco-2 and HT-29, using the MTT assay. These cell lines were selected to represent complementary aspects of intestinal epithelial biology: HT-29 cells are less differentiated and produce mucus, while Caco-2 cells differentiate into enterocyte-like cells that form tight junctions and a

functional barrier. This approach enables a comprehensive evaluation of propolis effects across different states of intestinal epithelial differentiation and function.

As shown in Fig. 1a, treatment of Caco-2 cells with increasing concentrations of the extract resulted in a clear, dose-dependent reduction in cell viability at all time points tested (24, 48, and 72 hours). At 24 hours, pronounced cytotoxic effects were noted at higher concentrations (≥ 50 µg/mL). At 48 and 72 hours, minor variations in viability were observed, but no consistent recovery occurred, indicating growth inhibition was maintained. At lower concentrations (2.5–0.01 µg/mL), cell viability remained similar to control levels across all time points.

These findings were corroborated by IC_{50} values measured at specific time points: 17.99 µg/mL at 24 hours, 24.56 µg/mL at 48 hours, and 26.90 µg/mL at 72 hours. This indicates a moderate, time-dependent decrease in sensitivity.

HT-29 cells also showed a concentration-dependent reduction in viability following extract treatment (Fig. 1b). Marked cytotoxicity was observed at higher concentrations, especially at 24 hours. Later time points showed a milder attenuation of this response. The IC_{50} values increased from 21.43 µg/mL at 24 hours to 26.99 µg/mL at 48 hours, and then to 33.28 µg/mL at 72 hours. This trend indicates a progressive decline in cellular responsiveness over time.

Comparative analysis showed that Caco-2 cells were more sensitive to the propolis extract than HT-29 cells. This difference was more pronounced during early exposure periods, as indicated by consistently lower IC_{50} values. The gradual increase in IC_{50} values over time may result from cellular adaptation, activation of survival pathways, or the selective persistence of more resistant subpopulations.

These findings are further supported by previous studies. For example, Calhelha et al. (2014) demonstrated dose-dependent cytotoxic effects of Portuguese ethanolic propolis extracts against several cancer cell lines, including MCF-7, NCI-H460, HCT-15, HeLa, and HepG2. Similarly, Choudhari et al. (2013) reported cytotoxic and pro-apoptotic activities of Indian propolis extracts against MCF-7, HT-29, Caco-2, and B16F1 cells.

Falcão et al. (2023) found that only red propolis significantly reduced HT-29 cell viability. This effect was seen at concentrations of 50 and 100 µg/mL, with viability falling below 50%. Green and brown propolis showed limited or no cytotoxicity. This variation highlights the importance of botanical origin and chemical composition in determining the anticancer potential of propolis extracts.

The anticancer activity of propolis is mainly due to bioactive compounds such as CAPE (Caffeic Acid Phenethyl Ester) and chrysin. These compounds regulate apoptotic signaling by activating caspases and inhibiting anti-apoptotic proteins (Bulavin et al., 1999; McEleny et al., 2004). Thus, the cytotoxic effects seen in this study are likely mediated, at least in part, by these molecular mechanisms.

3.9. Mass Spectrometry Results

Mass spectrometry was employed to identify and quantify the principal phenolic acids in Ankara propolis extract. Vanillic acid, caffeic acid, and p-coumaric acid were detected at concentrations of 0.4875 ± 0.0014 ppm, 223.50 ± 0.44 ppm, and 341.06 ± 1.43 ppm, respectively (Supplementary Figure S1-S3). Among these compounds, the concentration of p-coumaric acid exceeded that of both vanillic acid and caffeic acid, making it the most abundant phenolic constituent observed in the sample.

Similar regional variations in phenolic composition have been documented in previous research. Karagecili et al. (2023) analyzed propolis samples from the Berdav region of Ağrı province. They reported concentrations of 1.647 mg/g for vanillic acid, 21.358 mg/g for caffeic acid, and 16.911 mg/g for p-coumaric acid. Although differences in analytical units and extraction protocols preclude direct quantitative comparison, these results demonstrate considerable geographical variability in the phenolic profiles of Turkish propolis. The elevated levels of p-coumaric and caffeic acids identified in this study may underlie the pronounced antimicrobial and cytotoxic activities observed in Ankara propolis. Both compounds have been reported extensively to possess antimicrobial, antioxidant, and antiproliferative properties, supporting their potential contribution to the biological activities observed in this study (Svetikiene et al., 2024). Consequently, variations in phenolic composition attributable to regional and botanical factors are likely critical determinants of propolis bioactivity.

Conclusion

Propolis from the Mamak district of Ankara, Türkiye, was analyzed for antioxidant potential, antimicrobial activity, and effects on cell proliferation. The ethanol extract of Ankara propolis appeared to contain relatively high levels of phenolic and flavonoid compounds, which may be associated with its notable radical-scavenging capacity. Although sourced from one location, seasonal and annual changes in constituent proportions may affect its biological properties.

The extract exhibited strong antifungal activity against the tested fungal strain and inhibited the growth of the Gram-positive bacterium *S. aureus.* No inhibitory effect was observed against the Gram-negative strain *E. coli.* The resistance of *E. coli* may result from the protective barrier provided by the lipopolysaccharide layer.

LC/MSMS data established the phenolic profile of Ankara propolis. Among the phenolic acids identified, p-coumaric acid (341.06 ± 1.43 ppm) was most abundant. Caffeic acid (223.50 ± 0.44 ppm) and vanillic acid (0.4875 ± 0.0014 ppm) followed. The high level of p-coumaric acid indicates it likely contributes most to antimicrobial and cytotoxic effects.

The ethanol extract also showed significant cytotoxicity against colorectal carcinoma cell lines HT-29 and Caco-2 at 100 µg/mL. Heat treatment, in contrast, educed radical scavenging activity against DPPH and ABTS radicals. It also decreased antimicrobial efficacy. These results indicate the bioactive phenolic constituents are thermolabile.

In summary, Ankara propolis is a rich natural source of phenolic acids and flavonoids. It exhibits potent antioxidant, antimicrobial, and cytotoxic activities. Further research is needed to clarify the molecular mechanisms behind these effects in microbial systems and cancer models. Ankara propolis has strong bioactive potential, making it a promising candidate for new therapeutic applications. If extract standardization and dose stabilization are achieved, propolis could be considered an adjuvant to conventional cancer treatments. Its phenolic components may reduce oxidative stress, influence apoptotic pathways, and enhance therapeutic response. However, controlled *in vivo* studies and clinical evaluations are essential before any medical use can be advised.

Declarations

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process.

During the preparation of this work the author(s) used Generative AI and Grammerly AI-Pro in order to language editing purposes. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

Author Contribution

● Ü. Y. : Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft.● M. C. : Investigation, Formal analysis, Data curation, Writing – review & editing.● S.Y. : Methodology, Validation, Writing – review & editing.● F. T. B. : Supervision, Investigation, Writing – review & editing● A. G. S. C. : Supervision, Project administration, Writing – review & editing.

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

Ethics, Consent to Participate, and Consent to Publish declarations

Ethics, Consent to Participate, and Consent to Publish declarations: not applicable.

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Figures

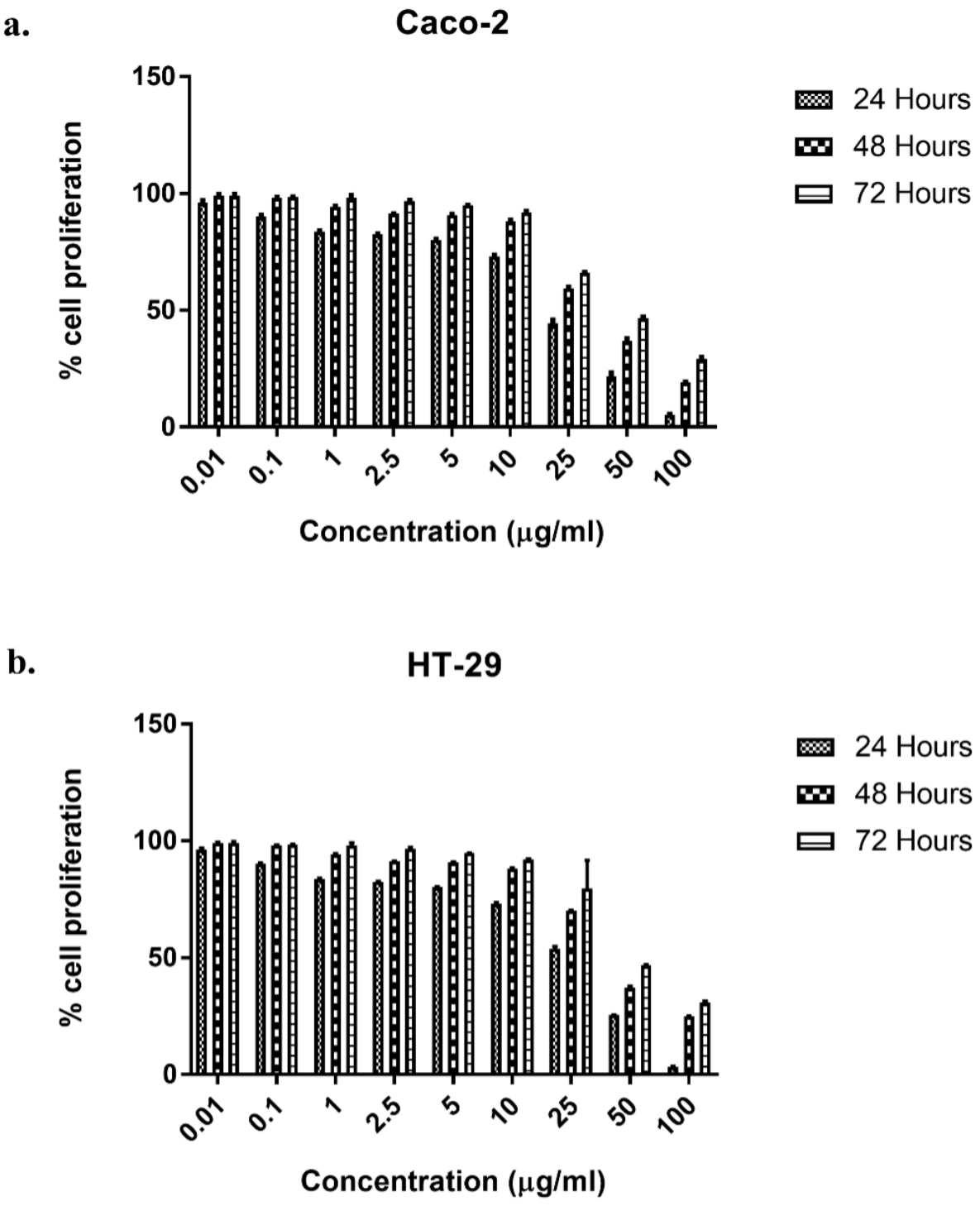

Figure 1

Effect of Ankara propolis extract on cell proliferation of Caco-2 (a) and HT-29 (b)

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